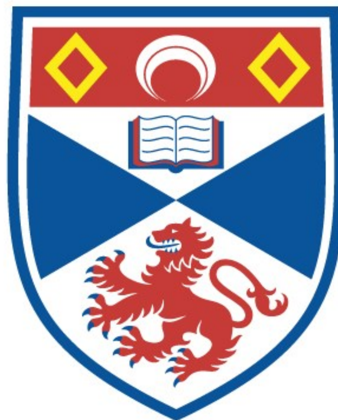
# THE EFFECTS OF EXTRACELLULAR SODIUM CHLORIDE ON THE ACTIVITY AND EXPRESSION OF NA,K-ATPASE IN PRIMARY CULTURES OF DOGFISH (SCYLIORHINUS CANICULA) RECTAL GLAND EPITHELIAL CELLS

Joanne Edwards

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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# The Effects of Extracellular Sodium Chloride on the Activity and Expression of Na,K-ATPase in Primary Cultures of Dogfish (*Scyliorhinus canicula*) Rectal Gland Epithelial Cells.

by

Joanne Edwards

Thesis submitted for the degree of
Doctor of Philosophy
in the University of St. Andrews.

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This thesis is dedicated to my family, particularly my mother, father, Mark and Andrea.

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Abstract

Dogfish, *Scyliorhinus canicula,* rectal gland epithelial cells were successfully cultured using two different techniques: 1) a perfusion based technique and 2) a modified Valentich's technique. The morphology of the primary rectal gland epithelial cell cultures was investigated using light, fluorescence and electron microscopy. These studies demonstrated that the cell cultures express most of the structural features of native shark rectal gland cells, including numerous mitochondria, complex tight junctions and extensive membrane folding. The cultured cells using the perfusion technique adopted an extremely flattened morphology when grown on collagen. These cells whether grown in suspension or on collagen, displayed a striking level of vacuole formation, these vacuoles were not associated with transport epithelia.

The rectal gland cell cultures were then used to investigate the effect increasing extracellular sodium chloride concentraton has on rectal gland cells Na,K-ATPase activity. Increasing sodium chloride concentration in the growth medium by 50 % (240 mM to 360 mM) resulted in a transient 3-4 fold increase in Na,K-ATPase activity in cell homogenates approximately 12 hours after the medium change. The response was dependent upon both sodium and chloride ions and was also inhibited by the loop diuretic bumetanide (0.1 mM within 30 minutes), indicating that entry of the ions into the cell is via the Na,K,Cl cotransporter. Incubation of cells in normal medium in the presence of the sodium ionophore monensin also resulted in a dose dependant sustained increase in Na,K-ATPase activity following a 12 hour incubation. The increase in Na,K-ATPase activity associated with increased extracellular sodium chloride concentration was only seen in cells grown on collagen and not in cells grown in suspension.

Increases in activity are sensitive to the protein synthesis inhibitor cycloheximide (10 μg/ml), but not the transcriptional inhibitor actinomycin D suggesting that up-regulation of the Na,K-ATPase occurs at the level of translational regulation. Unfortunately this result could not be confirmed using Northern analysis due to unforeseen difficulties in extracting sufficient RNA from the cell cultures.

Addition of bumetanide (0.1 mM) to cells grown in normal medium caused a rapid but reversible down-regulation (by 70 %) of basal Na,K-ATPase activity within 30 minutes. The anti-microtubular agent colchicine (0.1 μg/ml) inhibited the bumetanide induced down-regulation of Na,K-ATPase and also the recovery of activity following bumetanide removal.

The rectal gland cell cultures were used to investigate potential hormonal regulators of the shark rectal gland. The effect of the putative regulators of sodium chloride secretion scyliorhinin II and sCNP on intracellular concentrations of cAMP and cGMP was investigated. The cell cultures were shown be hormonally active as they responded with an increase in intracelllular cAMP concentration to forskolin, $PGE_1$ and $PGE_2$. When scyliorhinin II (10 μM) and IBMX (1 mM) was perfused through the isolated rectal gland a 2 fold increase in cAMP concentration was found in the perfusate after 8 minutes, however no increase was seen in cAMP levels when cell cultures were treated with scyliorhinin II. Shark CNP increased cGMP concentrations in the perfusates of the perfused rectal gland by up to four fold after seven minutes but there was no consistant effect on cGMP concentrations in the cultured cell monolayer. In conclusion it is believed that sCNP and scyliorhinin II mediate their actions on the regulation of sodium chloride secretion by the rectal gland at the vascular level, controlling the extent of perfusion of the gland.

This study showed that high salt levels in the medium of shark rectal gland cell monolayers increased the measurable Na,K-ATPase activity and that this response was dependent on protein synthesis but not transcription. It also showed that the response is inhibited by the loop diuretic bumetanide, indicating that entry of the ions into the cell is via the Na,K,Cl cotransporter and that the increase in Na,K-ATPase activity is presumably due to an increase in intracellular sodium concentration. The hormones sCNP and scyliorhinin II appear to mediate their actions on the regulation of sodium chloride secretion by the rectal gland at the vascular level controlling the extent of perfusion of the gland. In conclusion although sodium chloride transport in the dogfish rectal gland requires much more investigation, this study has hopefully proved that dogfish epithelial cell cultures provide a good model for further investigations involving the regulation of activity and expression of the sodium pump.

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## Abbreviations

| | |
|---|---|
| 1αOH-B | 1 α hydroxycorticosterone |
| AC | Adenylate cyclase |
| ACE | Angiotensin converting enzyme |
| ADH | Atrial natriuretic peptide |
| BNP | B-type natriuretic peptide |
| CNP | C-type natriuretic peptide |
| sCNP | Shark C-type natriuretic peptide |
| Ang I | Angiotensin I |
| Ang II | Angiotensin II |
| Ang II | Angiotensin III |
| cAMP | Cyclic adenosine monophosphate |
| AMP | Adenosine monophosphate |
| ADP | Adenosine diphosphate |
| ATP | Adenosine triphosphate |
| Ca | Calcium |
| CFTR | Cystic fibrosis transmembrane conductance regulator |
| sCFTR | Shark cystic fibrosis transmembrane conductance regulator |
| Cl | Chloride |
| DAG | Diacylglycerol |
| DNA | Deoxyribonucleic acid |
| GC | Guanylate cyclase |
| cGMP | Cyclic guanosine monophosphate |
| GMP | Guanosine monophosphate |
| GDP | Guanosine diphosphate |
| GTP | Guanosine triphosphate |
| EDTA | Ethylenediamine tetra-acetic acid |
| EGTA | Ethyleneglycol-bis-(β-aminoethylether)N,N,N-tetraacetic acid |
| $IP_3$ | Inosito, 1.4.5-triphosphate |
| IBMX | Isobutyl methyl xanthine |
| K | Potassium |
| MDCK | Madin-Darby canine kidney |
| NaCl | Sodium chloride |
| Na, K, Cl cotransporter | Sodium potassium chloride cotransporter |
| Na,K-ATPase | Sodium potassium adenosine triphosphatase |

| | |
|---|---|
| NBD | Nucleotide binding domain |
| NP | Natriuretic peptide |
| NPY | Neuro-peptide Y |
| ORCC | Outwardly rectifying chloride channel |
| $P_i$ | Inorganic phosphate |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PP1 | Phosphatase 1 |
| RAS | Renin angiotensin system |
| RIA | Radioimmuno assay |
| RNA | Ribonucleic acid |
| mRNA | Messenger ribonucleic acid |
| rRNA | Ribosomal ribonucleic acid |
| tRNA | Transfer ribonucleic acid |
| RTG-2 | Rainbow trout gonads-2 |
| TMAO | Trimethylamine oxide |
| Scy II | Scyliorhinin II |
| SR | Shark ringer |
| UI | Urotensin I |
| UII | Urotensin II |
| VIP | Vasoactive intestinal peptide |

# General Introduction

Elasmobranchs can be separated into two distinct groups; the selachii, which include sharks and dogfish; and the batodei, which include skates and rays. This study is concerned with the role of the rectal gland in the regulation of salt secretion in the European dogfish (*Scyliorhinus canicula).* In contrast to the teleosts, marine elasmobranchs eliminate the osmotic challenge presented as a result of the high osmolality of seawater by maintaining their plasma slightly hyperosmotic to the surrounding medium (Smith, 1936). This is mainly achieved by the retention of urea and TMAO to maintain the high plasma osmolality. Even although the plasma is iso-osmotic there is still a salt gradient present between the fish and its surrounding environment.

Plasma levels of sodium and chloride in elasmobranchs are typically between 280 and 300 mmol / l whereas seawater sodium and chloride levels are in excess of 450 mmol/l. Therefore the chemical gradient favours the influx of sodium and chloride ions across semi-permeable barriers (e.g. skin and gills) from seawater. However the greatest salt loads occur during feeding when the ionic content of the food (principally soft bodied invertebrates) plus the inevitable intake of seawater adds to the salt influx and results in a grossly increased absorption of sodium and chloride ions (Shuttelworth, 1988). Typically like many members of the elasmobranch family, *Scyliorhinus canicula* tend to gorge food taking in large quantities of mainly marine invertabrates over a short period of time followed by periods of starvation. Therefore the salt load associated with feeding is intermittent. *Scyliorhinus canicula* must therefore regulate salt and water balance in the face of intermittent acute sodium and chloride challenges associated with large changes in dietary intake (Shuttleworth and Thomson, 1980). To enable these fish to adapt to such situations they have evolved a highly specialised salt secreting organ known as the rectal gland. This organ excretes an isosmotic solution almost entirely composed of sodium chloride (Shuttleworth, 1988). MacKenzie (1996) has previously demonstrated that the activity of Na,K-ATPase (the only active ion transporter involved in rectal gland sodium chloride secretion) is increased 44-fold, 9-12 hours following a feeding episode. The aim of this study was to investigate ionic and hormonal factors associated with rectal gland regulation, following a feeding episode. Experiments were based on the isolation and establishment of cell cultures consisting mainly of secretory epithelial cells, which are responsible for the

translocation of sodium and chloride from the plasma into the external environment. These cells are the major cell type present in the dogfish rectal gland.

## 1.I SALT SECRETING EPITHELIA

Epithelia are sheets of cells that line body cavities such as the lumen of the intestine, the kidney tubules and the salivary glands. The differentiated phenotype of epithelial cells enables structural and functional specialisations of the plasma membrane which enable these cells to transport ions and fluids across the cell layer (Madara *et al.* 1992). Active vectorial sodium transport by epithelia is employed by many organisms inhabiting different environments as a method for controlling salt and water balance (Matlin and Valentich, 1989). This ion balance is a crucial requirement for the body to maintain internal fluid homeostasis. The active transport of sodium across the apical and basolateral cell surfaces establishes ion and electrical gradients across these cellular membranes and across the epithelia itself. The energy stored by these electrochemical gradients can be used to induce the secondary active transport of other ions and metabolites such as $Cl^-$, $HCO_3$, $K^+$, glucose and amino acids (Valentich, 1991). The formation of chemical gradients can also result in the production of osmotic gradients across the epithelia resulting in the net absorption or secretion of water which may be essential for survival in the face of a particular environmental demand e.g. allowing fish to be able to adapt to life in seawater. Active epithelial sodium transport is a central aspect of osmoregulation, an essential process which allows animals to inhabit environments of varying salinities and still be able to maintain body salt and water homeostasis. Different organisms have developed different methods for transporting sodium between internal and external environments and hence are able to maintain body salt and water balance e.g. mammalian kidney, the avian salt gland, the teleost chloride cell, and the elasmobranch rectal gland.

### 1.I a The mammalian kidney

The mammalian kidney consists of approximately 2 million nephrons which act as filtration units for blood plasma to remove waste products and retain important ions and substances essential for normal physiology (Valtin, 1983). Filtration begins at the glomerulus, a tight ball of capillaries which exhibit selective filtration properties. Reabsorption of essential substances filtered occurs in different regions of the nephron with the proximal tubule being quantitatively of most importance. Proximal tubule reabsorption is an obligatory process with between 70-90 % of reabsorption of

essential substances occurring in this region by a variety of both active and passive transport pathways (Brenner *et al.*, 1987).

Following the late proximal tubule and passing into the inner medulla, the loops of Henle, composed of the thin descending and thin and thick ascending limbs, have varying reabsorptive responsibilities depending upon which part of the loop is considered. Each limb has different permeability characteristics, allowing for selective reabsorption of solutes or water. The thin descending limb is impermeable to sodium and chloride but allows water to pass through into the interstitium allowing the concentration of solutes within the filtrate. On the other hand, the thin and thick ascending limbs are impermeable to water and permeable to sodium chloride (Brenner *et al.*, 1987).

A portion of the thick ascending limb lies in the outer medulla and a portion in the cortex. The medullar portion of the thick ascending limb functions to produce a maximal hypertonic environment by actively transporting sodium into the interstitium. Similarly, the cortical portion of the thick ascending limb functions to dilute the tubule fluid by actively transporting sodium into the interstitium via Na,K-ATPase. The thick ascending limb is characterised by a Na,K, Cl cotransporter located in the apical membrane and Na,K-ATPase located in the basolateral membrane. The active transport of sodium out of the cell into the interstitial fluid by way of the Na,K-ATPase creates a concentration gradient for sodium entry into the cell via the Na,K,Cl cotransporter. A potassium channel located in the apical membrane also acts to decrease the intracellular potassium concentration gradient by allowing potassium to diffuse back into the lumen. Chloride moves into the cell drawn by the sodium concentration gradient, leaving the lumen positively charged. This lumen positive charge functions to drive calcium, and magnesium through paracellular pathways into the interstitial fluid, leaving a very dilute fluid in the lumen (Brenner *et al.*, 1987).

The distal convoluted tubule follows the thick ascending limb as it re-enters the cortex of the kidney. Acting in much the same way as the proximal tubule, the distal convoluted tubule passively reabsorbs sodium across the luminal membrane because of the electrochemical potential gradient and then actively passes the sodium across the basolateral membrane by way of Na,K-ATPase. Chloride can follow passively. Water reabsorption depends mostly on the presence of anti-diuretic hormone (ADH) which stimulates production of cyclic AMP in the epithelial cells of late distal tubules, leading to the appearance, in the luminal membranes of these segments, of proteins that

function as water channels. Accordingly, in the presence of a high plasma concentration of ADH, the water permeability of the late distal tubules is very great and water reabsorption is maximal (Vander *et al.* 1989).

The end of the filtrate journey terminates after the collecting ducts. The collecting ducts which originate in the cortex, pass through the outer and inner medulla and end in the renal pelvis, where they empty into the urethra. Sodium absorption (1.5%) occurs in the outer and inner medulla by the same mechanism as in the distal convoluted tubule and proximal tubule. Sodium passively crosses the apical membrane because of an electrochemical gradient and is actively transported into the peritubular fluid by the action of the Na,K-ATPase located in the basolateral membrane. Chloride is again passively reabsorbed by the Na,K,Cl cotransporter (Brenner *et al.,* 1987). Water reabsorption is again sensitive to the presence of ADH and occurs up the osmotic gradient created by countercurrent multiplication (Brenner *et al.* 1987). The countercurrent multiplier system is the result of different transport permeablility in the loops of Henle. These loops, like the collecting ducts, extend into the medulla. The fluid in a loop flows first in one direction down the descending loop and then in the opposite direction up the ascending loop. The ascending loop actively transports both sodium and chloride and is itself relatively impermeable to water. In contrast, the descending limb does not pump sodium chloride and is highly permeable to water. Therefore, there is a net diffusion of water out of the descending limb into the more concentrated interstital fluid until the osmolarities inside this limb and in the interstitial fluid are again equal (Brenner *et al.,* 1987).

## 1.I b The avian salt gland

Avian renal tissue has mammalian-like nephrons (Schmidt-Nielsen *et al.* 1958 ) and the presence of this type of nephron in birds would lead one to expect the capability of production of concentrated urine. Birds do produce such a fluid, but cannot concentrate their urine to the same degree as mammals (Schmidt-Nielsen *et al.* 1958 ). This is perhaps the principle reason why marine birds, with a high dietary sodium intake, require a separate organ specifically designed to excrete excess sodium chloride with minimal water loss. The avian nasal salt gland is an example of a highly specialised extra-renal gland designed for this purpose (Schmidt-Nielsen *et al.* 1958; 1979). The avian salt glands are located on the top of the skull just above the orbit of each eye. These paired glands connect through a duct to the nasal cavity and contain lobules filled with secretory tubules which surround a central excretory canal. The

peripheral cells are located at the terminal end of the secretory tubule, away from the central excretory canal. These cells are small in size and function to secrete an isotonic solution into the lumen of the secretory tubule (Ellis *et al.* 1977). As the solution passes down the lumen, water is selectively reabsorbed down osmotic gradients through larger cells known as principal cells. The principal cells join in a manner that creates the luminal canal. The reabsorption of water results in the secretion of a concentrated salt solution. Likewise all estuarine and a few arid dwelling reptilian species have developed a salt gland specific in the secretion of sodium chloride (Schmidt-Nielsen *et al.* 1958).

## 1.I c The teleost chloride cell

Fish gills are semi-permeable membranes covering a large surface area maximising gaseous exchange, via the pavement cells, and are the most obvious site for extra-renal transfer of ions and fluid between the plasma and external environment. The "chloride" cells in teleost gills are large cuboidal cells which are responsible for active salt secretion in seawater (Forskett *et al.* 1982). Morphologically, the chloride cell has a characteristic apical crypt which comes in contact with the external environment. Just below the plasma membrane of the apical crypt is a thin layer of cytoplasm which contains microtubules, microfilaments, polyribosomes and various small vesicles. Some of these vesicles appear to be fused with the apical crypt membrane (Hootman *et al.* 1979). Evidence suggests the apical crypts develop during adaptation to seawater (Copeland, 1950: Philpott and Copeland, 1963), and it is thought that they play an important role in sodium chloride secretion. The chloride cell transports sodium and chloride from the internal to the external environments via various ion transport pathways including the basolateral Na,K-ATPase and the basolateral Na, K, Cl cotransporter (Forskett *et al.* 1982). The rate of NaCl excretion varies with the osmolality of the surrounding environment (Forskett *et al.* 1982). Teleosts are able therefore to maintain salt and water balance via controlled excretion of NaCl from the branchial epithelium. Hormones such as cortisol, thyroid hormones or the growth hormone have a hypoosmoregulatory effect which dilutes the body fluids when the fish move from fresh to sea water, essentially by accelerating ion excretion through the gill chloride cells (Hourdry, 1995). Cortisol acts by increasing the number of chloride cells in the teleost gill and produces additional chemical energy following an increase in the activity of Na,K-ATPase (McCormick *et al.* 1991). The thyroid hormone T3 may act by a similar method as cortisol or may exert its effect by upregulating the interrenal gland, sensitising it to ACTH, hence increasing plasma cortisol levels (Leloup and

Lebel, 1993). The mechanism of growth hormone action has not yet been defined although it has been discovered that growth hormone can upregulate the enzymatic deiodination of T4 into T3 the active thyroid hormone in teleost (MacLatchy and Eales, 1992). Prolactin on the other hand has a hyperosmoregulatory effect than enables migration from sea to fresh water. Prolactin limits osmotic water intake through the gills and digestive epithelium. It also increases the sodium retention capacity by inducing a more intense sodium and chloride ion intake by the chloride cells (Hourdry, 1995). The chloride cells in gill secretory epithelium of teleost in conjunction with the teleost endocrine system therefore enables these fish to maintain salt and water hemeostasis in environments of different osmolarities.

## 1.II OSMOREGULATION BY ELASMOBRANCHS

There are two major groups of marine fish, the teleosts and the elasmobranchs. Both groups have different osmo-regulatory strategies although they face broadly similar problems of ionic balance. The marine teleost retains a relatively low osmotic concentration of body fluids with respect to the external environment and have developed as "hypo-osmotic regulators." The elasmobranch copes with the high osmolality of seawater by elevating the osmotic concentration of their body fluids so as to become iso-osmotic, or slightly hyper-osmotic, to their marine environment. This elevation of body fluid osmotic concentration is mainly achieved by accumulation and retention of certain organic nitrogenous compounds such as urea and trimethylamine oxide (TMAO). For elasmobranchs, the basic problems of hydro-mineral regulation therefore centres on the retention of urea and related compounds and the elimination of excess inorganic ions. Although the elasmobranchs are iso-osmotic conformers the fish still has to cope with a large inwardly directed sodium chloride gradient. This situation favours passive diffusion of sodium and chloride into the fish from the seawater. However the major source of salt loading for the fish is due to ingestion of sodium chloride in association with feeding (MacKenzie, 1996). Elimination of excess sodium and chloride ions is achieved using the salt-secreting rectal gland, which specialises in excreting excess sodium chloride from the fish (Shuttleworth, 1988). This gland provides an extremely rich source of the enzyme Na,K-ATPase and has proved to be a good model system for studying transepithelial ion transport in general and the secondary active transport of chloride (Greger, 1996).

## 1.II a Urea and Trimethylamine oxide (TMAO)

The retention of urea and TMAO in the body fluids of elasmobranchs essentially solves the immediate osmotic problem experienced by these animals in the marine environment, however the retention of these nitrogenous compounds in the blood and tissues of elasmobranch fish does raise certain questions including how are such high concentrations of urea and TMAO maintained in the dogfish and what are the biochemical and physiological consequences of their presence? The urea gradient existing across the gills of marine elasmobranch represents one of the largest chemical gradients known in the animal kingdom. The gills are the major site of urea efflux from the animal, with renal losses representing only 4-5% of the total urea excreted, reflecting the extremely effective reabsorption of urea by the kidney tubule (Shuttleworth, 1988). Urea levels are replenished as it is a metabolite of the elsamobranch kidney (Shuttleworth, 1988). Rates of TMAO excretion from the dogfish reported by Goldstein and Palatt are similar to those of urea, however during prolonged starvation, plasma levels of TMAO are maintained by release from the large TMAO pool present in the muscle, supplemented by active reabsorption in the kidney tubule (Shuttleworth, 1988).

As urea is known to denature proteins, it might be expected that the presence of such high concentrations of urea in the body fluids would have severe consequences for metabolic processes in the animal, especially as the evidence suggests that most elasmobranch enzymes are no less sensitive to urea than those of other vertebrates (Shuttleworth, 1988). However the simultaneous accumulation of TMAO and other methylamines act as general protein stabilisers counteracting the adverse effects of urea (Yancey and Somero, 1980). However, this does not appear to always be the case, as some elasmobranch proteins have been shown to be urea-insensitive, and others actually require the presence of urea in order to demonstrate normal properties or activities (Shuttleworth, 1988).

## 1.II b Salt loading

The elasmobranch has a relatively high whole body diffusional permeability to water compared to marine teleosts; 3.5-6 $\times 10^{-5}$ cm $s^{-1}$ compared to 1-2$\times 10^{-5}$ cm $s^{-1}$ (Shuttelworth, 1988). This together with the fact that the body fluids are slightly hyper-osmotic to the environment, results in an uptake of water which, with that produced

metabolically, is sufficient to balance that lost in the urine and in the fluid secreted by the rectal gland (Shuttleworth, 1988). As such, and in marked contrast to marine teleosts, an osmotic requirement for ingestion of the seawater is absent and indeed Payan and Maetz (1973) reported that drinking was negligible in *Scyliorhinus canicula.*, although more reasontly Armour, 1990 reported that there is a very low intermittent drinking process present in elasmobranchi. Although drinking in elasmobranchs is not a continuous or regular process, a large volume of seawater is ingested when the elasmobranch is feeding.

Although only limited amounts of salt is taken up as a result of ions diffusing across the permeable parts of the elasmobranchs body surface, eg gills, the fish is presented with a large salt load via the gut following feeding (MacKenzie, 1996). The elasmobranchi often have spasmodic feeding patterns, going for prolonged periods before eating and therefore this produces a highly irregular and intermittent salt load to the fish, mainly caused by the ingestion of sea water when eating but also from the salt present in the food itself (Anderson, 1995). Mechanisms must therefore be in place to maintain body salt homeostasis in the face of both types of salt loading. Evidence suggests that ion excretion can take place at three principal sites - the kidney, the gills and the rectal gland. The gills, however, are the site of a significant net ion uptake and do not contribute to the overall elimination of the excess salt gained from the environment or the diet (Shuttleworth, 1988). Hence, the only routes available to the fish for the net excretion of excess ions are the kidney and rectal gland. The kidney of elasmobranchs is unable to produce a urine with ion concentrations greater than those of the plasma (Shuttleworth, 1988), whilst the fluid secreted by the rectal gland contains sodium and chloride at concentrations almost twice those of the plasma and is therefore is the major organ involved in the reduction of plasma sodium chloride concentrations.

## 1.II c The Rectal Gland

The earliest reference to the existence of the rectal gland is probably that in Severini's "Zootomia Democritae" in 1645. Since then the gland has been extensively studied both in terms of morphology and function and it is now recognised as an excellent model for studying epithelial chloride transport. The rectal gland is finger shaped, lying in the dorsal mesentery, opening into the dorsal surface of the rectum immediately anterior to the cloaca, in the region of the pelvic girdle. The fluid excreted from the rectal gland is colourless, nearly neutral in pH and contains relatively small amounts of

urea, magnesium, calcium, potassium, bicarbonate and sulphate, but contains sodium chloride at about twice their plasma concentration.

Morphological examination shows that the rectal gland consists largely of simple and branched, blind-ended secretory tubules that radiate from a central duct (Bulger, 1966). These tubules are composed principally of densely packed epithelial cells whose only known function is the concentration and secretion of sodium chloride into the tubular lumen. A cross section of the rectal gland demonstrates a central lumen surrounded by three concentric layers:- an outer fibro-muscular layer with small arteries, a middle glandular layer consisting of tubules and capillaries, and a central region, consisting of ducts and veins arranged around the central canal that terminates in the drainage duct to the rectum. The central duct presumably serves as a simple excretory conduit and is lined by a stratified epithelium some four to six cells thick. The simple columnar cells of the secretory tubules exhibit extensive basolateral plasma membrane interdigitations and a rich population of mitochondria (Burger, 1965). The extensive infoldings of the basolateral plasma membrane places a high density of the solely basolaterally located Na,K-ATPase in close contact with ATP-producing mitochondria. This cellular architecture is the structural basis for generating the inwardly directed transmembrane sodium gradient that provides the driving force to support the exceptionally high rates of secondary active chloride secretion by shark rectal gland epithelial cells (Valentich *et al.* 1996). At the apical pole, the rectal gland secretory cells interlock at regions containing tight junctions. This results in an extensive increase in the linear distance of the tight junction (Valentich *et al.* 1996).

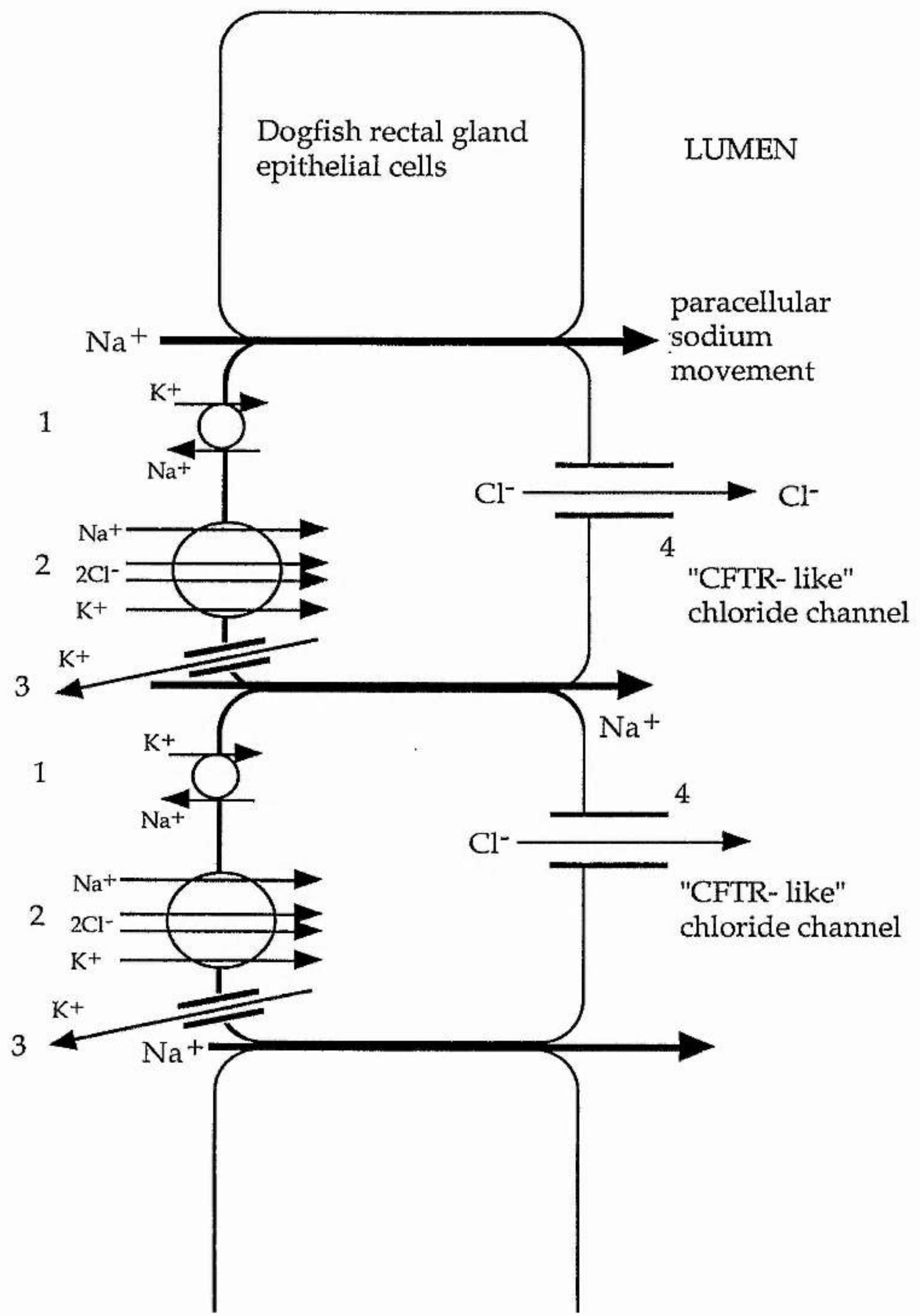
The rectal gland is a highly vascular gland and the main rectal artery arises from the dorsal aorta (Anderson, 1995). The artery enters the gland and branches to form a plexus of smaller vessels in the gland. The arterial system appears to be mainly peripheral. The venous system is mainly central although a few small branches can be seen externally at the proximal end of the gland (Valentich, 1991). The finest ramifications of the central component of the venous system are seen to join with other vessels and become larger and fewer in number until they ultimately converge to form a large central venous sinus or rectal vein which lies in the central wall of the central canal just beneath the transitional epithelium. The blood, therefore flows in the same direction as the excreted fluid and no counter current exchange mechanism is possible (Bulger, 1966).

## 1.III RECTAL GLAND EPITHELIAL CELLS

The shark rectal gland epithelial cells couple translocation of a least three different ions using several transporters and channels to accomplish the secretion of salt from the blood to sea water. Transport of the sodium chloride is thought be associated with prior elevation of adenosine 3'-5' cyclic mono phosphate (cAMP) which activates protein kinase A which subsequently phosphorylates and activates the apical chloride channel which is homologous to the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel expressed in mammals (Marshall *et al.* 1991). This activation allows the Cl to exit the luminal side of the cell down its chemical gradient. The resulting transient reduction in the intracellular Cl concentration is thought to activate the basolateral Na,K,Cl co-transporter which results in the inward flux of all three ions from the serosal side of the cell. The immediate energy supply for the overall process is maintained by the active transport of $Na^+$ back out of the cell across the basolateral membrane by the Na,K-ATPase. The excess K that has entered the cell via the co-transporter exits on the basolateral side through K channels. The Cl which is transported into the cell by the co-transporter restores the intracellular Cl concentration to above its electrochemical equilibrium with respect to the luminal surface so that exit through the CFTR channel can continue if its activation persists and the channel remains open. This transcellular chloride movement contributes substantially to the lumen-negative transepithelial potential that provides the driving force for sodium secretion through a paracellular pathway from blood to lumen (Silva *et al.* 1983). The net effect is $Cl^-$ transport through the cell via the integrated action of the cotransporter and chloride channel and passive Na diffusion between the cells via so called leaky "tight" junctions (Fig 1.1). The apparent paradox is that sodium chloride is actively moved in a vectorial fashion across the epithelium from blood to lumen without primary active transport of either $Na^+$ or $Cl^-$ in that direction. In fact the active $Na^+$ transport step is in the opposite direction across the basolateral membrane and $Cl^-$ moves by secondary active transport across the basolateral membrane, coupled to the $Na^+$ gradient by the cotransport and by passive transport across the apical membrane via the shark CFTR (sCFTR). The rate-limiting and primary regulatory step resides at the "CFTR-like" $Cl^-$ channel, which is optimally designed to respond to hormones employing cAMP as a second messenger (Valentich *et al.* 1996). The Na,K-ATPase maintains basic monovalent cation homeostasis in virtually all vertebrate cells and is normally basolaterally located in epithelia (Podevin and Parini 1989). It is recognised as an extremely important protein in the maintenance of normal transcellular ionic gradients

Figure 1.1

Figure 1.1 Schematic representation of dogfish rectal gland epithelial cells in the wall of the secretory tubules of the shark rectal gland. The principal molecules responsible for transcellular chloride secretion are indicated: The sodium pump (1), the Na,K,Cl cotransporter (2) and the potassium channel (3) on the basolateral surface and the chloride channel (4) on the apical surface.



BLOOD
Dogfish rectal gland
epithelial cells
LUMEN
Na+
paracellular
sodium
movement
K+
1
Na+
Cl-
Cl-
Na+
2
2Cl-
K+
4
"CFTR- like"
chloride channel
K+
3
Na+
K+
1
Na+
4
Cl-
Na+
2
2Cl-
K+
"CFTR- like"
chloride channel
K+
3
Na+

and ionic homeostasis and consequently it has been extensively studied in the dogfish rectal gland. In contrast, the K channel, which is also essential to rectal gland sodium chloride secretion, has not been characterised beyond its sensitivity to inhibition by Ba, which also blocks secretion (Silva *et al.* 1983). The CFTR chloride channel was first noted in humans 7 years ago and, because it is the site of the defect in cystic fibrosis, has been extensively studied since then (Riordan *et al.* 1989: Riordan, 1994). The Na, K, Cl, co-transporter has been characterised to some extent (Xu *et al.* 1994), but is still less well understood than the CFTR chloride channel. The functional characteristics of the sCFTR, the Na,K,Cl cotransporter and the Na,K-ATPase are described in the following section.

## 1.III a CFTR

Chloride channels play a pivotal role in salt and water transport in a number of epithelia including the lungs in mammals and other higher vertebrates (Marshall *et al.* 1991), as well as those in various exocrine and osmoregulatory glands. The CFTR chloride channels reside in the apical membrane of many transporting epithelia (Fig 1.1) and a functional compromise of this channel is thought to be involved in cystic fibrosis, a disease characterised by a fundamental defect in Cl permeability principally in the lung but also in other epithelial tissues (Riordan, 1994). Cystic fibrosis is caused by mutations in the gene encoding the protein termed CFTR. Studies have shown that expression of CFTR in transfected cells generates a cAMP-stimulated Cl conductance (Anderson *et al.* 1991, Kartner *et al.* 1991) and that mutation of charged residues (most predominant mutation Δ F508) in CFTR alters anion selectivity (Anderson *et al.* 1991). It is now well known that the CFTR gene encodes a large integral membrane protein which functions as a cAMP-regulated chloride channel (Bear *et al.* 1992).

There is now considerable biochemical and biophysical information about CFTR in mammalian tissues and derivative cell lines and in cells transfected with the CFTR coding sequence. Although regulated expression of CFTR mRNA has been reported in some tissues during growth and differentiation in several cell lines (Montrose-Rafizadeh *et al.* 1991, Morris *et al.* 1992, Sood *et al.* 1992, Trezise *et al.* 1992, Trezise *et al.* 1993), there is little information about whether the abundance of CFTR protein can be modulated under physiological conditions.

Chloride channels in the apical plasma membrane of cells in the dogfish rectal gland have been extensively studied (Sullivan *et al.* 1991, Hanrahan *et al.* 1993). A cDNA

clone for sCFTR was isolated from libraries constructed using RNA isolated from the rectal gland of *Squalus acanthias* (Grzelczak *et al.* 1990. Marshall *et al.* 1991) shortly after the cloning of the human CFTR gene (Riordan *et al.* 1991). Overall amino acid sequence identity between human CFTR and elasmobranch sCFTR is 72% (Marshall, 1991). Extensive heterologous expression and in vitro mutagenesis studies (Welsh and Smith. 1993), in addition to the purification and reconstitution of human CFTR (Bear *et al.* 1992), has subsequently provided convincing evidence that the protein constitutes a finely regulated low- conductance (5-10pS) chloride channel. Hence, in many epithelial tissues that are associated with cAMP-stimulated $Cl^-$ secretions such as mammalian intestine, CFTR is the major apical conduit as well as the main regulatory element for the secretory process. In the case of sCFTR, it has more recently been possible to assemble a stable full-length cDNA and to express this construct in an number of heterologous systems including insect somatic cells and *Xenopus oocytes* (Hanrahan *et al.* 1993). The sCFTR chloride channel exhibits biophysical and pharmacological characteristics similar to human CFTR. Hanrahan, *et al* 1993 demonstrated that expression of sCFTR in *Xenopus oocytes* produced a chloride channel with conductance of 4-5 pS. This channel had a linear current to voltage relationship and was stimulated by cAMP. It was therefore assumed that the sCFTR chloride channel was responsible for the cAMP-stimulated chloride secretion in the dogfish rectal gland as this secretion was time and voltage independent as was the sCFTR current.

The cAMP-stimulated chloride secretion in the rectal gland was therefore concluded to be due to the sCFTR channel, although there is also a 40 pS chloride channel present in the rectal gland cells which also is cAMP dependent. This 40 pS chloride channel however is voltage dependent and is not always stimulated during sodium chloride secretion by the rectal gland (Greger *et al.* 1985; 1987). The current observed during cAMP stimulated chloride secretion in rectal gland primary cultures has the same characteristics as those observed in *Xenopus oocytes* expressing sCFTR. There is also a 11 pS chloride channel present in the rectal gland epithelial cell, however this channel is not regulated by cAMP and is active in both non-transporting and transporting glands; it may account for the low chloride conductance observed when the gland is not stimulated by cAMP (Gogelein *et al.* 1987). The pronounced differences found between the apical chloride channels found in the shark rectal gland suggest that their concurrent presence in the same membrane may serve different physiological needs within the cell.

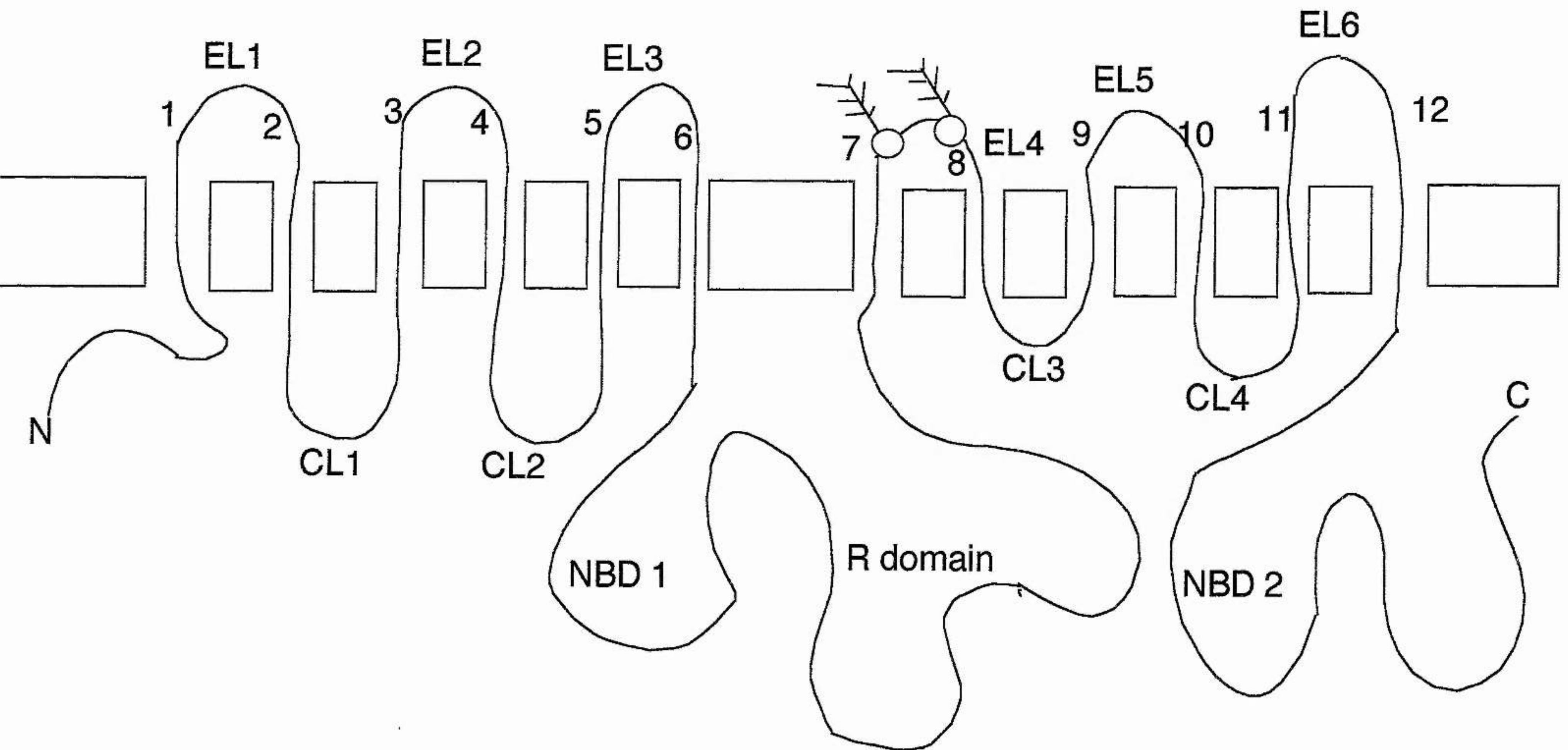
The best known regulator of the sCFTR is cAMP. Elevation of intracellular cAMP levels by the use of the adenylate cyclase activator forskolin rapidly causes dramatic and sustained alterations in the electrophysiological properties of shark rectal gland cells (Valentich and Forest, 1991, Yancey *et al.* 1991). These changes are characterised by depolarisation of apical membrane electrical potential difference ($V_a$) and basolateral membrane electrical potential difference ($V_b$), a large fall in fractional resistance of the apical membrane ($fR_a$) and a small increase in transepithelial voltage ($V_{ab}$) (Moran and Valentich, 1993). These alterations in membrane properties are with increased electrogenic Cl secretion. Following forskolin stimulation, superfusing the apical surface of shark rectal gland cells with low-$Cl^-$ shark ringer causes abrupt depolarisation of $V_a$ and an increase in $fR_a$, both of which are indicative of a cAMP-activated apical membrane Cl conductance ($G_a^{Cl}$) (Moran and Valentich 1993). In the absence of forskolin, low-Cl shark ringer has little effect on either $V_a$ or $fR_a$, because $G_a^{Cl}$ is very small. The large decrease in $fR_a$ from 0.97 to 0.1 in response to forskolin shows that the apical plasma membrane undergoes a major change in Cl permeability following cell activation.

The response of cultured shark rectal gland cells to Ca ionophores suggests that rises in intracellular Ca also activate $G_a^{Cl}$ and stimulate Cl secretion. The changes in $V_a$ and $fR_a$ induced when ionomycin (a calcium ionophore) was added to the apical superfusate are very similar to those observed in forskolin-stimulated cells, indicating that ionomycin and therefore presumably intracellular calcium concentration activates $G_a^{Cl}$ (Moran and Valentich 1993). One mechanism to account for the massive increase in $G_a^{Cl}$ in cAMP- or Ca- activated shark rectal gland cells involves secretagogue-stimulated exocytotic delivery to this barrier of Cl channels that previously were sequestered in cytoplasmic vesicles. The large number of vesicles in the cytoplasm near the apical surface of shark rectal gland cells supports this idea (Valentich *et al.* 1991). In addition, data from impedance analysis of cultured shark rectal gland cells are consistent with an increase in apical plasma membrane capacitance following forskolin stimulation (Kushman *et al.* 1992). Such a capacitance change is accountable by an increase in the area of the apical plasma membrane as would be expected following vesicular exocytosis (Valentich *et al.* 1991).

A schematic representation of the sCFTR molecule is shown if Fig 1.2. This two dimensional depiction was originally based primarily on hydropathy analyses (Riordan *et al.* 1989). The CFTR protein is composed of two repeats of six transmembrane spanning domains (repeat 1: M1-6 and repeat 2: M7-12). Between these repeats are

Figure 1.2

Figure 1.2 Topological model of CFTR indicating twelve putative transmembrane (TM) spanning helices, six extracytoplasmic loops (EL), four cytoplasmic loops (CL), two nuceotide binding domains (NBD 1 and NBD 2) and the R domain. Circles are two glycosylation sites.


EL1
EL2
EL3
EL4
EL5
EL6
1
2
3
4
5
6
7
8
9
10
11
12
N
C
CL1
CL2
CL3
CL4
NBD 1
R domain
NBD 2

interposed the first of two, nucleotide binding domains (NBD1) and a large hydrophilic central domain (R domain) containing several consensus sites for phosphorylation. The second nucleotide binding domain (NBD2) is at the carboxy end of the protein following the second transmembrane domain repeat (Fong and Jentsch. 1994) This topological disposition with respect to the lipid bilayer has been verified experimentally (Chang *et al.* 1994) and there is increasing evidence that several of the twelve transmembrane sequences contribute to the ion pore (Anderson *et al.* 1991, Tabcharani *et al.* 1993). The M2 and M6 comprise at least part of a central ion pore structure that could also include the M1 domain (Anderson *et al.*1991b). The work of Oblatt-Montal and colleagues (1994) supports this theory; mixtures of synthetic peptides with sequences corresponding to M2 and M6, but not M1, M3, M4 or M5, produce anion selective channels when introduced into lipid bilayers.

Regulation of the channel activity is extremely complex (Tabcharani *et al.* 1991). Channel activation is dependent on ATP not only as a substrate for the protein kinases that phosphorylate and modulate CFTR, but also as a direct modulator or cofactor which can regulate activity by binding (in a hydrolysed or non hydrolyysed form) to the NBDs (Baukrowits *et al.* 1994). Cyclic AMP induced activation of the $Cl^-$ secretion in the rectal gland is thought to occur as a result of phosphorylation of the R domain by protein kinase A (PKA) and inactivation of secretion is associated with dephosphorylation of the channel (Cheng *et al.* 1991). Some insight is beginning to be gained into the mechanism whereby phosphorylation of multiple sites in the R domain results in the activation of the channel (Dulhanty and Riordan, 1994 a,b). The current theory is that the R domain occludes the ion pore, and that phosphorylation of the R domain by cAMP-dependent PKA induces a conformation change which de-occludes the channel and allows chloride to enter the transmembrane pore. The R domain contains nine consensus sites for phosphorylation by PKA, and at least seven serine residues have been identified as potential phosphate acceptors. These sites are serines 660, 700, 712, 737, 768, 795 and 813. Since each one of these sites is part of a consensus PKA recognition sequence it suggests that cAMP acts to regulate CFTR (Cl channels) via PKA rather than by direct binding of cAMP to the channel itself (Cheng *et al.* 1991, Berger *et al.* 1991). Phosphorylation-dependent regulation of the CFTR $Cl^-$ channel is degenerate, (i.e. more than one site is normally involved in channel activation although not all sites need to be phosphorylated as no one site is essential) (Rich *et al.* 1991 and Riordan *et al.* 1994). Selective mutagenesis of individual potential phosphorylation sites suggests that phosphorylation of serine 660, may be inhibitory to activation (Riordan *et al.* 1994), hence, the mechanism of regulation by

PKA might be more complex than initially thought and may involve other as yet unidentified, regulatory sites. In addition, the R domain has been shown to include a number of potential phosphorylation sites for protein kinase C (PKC) (Cheng *et al.* 1991) and has been shown to be phosphorylated by PKC in vitro (Picciotto *et al.* 1992). However any possible role of PKC phosphorylation in the activation of CFTR is less clear than that of PKA (Tabcharani *et al.*1991), although it is thought that phosphorylation of serines 684 and 790 by PKC might potentiate the activation seen with PKA (Riordan, 1993). The additional layer of regulation arising from ATP interacting with the NBDs becomes most apparent in mutated CFTR variants where the R domain is either removed (Rich *et al.* 1991) or PKA phosphorylation sites are converted to residues which are already negatively charged i.e. aspartate or glutamate (Riordan *et al.* 1994) thus activating the channel. Hydrolysis of ATP when bound to NBD 1 potentiates the opening of the chloride channel, while hydrolysis of ATP bound to NBD 2 leads to the closure of the chloride channel.

## 1.III b The Na,K,Cl-Cotransporter

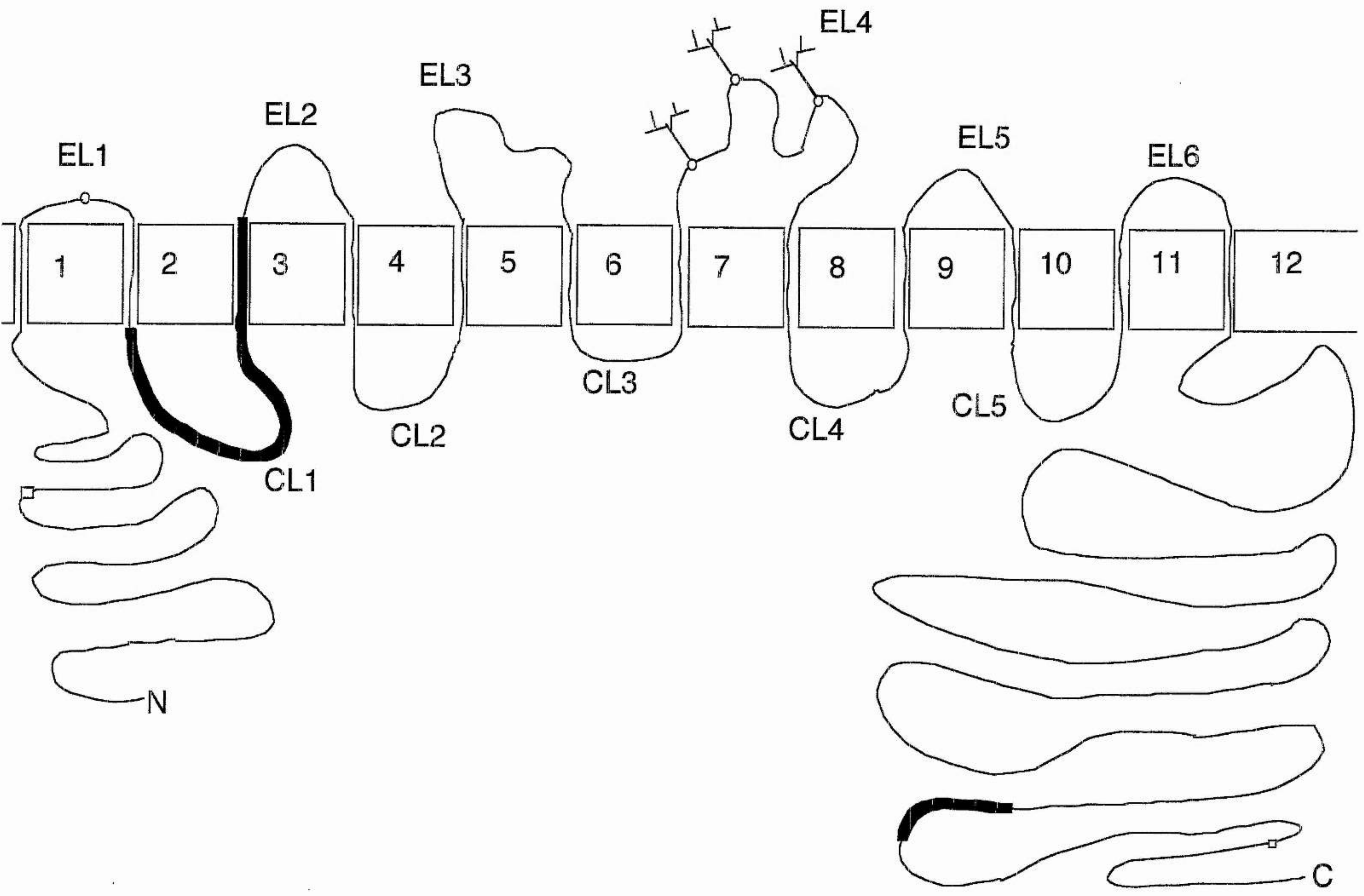
The Na,K,Cl-cotransporter operates in conjunction with the sodium pump, a K channel and a Cl channel to carry out transepithelial salt movement. There are numerous examples where these proteins act together to move sodium and chloride across epithelial barriers including the thick ascending limb of Henle's loop in the mammalian kidney, distal tubule of amphibian and mammalian kidney, the Necturus gallbladder, the shark rectal gland, the rabbit parotid gland and the flounder intestine (Haas, 1989). In other cells, including Ehrlich ascites tumour cells and red blood cells, the Na,K,Cl-cotransporter plays a role in the maintenance and regulation of cell volume (Haas, 1989). In excitable cells, such as squid axon and cardiac myocytes, this co-transporter regulates the ion gradients that determine the resting membrane potential (Haas, 1989).

The Na,K,Cl-cotransporter directly mediates the electrically neutral transport of the named ions across the membrane, demonstrating a stoichiometry of 1 $Na^+$, 1 $K^+$, and 2 $Cl^-$ (Haas, 1989). The exception to this is the squid axon, where there is translocation of positive charge due to a 2 $Na^+$, 1$K^+$ and 2$Cl^-$ stoichiometry which has been shown in influx and efflux directions (Altamirano *et al.* 1987). The direction of transport depends on the resulting gradients of all three ions across the cell membrane, and in the case of the squid axon the magnitude and polarity of the membrane potential.

A property of all Na, K, Cl co-transport systems is the inhibition of the transporter by loop diuretics, including furosemide and bumetanide (Palfrey *et al.* 1984). Bumetanide, which in most tissues half-maximally ($IC_{50}$) inhibits the cotransporter at concentrations ranging from 0.005-5 μM (species dependent), has become the inhibitor of choice for most studies of Na, K, Cl-cotransport. The co-transporter in the basolateral membrane of shark rectal gland epithelium requires 5 μM bumetanide for 50% inhibition, and 100 μM for complete inhibition (Palfrey *et al.* 1984). Even at such relatively high concentrations bumetanide is still specific for the Na, K, Cl co-transporter in shark rectal gland cells (Haas, 1989). Bumetanide binding to the co-transporter requires the simultaneous presence of $Na^+$, $K^+$, and $Cl^-$ in the medium. Removal of any one of these ions markedly reduces binding of diuretic compounds (Haas, 1989). Furthermore, the stimulatory effects of $Na^+$ and $K^+$ on diuretic binding appear to reflect binding of these ions to their respective sites on the co-transporter (Haas, 1989). Although low concentrations of chloride are required for diuretic binding, high concentrations of $Cl^-$ inhibit this binding, consistent with ion flux studies that suggest competition between loop diuretics and $Cl^-$ for a common site (Haas, 1989). These observations suggest that the loop diuretics preferably bind to a co-transporter where the $Na^+$ and $K^+$ ions and one of the $Cl^-$ ions have already bound and the loop diuretic would then compete for the second $Cl^-$ site (Haas, 1989). Properties of the Na,K,Cl cotransport protein discerned largely from its primary structure (Fig1.3). The predicted 1191 residue polypeptide has features consistent with its identity as an integral membrane glycoprotein. Analysis of the sequence using hydropathy algorithms suggests that there may be 12 membrane spanning sequences, as in CFTR. These are bracketed by long hydrophilic sequences at the N and C termini which, on the basis of the presence of verified phosphorylation sites could be cytoplasmically oriented. This topological disposition would provide five cytoplasmic and six extracytoplasmic loops separating the transmembrane segments. Of the total of nine consensus sequences for N glycosylation, one is present in the first and three in the fourth extracytoplasmic loop. The two positions which are phosphorylated in the secretory tubules in vivo are at residues T189 and T1114 in the N and C terminal hydrophilic domains respectively are indicated in Figure 1.3. There are ten complete putative sequences of homologous Na, K, Cl-cotransport proteins currently available from human, rabbit, rodent, shark, winter flounder, hawk moth, worm and yeast sources. They comprise 1002-1212 amino acid residues in length with varying degrees of homology (Table 1, Park and Saier, 1996).

Figure 1.3

Figure 1.3 Topological model of Na,K,Cl cotransporter indicating twelve putative transmembrane helices, six extracytoplasmic loops (EL), five cytoplasmic loops (CL) and large N- and C- terminal cytoplasmic domains. Thickened lines indicate regions of increased sequence similarity with the thiazide-sensitive Na,K cotransporter. Squares indicate biochemically confirmed phosphorylation sites.


EL1
EL2
EL3
EL4
EL5
EL6
1
2
3
4
5
6
7
8
9
10
11
12
CL1
CL2
CL3
CL4
CL5
N
C

| Source | Name | Size (amino acids) | % ID |
|---|---|---|---|
| Rat | Bumetanide sensitive Na,K,Cl co-transporter | 1095 | 100 |
| Rabbit | Bumetanide sensitive Na,K,Cl co-transporter | 1099 | 94 |
| Human | Bumetanide sensitive Na,K,Cl co-transporter | 1212 | 77 |
| Mouse | Bumetanide sensitive Na,K,Cl co-transporter | 1205 | 77 |
| Shark (*Squalus acanthias*) | Bumetanide sensitive Na,K,Cl co-transporter | 1191 | 73 |
| Winter flounder | Thiazide sensitive NaCl co-transporter | 1023 | 62 |
| Rat | Thiazide sensitive Na,K,Cl co-transporter | 1002 | 60 |
| Hawk moth | Bumetanide sensitive Na,K,Cl co-transporter | 1060 | 48 |
| Yeast | Hypothetical protein 124 KD | 1120 | 31 |
| Worm | Hypothetical protein KO2 A2.3 gene product | 1010 | 21 |

Table 1:- Protein members of the Na, K, Cl cotransporter family. % ID is the percentage homology for each of the proteins compared with rat Na,K,Cl cotransporter.

The shark rectal gland epithelial cells are among the richest known sources of the Na, K, Cl co-transporter (Haas, 1989). In the shark rectal gland secretory cell, co-transporter activity appears to be regulated by the level of intracellular $Cl^-$ through a process involving direct phosphorylation of the transporter protein, which is associated with increases in cAMP (Haas, 1989). Recent evidence suggests that the Na, K, Cl co-transport protein is regulated by a feedback control mechanism that sense and prevent increases or decreases in cytoplasmic Cl concentration ($[Cl]_i$) (Haas *et al.* 1993). How a change in $[Cl]_i$ is perceived and translated into a corrective Na,K,$Cl^-$cotransport flux is unknown. However in the rectal gland the protein appears to be regulated by reactions that control its state of phosphorylation. It is known that the rectal gland co-

transporter acquires phosphate at serine and threonine residues when activated by cAMP-dependent (forskolin) and cAMP-independent (cell shrinkage) stimuli (Lytle *et al.* 1992 and 1996). It has been hypothesised that when the epithelial cell is dormant and not involved in secretion, the cotransporter is maintained in an inactive, dephosphorylated state by an enzyme that has similar characteristics to protein phosphatase type 1 (PP1). Such protein phosphatases are potently inhibited by calyculin A but not okadaic acid (Alimirano *et al.* 1988). Inhibition of the shark rectal gland phosphatase by calyculin A produces a rapid conversion of the co-transport protein to its phosphorylated and activated state and secretion is activated (Alimirano *et al.* 1988). Hence, as in other cells, a type 1 phosphatase (PP1) appears to function continuously in the rectal gland cell to counteract a cAMP-regulatable kinase.

Activation of the rectal gland cotransporter can be brought about by treatments which are also known to elevate cAMP concentrations, such as application of vasoactive intestinal peptide (VIP) (Forbush *et al.* 1992). Such treatment can promote Na,K,Cl-cotransporter activity by as much as 15 fold. In addition VIP also stimulates maximal bumetanide binding 15 to 20 fold (Forbush *et al.* 1992). As said previously the cotransporter has been shown to be phosphorylated when activated by cAMP indicating that cAMP-dependent PKA is involved (Lytle *et al.* 1996). In several cell types cAMP elicits an increase in the number of specific bumetanide binding sites (Greger *et al.* 1984), an event that may reflect an increase in the number of functional cotransporters in the plasma membrane. Recently reported biochemical characterisation of the co-transporter protein in shark rectal gland and in avian erythrocytes indicates that activation of sodium chloride cotransport indeed correlates with the state of phosphorylation of the co-transporter protein, although whether the co-transporter itself is the substrate for the action of cAMP-dependent protein kinase remains to be established (Lytle *et al* 1992, Matthews *et al* 1993). In certain cases there is evidence that the cotransporter can be functionally regulated as a result of dynamic changes in F-actin which is known to associate with the transporter (Matthews *et al.* 1994). It has been suggested that the cytoskeleton provides a means for transducing volume regulatory signals to membrane bound co-transporter proteins (Matthews *et al.* 1994). Whether actin filaments directly associate with the co-transporter and influence its function has not yet been determined, however the cytoplasmic aspect of the plasma membrane of all eukaryotic cells is lined by a cortical meshwork consisting not only of actin filaments but also a number of actin associated proteins (Matthews *et al.* 1993). Dynamic changes in sub-membranous actin polymerisation may thus alter the kinetics and equilibrium of a number of biochemical reactions involving actin associated

proteins and various membrane proteins linked to the cytoskeleton. Several examples of ion transporting proteins that are regulated by the cytoskeleton have now been identified e.g. the Na, K-ATPase of renal epithelia (Matthews *et al.* 1993).

## 1.III c The Na,K-ATPase

In animal cells, the major ion-motive ATPase is the ouabain-inhibitable sodium pump or Na,K-ATPase, a membrane-bound enzyme which couples the free energy retained within the ATP molecule to the translocation of $Na^+$ and $K^+$ across the plasma membrane. The enzyme consists of a $\alpha\beta$ heterodimer which constitutes the minimal functional unit able to hydrolyse ATP (Skou *et al.* 1992). The Na,K-ATPase is one of the major users of cellular energy, responsible for up to 40% of the steady state energy consumption in certain cells (Skou *et al.* 1992). Three $Na^+$ ions leave the cell in exchange for two $K^+$ ions per molecule of ATP hydrolysed. The transport ratio of 3 $Na^+$ : 2 $K^+$ generates a potential across the membrane, positive to the outside. In the normal cell with its high permeability to $K^+$, the pumping of $Na^+$ and $K^+$ only adds a few millivolts to the resting membrane potential (Skou *et al.* 1992).

### 1.III c i Kinetic studies of the Na,K-ATPase

The Na,K-ATPase has two major conformations, denoted the $E_1$ and $E_2$ forms (Skou *et al.* 1992). In the $E_2$-form it is assumed that 2 potassium ions are bound to the Na,K-ATPase as two potassium ions are transported by the Na,K-ATPase. When in the $E_1$-form it is assumed that there are three sodium ions bound to the Na,K-ATPase as the enzyme transports three sodium ions. The rate of transition from $E_1$ conformation state (where sodium is bound to the enzyme) to $E_2$ conformation state (where potassium is bound to the enzyme) is relatively quick (300 $s^{-1}$) at 22°C, while the rate of transition from $E_2$ conformation state (where potassium is bound to the enzyme) to $E_1$ conformation state (where sodium is bound to the enzyme) is much slower (0.3 $s^{-1}$). However both rates are influenced by the concentration of cations both intra and extracellular and are also dependent upon the presence of ATP (Skou *et al.* 1992). The binding of these substrates involves conformational changes exposing ion binding sites to the intracellular and extracellular environments at different stages of the cycle. In the $E_1$ conformation state the protein exchanges cytoplasmic potassium for cytoplasmic sodium (Fig 1.4), once the sodium ions bind to the $E_1$ conformation state the Na,K-ATPase is phosphorylated by magnesium ATP (Jorgensen 1982). The sodium pump then changes to its $E_2$ conformation state where the protein then exchanges the bound

sodium ions for extracellular potassium ions in the extracellular medium (Fig 1.4) (Jorgensen 1982). In $E_2$ conformation state when potassium is bound, potassium ions are occluded and binding of ATP at the low affinity site accelerates release of enzyme bound potassium to the external medium (Jorgensen 1982). Magnesium ions are required for phosphorylation of the enzyme (the enzyme goes through a phosphorylated state as part of catalysis). Magnesium ions are also required for conversion between the phosphorylated $E_1$ conformation state and the phosphorylated $E_2$ conformation state, and for dephosphorylation (Jorgensen 1982).

### 1.III c ii The α-subunit of the Na,K-ATPase

The Na,K–ATPase enzyme is a complex of two polypeptides, α and β , and a number of lipid molecules incorporated into the lipid bilayer of the plasma membrane. The α-subunit contains the binding sites for sodium, potassium and ATP and for the specific inhibitor ouabain (a cardiac glycoside) (Ewart, *et al*, 1995). The α-subunit is an integral membrane protein that transverses the plasma membrane from 6 to 10 times, according to different models (Ewart, et al, 1995). The location of the N-terminal of the α-subunit is on the intracellular side of the membrane, and recent studies suggest that the C-terminus is also on the intracellular side of the membrane (Skou, *et al*, 1992). The first α-subunit to be cloned was a cDNA from the sheep kidney (Shull *et al.* 1985). The α-subunit cloned from the sheep kidney contained 1012 amino acids (Skou, *et al*, 1992). Since then, cDNAs encoding three tissue-specific isoforms of α, designated $\alpha_1$, $\alpha_2$, and $\alpha_3$ have been cloned from rat and other species (Pressley, 1992). Patterns of genomic DNA hybridisation indicate that the α-subunits are the product of different genes rather than the result of alternative mRNA splicing (Pressley, 1992). The $\alpha_1$-transcript is ubiquitous to all tissues and is typically 3.7 kb in size. In rat brain, skeletal muscle, and vascular smooth muscle two $\alpha_2$ transcripts of 5.3 kb and 3.4 kb have been identified which arise from differential polyadenylation sites and in the rat fetal heart the $\alpha_2$ transcript is 4.5 kb in size (Pressley, 1992). The rat $\alpha_3$ gene yields a transcript of 4.5 kb in most tissues and 6 kb in fetal brain, adult brain and heart (Ewart, *et al*, 1995).

The existence of different isoforms of the α-subunit has suggested that they may either possess distinct kinetic property or may be differentially regulated by factors including hormones. In rodents, but not in primates, the $\alpha_1$ isoform has a much lower sensitivity to the cardiac glycoside ouabain than either the $\alpha_2$ or $\alpha_3$ isoforms (Bertorello *et al.* 1993). Transfection of specific subunits into Hela cells has led to the

determination of the specific affinities for ions of each isoform. The $\alpha_1$ and $\alpha_2$ subunits have a similar apparent affinity for extracellular potassium which is 2.5 times lower than that of $\alpha_3$ (Pressley, 1992). The $\alpha_1$ and $\alpha_2$ subunits also have a similar affinity for intracellular Na which is about three times higher than that of $\alpha_3$ (Ewart, *et al,* 1995). These different kinetic properties of the $\alpha$ isoforms suggest that the expression of more than one $\alpha$-subunit isoform within a given cell, as occurs in muscle, heart and nerve cells, is not a redundant feature and may provide a basis for fine tuning of pump activity in these tissues (Ewart, *et al,* 1995).

Amino acid residues of the Na,K-ATPase $\alpha$-subunit involved in cation and ouabain binding have been studied using site directed mutagenesis and by expression of the altered proteins in cells. The Ser 775 residue has been shown to be associated with the $K^+$ binding site, but not the $Na^+$ binding site (Lingrel *et al.* 1996). Upon substitution of all the negatively charged transmembrane residues, only two charges appear to be absolutely required for cation translocation, Asp 804 and Asp 808 (Lingrel *et al.* 1996). Ouabain binding to intact cells expressing these mutants suggests that normal amounts of enzyme are produced and inserted into the membrane. It appears likely that these residues are involved in potassium binding and possibly sodium binding during translocation (Lingrel *et al.* 1996). Substitutions of several amino acids alter ouabain sensitivity and interestingly these residues fall into two regions, the H1-H2 and H5-H6 domains (where H denotes transmembrane spanning regions). Since Ser 775, Asp 804 and Asp 808 are located in the H5-H6 domain, it has been suggested that binding of ouabain to this region is the basis of the inhibition of Na,K-ATPase by the cardiac glycosides. The drug interactions may restrain the movement of the H5-H6 domain which is thought to be highly flexible during the changes in conformation associated with the binding and/or release of substrates (Lingrel *et al.* 1996). It has also been suggested that the extracellular charged residues in the first extracellular loop connecting transmembrane H1 and H2 regions are also implicated with ouabain binding (Yamamoto, *et al,* 1996). Recent sequence analysis and site-directed mutagenesis have indicated that the Cys at 106 in the human $\alpha$-subunit or at 104 in the sheep $\alpha$-subunit (both in the H1-H2 domain) play critical roles in determining the affinity for ouabain (Yamamoto, *et al,* 1996). The cation binding sites have not yet been identified, but sodium seems to be related to the structure of a 19-kD tryptic fragment having an amino-terminal end at residue 831 (Ewart, *et al,* 1995). It also has been suggested that the ouabain binding site is allosterically linked to a site involved in $K^+$ binding or

transport, or alternatively, it is possible that the Cys (106/104) substitution alters this conformation in such a way that $K^+$ binding is also affected (Yamamoto, *et al*, 1996).

### 1.III c iii The β-subunit of the Na,K-ATPase

The β-subunit has one membrane-spanning segment near the intracellular N-terminal of the protein, and the extracellular C-terminus is highly glycosylated. The degree of glycosylation is tissue specific. The apparent molecular mass of the β-subunit, calculated from mobility in sodium dodecyl sulphate polyacrylamide gel electrophoresis, ranges from 42 to 55 kDa when fully glycosylated, but its protein core corresponds to only 32 kDa (Ewart, *et al*, 1995). Three isoforms of the β-subunit designated $\beta_1$, $\beta_2$ and $\beta_3$ have been identified, with the $\beta_1$-subunit being widely distributed, occurring in most cell types. The $\beta_2$ subunit had been cloned from rat and mouse brain (Pressley, 1992) and a $\beta_3$ isoform has been cloned from the amphibian nervous system. Variable sizes of $\beta_1$ and $\beta_3$ gene transcripts are found, which arise from multiple initiation sites whereas only one transcript of 3.4 kb size is found for $\beta_2$. Interactions between the β-subunit and the α-subunit appear to include the extracellular domain of the β polypeptide and the extracellular loop between the putative transmembrane domains H-7 and H-8 of the α-subunit. This association between α and β subunits is necessary for efficient assembly of functional pumps in the golgi and their translocation and expression on the cell surface (Beguin, *et al*, 1996). Several studies have provided evidence that by associating with newly synthesised α-subunits the β-subunit facilitates the formation of the active αβ complex and regulates the level of enzyme transported to the plasma membrane (Proverbio *et al.* 1991). A role in the stability of the complex and in $K^+$ binding has also been proposed for the β-subunit (Ewart, *et al*, 1995). The $\beta_2$ isoform has an additional function as an adhesion molecule in glial cells, where it is found tightly bound to the $\alpha_2$ subunit (Bertorello, *et al*, 1993). Therefore its role as and adhesion molecule might be associated with the Na,K pump function. It has been suggested that the $\beta_2$ subunit could interact with receptors on adjacent cells restricting the pump units to specific regions of the plasma membrane resulting in a polarised distribution (Proverbio *et al.* 1991).

### 1.III c iv Regulation of Na,K-ATPase activity

Various hormones have been implicated in sodium pump regulation, both over short and long term periods. These effectors produce alterations in pump activity by either

changing sodium pump turnover, by changing the sodium pumps affinity for substrates or by changing its abundance at the cell surface either as a result of translocation of preformed units from an intracellular storage compartment or by increasing transcription and translation of new sodium pumps. Hormones which are know to increase Na,K-ATPase activity include aldosterone, insulin, thyroid hormone, corticosteroides and catecholamines (Bertorello *et al.* 1993, Beguin *et al.* 1996 and Ewart *et al.* 1995). Hormonal regulation of the sodium pump will be discussed in more detail in section 3.I., however regulation of the Na,K-ATPase via phosphorylation will be discussed in this section. Current evidence suggests that the sodium pump can be phosphorylated by protein kinases but there is controversy as to whether this phosphorylation alters enzyme activity as some workers have reported increases, whereas others either find no change or decreases in activitiy following phosphorylation (Feschenko and Sweadner 1996, Blanco *et al.* 1996, Borin 1996 and Aperia *et al.* 1996). Possible functional roles for phosphorylation of the $\alpha$ subunit of Na,K-ATPase by protein kinase A (PKA) and protein kinase C (PKC), has been evaluated in the rat using site-directed mutagenesis of potential regulatory sites. Phosphorylation of Na,K-ATPase by PKC have been reported to occur at two sites near the N-terminus of the rat $\alpha_1$ subunit: Ser 11 and Ser 18 (Feschenko and Sweadner 1996). Serine 18 is the principal site phosphorylated by PKC in the rat $\alpha_1$ subunit in L6 cell cultures, this is absent in both $\alpha_2$ and $\alpha_3$ sequences. Phosphorylation of the $\alpha_1$ subunit by PKC however was reported not to affect Na,K-ATPase activity (Feschenko and Sweadner 1996). Contrary to these results Aperia and coworkers (Apreria *et al.* 1996), have reported that phosphorylation of the rat $\alpha$ 1 subunit by PKC in COS cells resulted in the inhibition of Na,K-ATPase activity. Increasing this controversy, Borin (1996) has reported that PKC activates sodium pump activity in vascular smooth muscle cells from rat aorta, whereas cAMP dependent protein kinases (PKA) inhibit the activity.

Rapid changes in Na,K-ATPase activity can be brought about by sudden variations in substrate concentrations (mainly sodium) (Bertorello *et al.* 1993). Under normal steady-state conditions, the Na,K-ATPase is not saturated by the prevailing intracellular sodium concentrations (Beguin *et al.* 1996). Therefore any increase in intracellular sodium will drive its own extrusion simply by providing more substrate for the unsaturated pump (Beguin *et al.* 1996). This change in Na,K-ATPase activity in response to variations in intracellular sodium is not strictly speaking, a regulatory response, but it has also been reported that the availability of the intracellular sodium

can regulate activity of the Na,K-ATPase via transcription and turnover responses in the long term (Bertorello *et al.* 1993).

## 1.III d The basolateral potassium channel

Under physiological conditions, the basolateral potassium channel in rectal gland epithelial cells allows for potassium recycling from the cell to the peritubular space. It is required for the mechanism of sodium chloride secretion as described above in section 1.III. The $K^+$ selective channel has a unitary conductance of approximately 20 pS under normal ionic conditions (Greger *et al.* 1987). This channel permits potassium efflux when a force is created by sodium chloride transport. Single channel kinetic analysis reveals burst appearance with only one open time-constant of the order of 3 ms (Greger *et al.* 1987). In this respect, the channel is comparable to the $K^+$-channel in the basolateral membrane of the human proximal tubule (Greger *et al.* 1987). Microelectrode studies indicate that there is only one type of $K^+$ channel present in the basolateral membrane of the rectal gland epithelium, and that these $K^+$ channels are demonstrable in both secreting (hormonally stimulated) and in resting cells (Greger *et al.* 1987). Specifically, this channel functions as an outward conductor under physiological conditions.

The kinetic appearance of this channel in the shark rectal gland, with burst phases interrupted by flickering to the closed state and with long interburst pauses (Greger *et al.* 1987), is similar to that observed in $K^+$ channels of other tissues (Coloquhoun *et al.* 1985). Bursting behaviour, interrupted by long gaps between bursts, can be explained by binding of agents to the channel protein, leading to activation of the channel. Such agent can be neurotransmitters, as in the case of acetylcholine-activated channels, or it can be $Ca^{2+}$ ions as seen in $Ca^{2+}$ dependent $K^+$ channels (Greger *et al.* 1987). The $K^+$ channel described here is probably not $Ca^{2+}$ activated as reduction of the calcium activity on the cytosolic side to less than $10^{-9}$ M has no detectable inhibitory effect (Greger *et al.* 1987). Conversely, increases in calcium activity to some $10^{-3}$ M reduced the activity of the channel (Greger *et al.* 1987). Factors which activate and regulate this channel are unknown at present, although it is known that the channel is barium sensitive and natriuretic peptides (NP) may inactivate the channel by an unknown mechanism (Valentich *et al.* 1991)

In conclusion, there is a potassium-selective channel in the basolateral membrane of the rectal gland cell. This channel has a permeability to $K^+$ of the order of $0.25 \times 10^{-12}$

$cm^3$ /s and it permits $K^+$ outward currents. It is highly selective for $K^+$ over $Na^+$ and is barium sensitive.

The complete description of this overall cellular symport system that accomplishes sodium chloride secretion requires not only a characterisation of each of the transporter and channels involved, but also of how their activities are coordinately regulated.

## 1.IV REGULATION OF SHARK RECTAL GLAND ACTIVITY

In the rectal gland it appears that important relationships exist between secretory activity and the rate of blood flow to the gland, secretory activity and volume regulation and secretory activity and salt loading to the fish. It seems probable that hormones and neurotransmitters may co-ordinate the interactions between these different factors to regulate secretory activity.

Biochemical analyses of secretagogue-stimulated second messenger formation and addition of exogenous mediators to solutions bathing shark rectal gland cell monolayers demonstrate that cAMP, cGMP and diacylglycerol (DAG) are important intracellular messengers involved in activating chloride secretion (Valentich, 1991).

### 1.IV a Hormonal regulation of the shark rectal gland

#### 1.IV a i sCNP

The natriuretic peptides (NPs) are known to have vasodilatory effects and have been implicated as having an important role in salt and fluid metabolism in many organisms (Karnaky *et al.* 1991). Atrial natriuretic peptide (ANP) has a stimulatory effect on sodium chloride secretion from the isolated perfused rectal gland of the spiny dogfish however the mode of action for this is unknown (Karnaky *et al.* 1991). It was also found that mammalian ANP causes an increase in intracellular cGMP concentrations and stimulates sodium chloride secretion in freshly isolated and cultured shark rectal gland tubular epithelial cells (Karnaky *et al.* 1991; Lear *et al.* 1990). ANP has however also been associated with an increase in intracellular inositol phosphate concentrations, and in addition, PKC inhibitors block ANP activation of the Cl current by 50% without modifying forskolin-stimulated Cl secretion (Valentich *et al.* 1996). Procaine (local anaesthetic) which has well-documented inhibitory effects on Ca-dependent K channels by blocking Ca mobilisation in several cell types, also abolishes the characteristic ANP-

induced Cl current (Valentich *et al.* 1996). These data suggest that ANP activation of chloride secretion in shark rectal gland cells involve a complex interplay between at least two second messenger systems ($IP_3$ and cGMP) (Valentich *et al.* 1996). ANP-activated cGMP formation alone may be incapable of stimulating shark rectal gland Cl secretion. The need to co-ordinate several intracellular signalling events and membrane transporters both temporally and spatially may contribute to the characteristic transient oscillations in Cl current following ANP activation of shark rectal gland cells (Valentich and Forrest, 1996).

Molecular cloning, together with peptide purification and amino acid sequencing show that heart tissue from the spiny dogfish (*Squalus acanthias*) and the European dogfish (*Scyliorhinus canicula* ) contains a C-type natriuretic peptide (sCNP) (Schofield *et al.* 1991). sCNP has a steep dose-response relationship for activation of $Cl^-$ secretion in both the perfused shark rectal gland and shark rectal gland cell cultures, and this seems to be through an increase in intracellular cGMP levels as sCNP potently stimulates shark rectal gland particulate guanylate cyclase (Valentich and Forrest, 1996).

ANP and CNP have been reported to activate both basolateral and apical receptors in shark rectal gland epithelial cells cultures (Valentich *et al.* 1991). This is unusual as the peptide should be unable to gain access to the receptors on the apical side in the native tubule as shark rectal gland tubules are blind ended and there is no known cellular source that could secrete NPs. It has been suggested that shark rectal gland tissue contains NP-like molecules capable of activating Cl secretion through autocrine or paracrine mechanisms, therefore if the epithelial cells are capable of synthesising NPs and secreting them to the luminal surface then this would explain how the apical receptors were activated (Valentich *et al.* 1996). However, as yet, NPs have not been detected in the tubular fluid and the secreted fluid was found to be protein and peptide free (Valentich *et al.* 1996).

### 1.IV a ii VIP

Vasoactive intestinal peptide (VIP), a 28-amino acid polypeptide, is a specific and potent stimulus for rectal gland secretion in *Squalus acanthias* (Stoff *et al.* 1979). VIP increases sodium chloride secretion by increasing cAMP levels in the shark rectal gland epithelial cell (Shuttleworth *et al.* 1984). VIP-stimulated chloride secretion is inhibited by an unknown mechanism by the cyclic tetra deca peptide somatostatin (Stoff *et al.* 1979). Rectal gland cells isolated from the *Squalus acanthias* have been shown to

express high affinity receptors for VIP and binding of the hormone to these sites is rapid and saturable (Silva *et al.* 1987). However, despite the unequivocal presence of VIP receptors and actions on the secretion in the rectal gland of *Squalus acanthias*, VIP even at high concentrations (e.g. $10^{-6}$ M), is completely without an effect in the isolated rectal glands of two other elasmobranchs species, *Scyliorhinus canicula* and *Raja clavata* (Shuttleworth and Thorndyke, 1984). Therefore although VIP may be a regulator of secretory activity in the *Squalus* rectal gland it can not be considered as a general stimulator of secretory activity in the rectal gland of all elasmobranchs.

### 1.IV a iii Scyliorhinin II

Scyliorhinin I and II are two novel tachykinins isolated from the dogfish gut (Conlon *et al.* 1986), which show similar biological activities. Scyliorhinin I has also been isolated from the brain of *Scyliorhinus canicula* (Waugh *et al.* 1993). Scyliorhinin II which is synthesised in the dogfish gut (Conlon *et al.* 1986) exhibits a dose-dependent stimulation of sodium chloride secretion in the perfused rectal gland of *Scyliorhinus canicula* (Anderson, 1995). It has been proposed that scyliorhinin II is the uncharacterised peptide called rectin previously suggested as a gut peptide modulating rectal gland secretion in *Scyliorhinus canicula* (Anderson *et al.* 1995). Rectin is an intestinal peptide factor, the partial sequence of rectin identified is identical to that of scyliorhinin II. Rectin increased oxygen consumption in rectal gland slices of *Scyliorhinus canicula* as does scyliorhinin II (Anderson *et al.* 1995). However there is no current information available on second messenger systems involved in scyliorhinin II- or rectin- stimulated sodium chloride secretion.

### 1.IV a iv Adenosine

Adenosine is a potent autocoid regulator with diverse physiological functions (Epstein *et al.* 1990, Newby, 1984 and Berne, 1986). In the dogfish rectal gland adenosine has two distinct membrane bound adenosine receptors: a stimulatory receptor ($A_2$) with low, micromolar, affinity for adenosine and an inhibitory receptor ($A_1$) with 1000-fold greater affinity (nanomolar) for adenosine. The signal transduction mechanism of the receptors is considered to be through G-protein coupled stimulation (Kelley *et al.* 1990). Adenosine has been shown to stimulate sodium chloride transport in the rectal gland of the *Squalus acanthias* via the $A_2$ receptor (Kelley *et al.* 1990). The $A_2$ receptors principle mechanism of action is to increase intracellular cAMP levels (Kelley *et al.* 1990).

## 1.IV a v Somatostatin

Somatostatin is a cyclic peptide containing 14 amino acids. In mammals somatostatin is found in the hypothalamus, the nervous system, the gut and various endocrine and exocrine glands (Wingard *et al.* 1991). Somatostatin-related peptides are widely distributed in cells throughout the body of elasmobranchs (Silva *et al* 1985). Somatostatin in mammals has been found to inhibit various secretory processes in endocrine glands and epithelial tissues (Silva *et al* 1985). It acts via somatostatin specific receptors that are present in pituritary plasma membranes, brain synaptosome membranes and pancreatic islet cells via the $IP_3$, resulting in a increase in cytosolic calcium, but the mechanisms by which somatostatin exerts its inhibitory effect in elasmobranchs are unclear (Wingard *et al.* 1991). In *Squalus acanthias*, somatostatin has been found to be more potent than adenosine in inhibiting adenylate cyclase activity and cAMP accumulation in the rectal gland and somatostatin is able to inhibit VIP-stimulated chloride secretion (Silva et al 1985). The inhibitory action of somatostatin is by no means confined to the sodium chloride secretion produced by VIP e.g. inhibits adenosine stimulation and effects can be distal as well as proximal to the generation of cAMP (Silva *et al.* 1985). Somatostatin inhibits both adenosine ($A_2$ receptor), and also forskolin, stimulated adenylate cyclase activities in the shark rectal gland (Silva *et al.* 1985) and effectively inhibits rectal gland secretion stimulated by exogenous additions of cAMP analogues i.e. dibutyryl cAMP in combination with a phosphodiesterase inhibitor (Silva *et al.* 1985). The latter results indicate that a major site of inhibition in the rectal gland cell must be downstream from cAMP production (Silva *et al.* 1985).

## 1.IV a vi Renin angiotensin system

Angiotensin II (Ang II) and atrial natriuretic peptide (ANP) are potent extracellular regulators of vertebrate fluid homeostasis (Rang and Dale, 1990). Angiotensin I (Ang I) has been isolated and characterised from the plasma of *Tryakis scyllia* (Takei *et al.* 1993). The renin angiotensin system (RAS) consists of a variety of peptides with Ang II being the principle biological effector and angiotensin III (Ang III) having limited biological activity.

The basic structure of Ang II is highly conserved between species. Ang I is structurally more diverse and the dogfish Ang I is 20 times more potent at raising arterial blood

pressure in *Tryakis scyllia* than rat Ang I (Takei *et al.* 1993). This action was known to be due to Ang II and not Ang I as the ACE inhibitor captopril was found to inhibit the Ang I-induced increase in arterial pressure of *Tryakis scyllia* in vivo, but had no effect on Ang II-induced increase in blood pressure (Takei *et al.* 1993). As the RAS is now known to be present in the elasmobranch it is possible that one or more of these vasoactive peptides is involved in the regulation of rectal gland secretion. In addition renin-like activity has also been found to be present in rectal gland homogenates (Takei *et al.* 1993). Immunohistochemical studies have shown that Ang II-like and ANP-like peptides are mainly localised at the blind ends of the tubules in the subcapsular zone of the rectal gland in close contact with circumferential vessels suggesting that both peptides may have a role in rectal gland regulation (Takei *et al.* 1993). The close localisation and possible interactions between these two peptides in the subcapsular region of the gland is very intriguing. Further investigation of the effect of these peptides in the gland is needed before any a definite statement can be made about their function.

#### 1.IV a vii Steroids

1α-Hydroxycorticosterone (1αOH-B) has also been implicated in the control of rectal gland secretion in elasmobranchs (Holt and Idler,1975). It has been shown that 1αOH-B decreases plasma sodium levels (Armour *et al.* 1993 a, b) and it can increase chloride secretion rate by the rectal gland (Holt and Idler,1975). However no more is known about 1αOH-B and clearly further work is require to determine whether it plays a significant role in the physiological regulation of rectal gland secretion.

#### 1.IV a viii Other factors

Bombesin, which stimulates anion transport in mammalian ileal mucosa, is also present in the tubules of the shark rectal gland (Masini *et al.* 1994), indicating a possible role for this peptide in the secretion of sodium chloride by the rectal gland. Likewise glucagon has also been shown to be localised in the rectal gland capsule and it is thought that the capsule may not be a mere envelope, but it is probably involved, and may actively participate in glandular activity (Masini *et al.* 1994). Glucagon exerts a stimulatory effect on secretion in the isolated perfused rectal gland of *Scyliorhinus canicula* (Anderson, 1995). Neuropeptide Y (NPY) inhibits active chloride secretion in the VIP-stimulated dogfish rectal gland at some point distal to cAMP generation as dibutyryl cAMP stimulate chloride secretion is also inhibited (Forrest *et al.* 1992).

## 1.IV b The vasculature of the dogfish rectal gland

In the elasmobranch rectal gland, low levels of catecholamines, acting via $\alpha$-adrenergic receptors, induce pronounced vasoconstriction of blood vessels reducing flow through the secretory parenchyma of the gland (Shuttleworth, 1988). As this effect occurs within the physiological range of noradrenaline concentrations in the plasma, it is likely that blood flow through the gland is normally restricted by circulating catecholamines (Shuttleworth and Thorndyke, 1984). This vasoconstriction of the rectal glands vascular is, however, abolished by the presence of known stimulators of chloride secretion, such as VIP or adenosine, effectively shifting the normal noradrenaline dose-response curve to the right so that essentially all physiologically significant catecholamine concentrations have no effect (Solomon *et al.* 1984). This action of the secretory hormones on catecholamine induced vasoconstriction is thought to be the basis of an increase in blood flow to the gland associated with stimulation of secretion in vivo (Solomon *et al.* 1984). The evidence suggests that the observed vasoactive activities of the secretory agents, which are independent of their action(s) on the secretory epithelial cells, are mediated via direct effects on vascular smooth muscle, involving increases in intracellular cAMP levels. The vasoconstriction effect of the catecholamines can be reversed by the addition of cAMP and theophylline, providing evidence to support the view that the reversal of the vasoconstrictor effect observed when the gland is stimulated is due to an increase in cAMP (Solomon *et al.* 1984). Adenosine is also able to reverse the vasoconstriction observed in the rectal gland in response to noradrenaline (Shuttleworth, 1988). Adenosine is therefore thought to mediate its vasoactive effect via $A_2$ stimulatory adenosine receptors, which are linked to increases in adenylate cyclase activity and increased intracellular cAMP concentrations. The vascular effects of the secretory agents form an important integral part of the overall physiological control of secretion by the gland in vivo although secretion is not thought to be entirely regulated by the control of blood flow through the gland (Shuttleworth, 1988).

## 1.IV c Volume expansion

The rectal gland of *Squalus acanthias* responds to systemic volume expansion by increasing the rate of fluid secretion several fold (Solomon *et al.* 1984). The increase in fluid secretion by the gland that follows volume expansion is accompanied by a large increase in blood flow through the gland. Neither the mechanism responsible for this

increase in blood flow nor the relation it has to the increase in fluid secretion is known. The vasodiltation that accompanies the secretory response to volume expansion appears to increase the number of active tubules in the rectal gland and therefore increases the overall secretion rate. The vasodilatation also appears to be necessary to supply the gland with the oxygen required for the increase in epithelial transport (Solomon *et al.* 1984). As neither the pressure in the dorsal aorta nor rectal gland arterial pressure is affected by volume expansion (Solomon *et al.* 1984), the increase in rectal gland blood flow that coincides with increases in secretion must occur as a result of changes in vascular resistance within the rectal gland itself. Although the mechanisms responsible for this response are not established, it is known that the volume expansion is accompanied by an increase in cAMP levels in rectal gland extracts, this increase in cAMP may result in vasodilation by the method described above (Solomon *et al.* 1984).

How volume expansion induces glandular secretion is still unknown, whether volume expansion releases a mediator from a storage site in the gland or from another organ such as an endocrine gland or neuronal network remains to be determined. In summary, volume expansion may be involved in physiologically modifying rectal gland secretion, although this is not believed to be the only stimulus involved in regulating secretion of sodium chloride. It seems much more likely after reviewing the literature available, that upon salt loading a hormone is release from the gut (possibly scyliorhinin II) which acts on the rectal gland epithelial and/or smooth muscle cells via receptors to elevate second messenger systems (e.g. cAMP, cGMP or $IP_3$/DAG). Activation of these systems combine to increase blood flow and recruitment of secreting tubules and activation of individual proteins involved in the secretory pathway and hence induce stimulation of salt secretion by the rectal gland.

The first aim of the current study was to establish a primary culture of dogfish rectal gland epithelial cells in order to further investigate the effect of sodium chloride loading on rectal gland activity. It was previously reported in our laboratory that Na,K-ATPase activity in *Schliorhinus canicula* rectal glands increased 44 fold 9-12 hours following a feeding episode with 6% sodium chloride pellets (MacKenzie, 1996). The present study investigated if this effect was a direct sodium chloride effect using the rectal gland cultures. The mechanism by which sodium chloride exerts this effect was then investigated and actions of known activators of rectal gland sodium chloride secretion on various cyclic nucleotide levels were measured.

# Culture of Rectal Gland Epithelial Cells

## 2.I INTRODUCTION

It is only within the last thirty years that experimental approaches to the study of cellular physiology have progressed to the use of tissue dispersion techniques for the isolation and subsequent use of fully differentiated primary cell populations. In some cases these primary cells can be grown or maintained in culture for a number of passages before eventually losing their differentiated characteristics. Prior to the development of these techniques experiments were limited to either studies in the whole animal, or short-term studies either using intact organs or tissues or slices from these tissues. These whole animal studies have provided our basic understanding of system physiology, but this approach was limited because measurements in vivo represented the net effect of many homeostatic changes that occurred usually in many cell types during the experiment. What was needed was a new experimental approach to the study of cellular physiology in which a functionally homogeneous population of cells could be maintained in culture under conditions that would allowed direct manipulation of the environment and the subsequent measurement of the resulting changes in cell function. The development of techniques for the isolation and maintenance of cells has proven to be of major scientific importance in many different fields of study, and it is now universally accepted that in vitro methods are indispensable to the study of many aspects of cellular physiology, biochemistry and pharmacology. The term "tissue culture" is widely used and is not only concerned with the study of cells, but also includes tissues and organs maintained in vitro. It encompasses:- a) organ culture - the growth in vitro of tissues, the whole organ or parts of an organ are maintained in conditions which allow the differentiation and preservation of the original architecture and/or function: b) tissue culture - the growth in vitro of tissue fragments in conditions which does not necessarily preserve the original tissue morphology but heterogeneous and homogeneous cell - cell contacts are maintained; and c) cell culture - the growth in vitro of cells which are usually homogeneous in nature and are no longer organised into tissues (Paul 1970). Such systems allow the study of cellular or tissue processes in a controlled environment, free from the normal whole-body homeostatic mechanisms including hormonal and neuronal influences.

One of the first steps in the direction of tissue culture was taken in 1866 by F.D von Recklinghouse, who kept amphibian blood cells alive in sterile containers under a

variety of environmental conditions for 35 days. The first successful culture of organised tissue was carried out by Roux in 1885 who maintained the neural plate of developing chick embryo in warm saline for several days, and proved that the closure of the neural tube was controlled by the constituent cells and not by structures normally surrounding it in vivo (Parker 1961). In 1903, Jolly performed experiments which marked the first detailed observation of cell survival and cell division in vitro. He maintained leukocytes from the salamander for up to a month. This study was followed three years later in 1906, by a paper by Beebe and Ewing which recorded a genuine attempt at tissue culture. These authors described the cultivation of an infectious canine lymphosarcoma in blood from animals resistant to the infection or in animals susceptible to the infection (Paul, 1970). Since the initial attempts at cell culture, methods have evolved rapidly, with researchers in the field recognising that the establishment of even short-term cultures in vitro required the design of a basic chemically defined synthetic media as a replacement for the normal extracellular fluid in the animal. This media needs to be maintained at the correct pH and osmolality, it also needs the correct concentrations of essential inorganic salts, vitamins, amino-acids and various growth factors. Tissue fragments and cells isolated from many parts of the body have been cultivated, following the development of suitable media. Pioneers such as Harry Eagle, and Richard Ham, led the efforts to provide all the workers of this field with formulations useful for specific cell types or purposes, and other formulations of synthetic media that were useful with a wide variety of cell types.

As these types of studies progressed, attention began to turn toward the problem of the most undefined component used in tissue culture media, namely the animal serum or plasma supplement. For a variety of cells in culture, it was widely accepted that serum or plasma supplements were essential for growth. Serum additions were noted to be growth-stimulatory for some cell types, but what was less obvious was that the serum supplement could be toxic to other cell types or inhibitory to some of the cell functions to be investigated (Barnes *et al.* 1984). In nearly all cases, serum was the source of both growth-promoting and growth-inhibiting substances and, taken as a whole, serum-supplemented media greatly favour proliferation of cells of mesenchymal origin over growth of other cell types (Barnes *et al.* 1984). Most media now consist of the essential inorganic salts, glucose, vitamins, amino-acids and the various growth factors that are essential to growth of a particular cell type. Some cell types have an essential requirement for media supplemented with serum whereas others can grow without serum (Paul, 1970). In most cases antibiotics such as penicillin, streptomycin and/or kanamycin are added to the growth media. Antibiotics provide a convenient

means of killing any bacterial infections that might contaminate the cells, however it should be noted that certain antibiotics can interact with some cell systems and alter specific functions such as signal transduction (Barnes *et al.* 1984).

Other environmental factors also must be taken into consideration when growing cells. For example, the temperature and the culture substrata are both very important for optimal growth of cells (Parker, 1961). One of the characteristics of living cells is that they are rapidly destroyed by temperatures slightly in excess of those at which they best operate. In the case of most mammalian and avian tissues, the optimum temperature is between $37^0$C and $38.5^0$C (Paul, 1970). If the temperature is raised as high as $45^0$C the cells are killed within an hour. Cells from cold blooded animals however can only function at much lower temperatures than this (Paul, 1970). Cells will normally however survive at much lower temperatures than normal. Cells which normally require a temperature of $37^0$C for rapid growth will continue to grow slowly at temperatures of $20^0$C to $25^0$C (Paul, 1970). Cells may even be frozen slowly to $-170^0$C and can be stored for months ( only if glycerol or dimethylsulphoxide is added to the medium as a cryo-protecting agent against the formation of ice crystals within the cytoplasm) (Barnes *et al.* 1984). The surface to which cells attach is also an important consideration when dealing with tissue culture. Most normal cells are anchorage-dependent and require an adequate substrate on which to attach, grow and function. Transformed, tumour-like cells are less dependent on the culture substrate. However, normal anchorage independent cells and tumour cells are often cultured on a fixed substrate as it is difficult to prevent them from attaching to their culture vehicle (Jones, 1990). Cellular shape is determined by the interaction of cells with each other and the underlying substratum. The chemical composition of the substratum can modulate cell morphology and in turn control cellular proliferation and differentiation both in vivo and in vitro (Barnes *et al.* 1984). The extra cellular matrix is the natural substrate upon which mesenchymal and epithelial cells rest and proliferate. The basement membrane is a specialised form of the extracellular matrix that is synthesised by epithelial cells and separates epithelial and mesenchymal cells in a variety of tissues (Matlin and Valentich, 1989). Components associated with the basement membrane include type IV collagen, glycoproteins such as laminin, entactin and fibronectin as well as proteoglycans and structural carbohydrates such as hyaluronic acid (Barnes, *et al,* 1984). Attachment of specific types of cells to different types of collagen is a pre-requisite for their subsequent growth. This attachment is mediated through the interaction of cells with the extracellular matrix by their subsequent recognition of specific collagen types. It is therefore necessary with some cell types to coat the petri dish or flask in which the cells

are going to be introduced with the appropriate collagen matrix (Barnes *et al.* 1984). However all cells do not need the presence of collagen to attach and grow; negatively charged polystyrene is currently the most common plastic used in the manufacture of tissue culture ware and therefore the most common cell culture substrate (Barnes *et al.* 1984). This evolved empirically as a practical substitute for the negatively charged glass substrate that was the initial substrate used in the culture of mammalian cells. In recent years, chemical modifications to result in positively charged substrates for cell culture have been employed for specific applications. Attempts to modify polystyrene chemically to yield a positively charged surface usually results in a loss of optical clarity. However, a simple coating of various tissue culture substrates with basic polymers ( e.g. polylysine) results in improved attachment and cell proliferation rate and still preserves the optical clarity of the polystyrene substrates (Barnes, *et al*,, 1984). Finally, cells require oxygen for respiration so the air space that surrounds the growth medium normally needs to be gassed with 95% air / 5%$CO_2$. The latter component is required to maintain the pH of the media if a bicarbonate buffered growth medium is used.

A culture initiated from cells, tissues or organs taken directly from an organism is termed a "primary culture". Cells freshly isolated from a particular tissue may survive for days or months either dividing or non-dividing and then eventually die, or may divide repeatedly and require sub-culturing or passaging (the removal of cells to a new culture vessel with fresh medium) (Paul, 1970). The culture is then termed a "primary cell line" which may die after several passages or may become an "established cell line", with the apparent potential to be sub-cultured indefinitely. This transition between the primary and established state may occur gradually, or suddenly as a result of a "transformation" which usually can be monitored by the appearance of a few colonies of rapidly-dividing cells which become the predominant cell type (Parker, 1961). The cells often differ from the primary cell line in several ways e.g. abnormal chromosome number, loss of contact inhibition, shorter population doubling times and sometimes a loss of specialised functions (Fong and Jentsch, 1995). Transformation can be either spontaneous or can be induced by the application of agents such as viruses, radiation or carcinogenic chemicals (Barnes *et al.* 1984). In addition, primary cell lines can be established from tumours which already have the behavioural characteristics of an established cell line. Paradoxically, many cell lines established from tumours frequently show better retention of specialised function than those derived from normal tissue (Paul 1970).

They are several different methods for establishing primary cultures. Rous and Jones (1916), described the use of trypsin to digest away the plasma clot in which a tissue culture was grown and showed that the cells could be replated on coverslips after they had been dispersed in this way (Paul, 1970). At that time the method seemed to have little application. However many years later, in 1952, a paper was published by Moscona, as cited in Parker, (1961) in which he described the disaggregation of embryonic limb-bud cells by treating them with trypsin and the sub-culturing of the isolated cell suspensions. In the same year Dulbecco described a method for preparation of replicate cultures of chick embryonic tissues by digestion of the whole embryo with trypsin as cited in Parker (1961). This tissue dispersion technique has now been successfully applied a large number of many embryonic and adult tissues. The composition of the intercellular matrix varies between different tissues with different types and amounts of mucopolysaccharides and fibrous proteins making up the major constituents. In addition to carbohydrate and protein elements, inorganic salts are important matrix components, as in bone. Thus it might be expected that different tissues would require different treatments in order to induce disaggregation. However most tissues can be disaggregated by one of three techniques or a combination of these; physical disruption, enzymatic digestion and treatment with chelating agents. Physical disruption is not often used alone, mainly because it is difficult to obtain uniformed suspension without cell damage. Viable crude suspensions of chick embryonic tissue can be obtained by expressing the embryo through the nozzle of a syringe (Paul, 1970). Also viable suspensions of cells from human breast carcinomas can be obtained by slicing and chopping tissue fragments with a sharp knife to release the cells from the fibrous stroma (Lasfargues, 1957). However, physical disruption is usually combined with enzymatic digestion or chelating agents. The most successful enzymes that may be used to disaggregate tissues are collagenase and trypsin. Depending on the tissue, you may use only one of these enzymes or a combination of them. Trypsin is usually the enzyme of choice for separation of cells in established cultures however for initial isolation of cells collagenase is more effective as some tissues, especially from adult animals, are refractory to trypsin treatment because of their collagen content (Paul,1970). Certain tissues, especially epithelial tissues, seem to require divalent cations, particularly calcium and magnesium, for their integrity. If these ions are removed by substances which bind them (chelating agents) the tissue may be disaggregated very easily. Chelating agents most commonly used are ethylene-diamine-tetra-acetic acid (E.D.T.A.) and ethyleneglycol-bis-amino-ethyl ether N,N-tetra-acetic acid (E.G.T.A.) (Barnes *et al.* 1984). These agents are rarely used alone for tissue dispersion, however

their use in combination with enzymatic digestion is more common. The ways that enzymes and chelating agents are used to form a cell suspension are similar. The two main methods are:- a. the tissue (which is usually sliced or chopped) is placed in a solution containing an enzyme or chelating agent or both and rocked or stirred for various lengths of times before removing the single cells from the solution, or b. perfusion of the tissue with the solution containing the enzyme chelating agent mixture and then mechanically dispersing the tissue to produce a single cell suspension (Paul, 1970). It is also common practice to treat or perfuse the tissue with a chelating agent before the use of an enzyme for further digestion (Paul, 1970).

The preliminary aim of this project was to establish a primary cell line of epithelial cells from the dogfish *(Scyliorhinus canicula )* rectal gland. Epithelia are tissues that line the body and organ surfaces and regulate the exchange of substances with the outside environment and between tissue compartments. Because epithelial cells are present in many different tissues they possess a diverse number of functional properties. However, all epithelial cells share some common characteristics. The cells possess a morphological polarity, i.e. a plasma membrane organised into apical and basolateral domains. Within these surface membrane domains, enzymes, transport proteins, hormone receptors, and lipids are localised in a polarised fashion. Apical membranes often face the external compartment and are composed of membrane proteins with specialised properties related to the cells primary function (e.g. absorption) and the basolateral membrane domain usually faces the internal milieu. The difference in lipid composition of the apical and basolater membranes is responsible for large physiochemical differences between the two membrane domains and influences the function of numerous membrane proteins (Jones, 1990). The establishment and maintenance of apical and basolateral membrane protein and lipid asymmetry is essential for the normal functioning of polarised epithelial cells. For example, sodium reabsorption by renal proximal tubular cells is dependent on the polarised localisation of specific carrier proteins such as the sodium / hydrogen antiporter, the sodium dependent glucose cotransporter and sodium dependent amino acid cotransporter to the apical membrane, and the polarised distribution of Na,K-ATPase to the basolateral membrane (Gumbiner, 1992).

The lateral membrane domains between epithelial cells possess specialised intercellular junctional complexes composed of zonula occludens ( tight junctions), zonula adherens (intermediate junctions), desmosomes, gap junctions and cell adhesion proteins (Cross and Mercer, 1993). The components of the junctional complex have four important

functions:- a. to regulate initial cell to cell recognition and adhesion (cell adhesion proteins): b. to maintain the cohesive structural integrity of the epithelial monolayer (intermediate junctions and desmosomes); c. to allow for intercellular communication (gap junctions); and d. to regulate the permeability of the paracellular pathway ("gate" function) and maintain the protein and lipid asymetries between apical and basolateral membrane domains ("fence" function, the tight junction) (Gumbiner,1992).

The basement membrane contains specialised proteins that regulate cell-substratum interactions. Cell attachment to the substratum occurs via specific receptors to type IV collagen, certain proteoglycans or laminin. The type of cell attachment is tissue specific, and is important for epithelial morphogenesis, maintenance of the differentiated state, and tissue-specific gene expression (Barnes, *et al* , 1984).

The structural organisation of epithelial cells is dependent on the polarised nature of the cytoskeleton and its interaction with the surface membrane (Gumbiner,1992). Actin microfilaments and their associated proteins interact with surface membrane proteins via direct or indirect linkages to specialised cytoskeleton proteins including, fodrin (termed spectrin in erythrocytes), ankyrin, vinculin, talin and the 110-kD protein (brush border myosin) of microvilli. For example, ankyrin binds with high affinity to $Na^{+}$,$K^{+}$-ATPase, and a complex containing $Na^{+}$,$K^{+}$-ATPase, ankyrin, and fodrin has been detected in extracts of Madin-Darby canine kidney (MDCK) epithelial cells, demonstrating a direct linkage of cytoskeletal proteins to a specific, integral membrane protein (Gumbiner, 1992) Cyto-keratin intermediate filaments, composed of bundles of protein filaments, attach to the surface membrane at desmosomes and hemi-desmosomes, and provide a structural continuum between adjacent cells and with the substratum (Cross and Mercer, 1993). Microtubules are also aligned with the apico-basal axis of the cell, and they determine the spatial orientation of the endoplasmic reticulum, Golgi appparatus, lysosomes, and the distribution of actin microfilaments within the cell (Jones, 1990)

Confluent cultures of a number of established epithelial cells lines have shown the ability to transport ions across the epithelial monolayer as is characteristic of the cells in vivo. Transepithelial ion transport in cell culture is visible by phase contrast microscopy as osmotic movements of water cause dome (or blister) formation. Dome formation is found in both the MDCK cell line and the LLC-$PK_1$ cell line, where the epithelial cell monolayer detaches from the substrata as a result of active transport of ions and water across the cell monolayer, in an apical to basolateral direction, leading to

a trapped bubble of fluid between the cell layer and the impermeable culture dish (Fig 2.1).

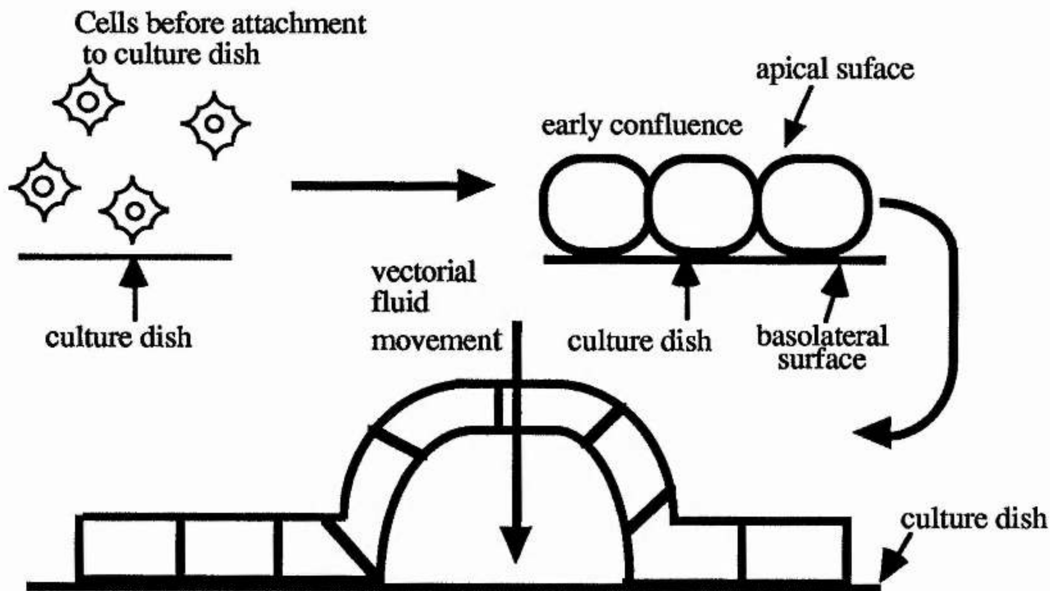



Figure 2.1 Diagrammatic explanation of dome formation. The apical cell surface is shown in contact with the medium; the basolateral surface is shown facing the culture dish.

Domes do not occur in cultures grown on porous supports such as collagen-coated mesh or nitrocellulose filters since fluid accumulation on the basolateral side is prevented (Taub, 1985).

The domes that are formed are dynamic structures that are in continuous formation till then eventually collapsed. A dome or blister in MDCK cells will only form if both the pumping force and the resistance of the occluding junctions prevail over the firmness of the attachment of MDCK cells to the dish. The resistance of the occluding junctions prevents escape of the fluid that has been pumped to the basolateral surface. However, blistering and nonblistering regions in the monolayer seem to have identical properties. If the strength of attachment of the cells to the substratum is reduced, the size and number of domes increase significantly (Taub, 1985). The fact that at any given moment, only a fraction of the total area (usually 10 to 20%) exhibits signs of blistering has been attributed to the prevalence, in nonblistering areas, of the attachment forces

over the resistance of the junctions. As a consequence, the cell junctions rupture in such nonblistering areas (Taub, 1985). MDCK cells have been used extensively for the study of selective ion transport across the epithelial monolayer. Physiologists have localised a variety of plasma membrane transport proteins to either the apical or basolateral membrane of the epithelial cells. These proteins are now being isolated and characterised at the molecular level. Despite these advances, a complete understanding of epithelial cell function requires detailed knowledge of the regulatory mechanisms that modulate transport activity.

The dogfish rectal gland is almost exclusively composed of epithelial cells which have very high Na,K- ATPase (sodium pump) activity. Primary cell cultures isolated from the dogfish rectal gland provide one of many suitable models available for studying ion transport across epithelium. However this particular model for ion transport is also of interest in itself as little is known about ionic or hormonal regulation of transport or expression of the endogenous ion transporters present in the rectal gland. The function of the rectal gland is to work in conjunction with the kidney and gill to excrete excess sodium chloride from the fish and hence maintain electrolyte balance. In order to maintain electrolyte balance in the dogfish (that is under constant change due to the entry of sea water through the gill and gut see genral introduction), the rectal gland excretes a fluid into the sea of approximately 0.5M NaCl (Hannafin,1983). This gland has a rapid on / off switch thought to be controlled by the high and intermittent salt loads associated with the sporadic feeding habits of the fish (Shuttleworth, 1988). The rectal gland needs to be able to cope with such loads and quickly excrete the excess sodium chloride absorbed from the gut following feeding (Greger and Schlatter, 1984). The endogenous signalling mechanisms which regulate this rapid on / off activity of the gland are unknown however several theories exist. The rapid control of the gland may be due to changes in rectal gland blood flow or may be the result of direct regulation of the epithelial ion transport, or more than likely a mixture of both (Kelley *et al.* 1991). However further information is needed to elucidate the hormonal and ionic factors involved in regulation of the rectal gland in vivo. A primary culture of rectal gland epithelial cells would allow direct investigation of Na,K-ATPase activity in response to sodium chloride loading without the in vivo complications eg hormonal inervation.

The first immortalised fish cell line, RTG-2, was established from rainbow trout gonads by Wolf in 1962. The trout cells were isolated by finely cutting the gonads with scissors to yield tissue pieces of a millimetre in width, and then digesting the

tissue with trypsin until individual cells and small cell aggregates of were released. These cells and cell aggregates were sedimented by differential centrifugation and resuspended in growth medium before they were plated on to culture dishes. A permanent cell line was formed as a result of a spontaneous mutation after a period where the cells were repeatedly passaged. Since then many other methods have been established for isolating and culturing fish cells including primary epithelial cells from the rectal gland of the dogfish shark (*Squalus acanthias* ) a member of the elasmobranch family (Valentich, 1991). This method was based on a collagenase dispersion technique where tissue slices were disaggregated into individual tubules comprising clumps of incompletely digested columnar epithelial cells. Once the tubules attached to the culture substrata, cells migrated and spread from the attached tubular segments. Although this technique did not prove suitable for the isolation and culture of large quantities of viable epithelial cells from *Scyliorhinus canicula* in this study, it provided the basis for the development of a more efficient perfusion technique which was then optimised for maximal cell yield and viability.

# 2.II MATERIALS AND METHODS

## 2.II a Preparation of epithelial cells from the rectal gland of the the European dogfish (*Scyliorhinus canicula*)

The fish used were caught off the west coast of Scotland either by net or long-line fishing and were maintained in 150 L of seawater in through flowing tanks maintained at ambient temperature (3 - 16°C) at the Gatty Marine Laboratory, St Andrews. The fish were kept under a 12 hour light : 12 hour dark photo period and were not fed.

All routine tissue culture procedures were carried out using standard aseptic techniques in a "Gelaire Class 100" laminar air flow cabinet (Gelman Instruments). All sterile glassware was autoclaved at 121°C (15 psi) for 90 minutes before use. All solutions were sterilised by passage through a 0.22 μm Millipore filter and stored at 4 °C in sterile bottles.

### 2.II a i Preparation of culture substrata

Coating culture dishes with collagen.

Using a sterile pipette, 0.6 ml of collagen solution of 0.1 % (w/v) acetic acid (type 1 from calf skin, Sigma) was placed in 90 mm diameter petri dishs and shaken to produce an even covering. Plates were left at room temperature (20 - 22 °C) for 4 hours to dry in the tissue culture cabinet. The pH of the collagen coating was equilibrated to that of the medium by overnight incubation with 7 ml/plate of culture medium (1:1 Dulbecco's minimum essential nutrient mix/Ham's F-12 with 10 % L-glutamine and 15 mM HEPES, pH 7.4 (Gibco BRL) supplemented with 100 mM NaCl, 21 mM $NaHCO_3$, 3.9 mM $CaCl_2$, 2.5 mM $MgCl_2$, 300 mM Urea, 150 mM TMAO, 0.025% Nu-serum (Collaborative Biomedical Products) 0.01% ITS+ (Collaborative Biomedical Products); and 5000 units/ml of penicillin / 5 μg/ml streptomycin (Gibco BRL)). The same method was used to coat culture dishes of various sizes with collagen using the volumes listed in table 2.1.

Table 2.1

| Culture dish | Volume of collagen solution/well (ml) | Equilibration medium (ml) |
|---|---|---|
| 96 well plate | 0.1 | 0.25 |
| 35 mm petri dish | 0.4 | 1 |
| 90 mm petri dish | 0.6 | 7 |

The 24 well plates, 12 well plates and 25 $cm^2$ Roux flask used in the experiments were pre-treated with collagen (type 1) by the manufacture (Iwaki).

Collagen coated glass cover slips

Coverslips were sterilised by autoclaving at 121°C (15 psi) for 90 minutes and then placed in tissue culture grade 35 mm petri dishes. The culture dishes and associated coverslips were coated with collagen, allowed to dry, and equilibrated over 12-24 hours in the tissue culture cabinet as described above. The media was removed before plating with the cell suspension.

### 2.II a ii Method I

This method for the isolation and culturing dogfish rectal gland epithelial cells was based on the method previously reported by Valentich, (1991).

A. Removing the rectal gland (applies to method I and method II)

The fish was killed by a sharp blow to the head and then pithed to destroy the spinal column and the body washed with tap water and then 70 % ethanol. The fish was then taken to a sterile air flow cabinet (Bassaire Ltd., Southampton) and was placed on a foam cradle. Using a sterile scalpel, an incision was made in the skin 2 inches anterior to rectal opening. This incision was extended anteriorly for 5 inches and posteriorly for 1 to 2 inches. The flaps of the abdominal incision were retracted away from the exposed body cavity. Using sterile, blunt forceps, the overlying organs were moved to expose the rectal gland. The gland was removed by cutting the artery, vein, and any adherent mesentery with small sterile scissors. All subsequent procedures were carried out under sterile conditions. The rectal gland was placed in a centrifuge tube containing ice-cold sterile shark Ringer (SR) (NaCl, 240 mM; KCl, 7 mM; $CaCl_2$ 10 mM; $MgCl_2$, 4.9 mM; $NaHCO_3$, 23 mM; $Na_2HPO_4$, 0.5 mM; Urea, 360 mM; trimethylamine oxide

(TMAO), 60 mM; glucose, 1% (w/v); gassed with 95% air and 5% $CO_2$, pH 7.6) for transfer to Gelaire Laminar Air Flow cabinet (class 100, Gelman Instruments, Northampton).

B. Collagenase dissociation of rectal gland tubules

Using sterile forceps, the rectal gland was transferred to an ice-cold, 100 mm glass petri dish containing 10 ml of ice-cold SR. Using a scapel, the rectal gland was cut into transverse slices approximately 1 to 2 mm thick. The two ends of the gland which contained a large amount of connective tissue were discarded and the slices were transferred to a new ice-cold, 100mm glass petri dish using fine forceps. This petri dish did not contain SR. The tissue was minced using a criss-cross action so that all pieces were $1mm^3$ or less in size. Ice-cold SR (7 ml) was added to the mince and the tissue dispersed by 7 passages of the suspension through a spinal biopsy needle (1 mm in diameter) fitted to a 10 ml syringe. The suspension was transferred to an ice-cold, 15 ml polystyrene centrifuge tube using the needle and syringe and the petri dish washed with additional SR (7 ml) and any remaining pieces of tissue was added to the centrifuge tube. The minced tissue was washed 3 times with 20 ml of ice-cold SR by allowing the pieces to settle under gravity and aspirating the supernatant. By the third rinse the supernatant was relatively clear. Once the supernatant was clear the tissue was resuspended in approximately 7 ml of fresh, ice-cold SR and centrifuged for 30 s at 1000 rpm (200 x $g_{max}$) at 4°C (MSE Coolspin). The supernatant was aspirated and the pellet resuspend at room temperature in 7 ml of SR containing 2 mg/ml collagenase (type D, Boehringer-Mannheim) and 100 μM $CaCl_2$. The air space in the 15 ml centrifuge tube was gassed with 95% air / 5%$CO_2$ and the tube was placed on its side on the top of a reciprocal shaking platform and shaken vigourously for 45 minutes at room temperature. The tissue was then dispersed by passaging 10 times with a spinal biopsy needle ( 1mm in diameter) and 10 ml syringe. The tube was re-gassed as described above, and then returned to the rocking platform for an additional 45 minutes. On removal from the rocking platform the tissue was again dispersed 10 times with the wide needle and syringe. Undigested tissue fragments were allowed too settle for approximately 30 s under gravity and then the supernatant, which contained individual dispersed tubules, was transferred to a new ice-cold, 15 ml polystyrene centrifuge tube. Ice-cold SR (7 ml) was used to wash the remaining tissue using 7 dispersions of the spinal biopsy needle and syringe. The fragments were again allowed to settle under gravity, and the supernatant transferred to the fresh tube containing the already collected tubule suspension. Washing of the tissue was then repeated once

more and then the combined tubule suspension was centrifuged at 1000 rpm (200 x $g_{max}$) for 45 s at 4 °C (MSE Coolspin). After removal of the supernatant by aspiration the tubules were washed by resuspending them in 7 ml of ice cold SR and centrifuged at 1000 rpm (200 x g max) for 45 s at 4 °C (MSE coolspin). The wash step was repeated twice.

C. Plating the tubules

The pelleted tubules were resuspended in 3 ml of culture medium which comprised a 1:1 Dulbecco's minimum essential nutrient mix/Ham's F-12 with 10 % L-glutamine and 15 mM HEPES (Gibco BRL) with the addition of 100 mM NaCl, 21 mM $NaHCO_3$, 3.9 mM $CaCl_2$, 2.5 mM $MgCl_2$, 300 mM Urea, 150 mM TMAO, 0.025% Nu-serum ( Collaborative Biomedical Products), 0.01% $ITS^+$ (Collaborative Biomedical Products), and 5000 units/ml of penicillin / 5 μg/ml streptomycin (Gibco BRL) and this suspension was used to seed two collagen coated plastic 35 mm petri dishes (Corning) (coated as described in section 2.II ai). The isolated tubules were boxed in a plastic sealed box (15 cm wide x 10 cm long x 7 cm deep) and gassed with 95% air / 5% $CO_2$ and maintained in an incubator at 18°C. The medium was replaced every 3 or 4 days.

### 2.II a iii Method II

A. Removing the rectal gland

The fish was killed and the body cavity opened and rectal gland exposed as described in section 2.II a ii part A. The gut was tied off with thread approximately 2 cm posterior to the gland. Using small sterile scissors an incision was made about 1mm in diameter in the rectal gland vein which runs from the gland along the gut. The vein was cannulated using a sterile intravenous cannula (size 2 fg) and tied off against the whole of the gut. The gland and part of the intestine were removed from the fish by cutting the artery and any adherent mesentery tissue with small sterile scissors. The rectal gland was placed in a sterile plastic 25 ml Universal tube and connected to a two-way tap which itself was attached to two sterile 5 ml syringes.

B. Collagenase digestion of the rectal gland

The gland was perfused at room temperature (20 -22 °C) with a head height of 300 mm giving a perfusion rate of 210 µl ± 24 µl/min (n=20 experiments), first with 5 ml of calcium free SR containing 1mM EGTA, and then 5 ml of 2 mg/ml collagenase (type D Boehringer-Mannheim) in SR containing 100 µM $CaCl_2$. The gland was then placed in a ice-cold sterile Universal tube using sterile forceps and taken to a Gelaire laminar air flow cabinet (class 100, Gelman Instruments, Northampton) and then transferred into a 100 mm glass sterile petri dish. All subsequent procedures were carried out under sterile conditions. Using a scalpel, the end of the gland was cut of and the digested tissue gently squeezed out on to the petri dish using the blunt end of the scapel blade. Shark ringer (7 ml) was added to the petri dish and the suspension was triturated by 15 repeat passages through a spinal biopsy needle (1 mm in diameter) and 10ml syringe. The resulting cell suspension was then filtered though 4 layers of sterile gauze to remove any large clumps of undigested tissue. The filtered suspension was transferred to a sterile plastic centrifuge tube (15 ml) and the cells sedimented by centrifugation at 1000 rpm (200 x $g_{max}$) for 90 s at 4 °C (MSE Coolspin). The supernatant was aspirated and the pellet resuspended in 7 ml of SR to wash the cells. The centrifugation and wash step was repeated twice.

C. Plating the cells

After the final wash the pellet was resuspended in 10 to 15 ml of media (1:1 Dulbecco's minimum essential nutrient mix/Ham's F-12 with 10 % L-glutamine and 15 mM HEPES (Gibco BRL) supplemented with 100 mM NaCl, 21 mM $NaHCO_3$, 3.9 mM $CaCl_2$, 2.5 mM $MgCl_2$, 300 mM Urea, 150 mM TMAO, 0.025% (v/v) Nu-serum (Collaborative Biomedical Products), 0.01% $ITS^+$ (Collaborative Biomedical Products), and 5000 units/ml of penicillin / 5 mg/ml streptomycin (Gibco BRL), depending on the size of the gland. Even distribution of cells throughout the medium was ensured by forceful dispersion of cells with a 10 ml syringe and spinal biopsy needle as described above. The cells were then plated (approximately 0.15 ml of medium per $cm^2$) onto collagen coated plastic prepared as described in section 2.II a i. and evenly distributed on the culture substrate, before being incubated undisturbed at 18 °C in an atmosphere of 95% air / 5 % $CO_2$ for 3 or 4 days.. Thereafter the cells were viewed by a microscope and the media was replaced every 3 to 4 days. The volume of cell suspension seeded into culture dishes or flasks varied according to the size of the container. The following table (table 2.2) describes the cell suspension

volume required for the different tissue culture dishes and flasks, the cell density is unknown as a single cell count was not possible as the cells were isolated in small clumps or aggregates. The volume of medium each gland was suspended in varied from 10-15 ml according to the size of the rectal gland.

Table 2.2

| Type of tissue culture dish | Volume of cell suspension required for appropriate cell seeding density |
|---|---|
| 96 well plate (Sterling) | 200 µl / well |
| 24 well plate (Iwaki) | 0.5 ml / well |
| 12 well plate (Iwaki) | 3 ml / well |
| 9 cm petri dish (Nunc) | 7 ml |
| 3.5 cm petri dish (Corning) | 2 ml |
| 3 cm Cellagen inserts (ICN) | 2 ml |
| 25 $cm^2$ Roux (Iwaki) | 5 ml |

Cells were also grown in suspension using the same isolation technique as described above, except that the cells were plated onto collagen free tissue culture graded dishes. Cells did not adhere to the plastic but continued to grow and divide in suspension as large clumps of cells.

### 2.II b Trypsinization and counting the cells

The growth medium was poured off confluent monolayers of shark rectal gland cells and the cells rinsed twice with approximately 1 ml (for a 35 mm petri dish) trypsin solution (1 % trypsin and 1 mM EDTA in calcium-and magnesium-free SR). Trypsin solution (1 ml) was then added to the 35 mm petri dish which was then boxed, gassed with 95 % air / 5 % $CO_2$ and incubated at 18 $^0$C for 2 hours on a vibration shaker until the cells were detached. The trypsin solution was then neutralised with the addition of 1ml culture media, and a single cell suspension was prepared by the forceful dispersion of the suspension through a spinal biopsy needle and 10 ml syringe. A 1ml aliquot of suspension was then added to 9 ml of SR and the cell number determined using a Coulter Counter Model ZM with Channelyzer (C1000). The same method was used to trypsinize cells grown in various sized dishes using the volumes listed in Table 2.3.

Table 2.3

| Tissue culture container | Volume of trypsin solution |
|---|---|
| 96 well plate | 100 μl |
| 24 well plate | 300 μl |
| 9 cm | 4 ml |

**2.II c Preparation of the dogfish *(Scyliorhinus canicula)* rectal gland tissue sections for light microscopy.**

2.II c i Fixing the gland

The rectal gland was removed from the fish as described in section A method 1 and immediately submerged in Bouin's fluid (BDH). However dropping the whole gland into the fixative could not ensure rapid fixation of the central portion of the gland therefore after a 10 minute incubation at room temperature in Bouin's fluid the gland was removed and sliced radially down the centre before being replaced in the Bouin's fluid and incubated for a further 5 hours at room temperature.

2.II c ii Sectioning the gland

The gland was removed from the fixative and placed in a series of alcohol and chloroform solutions to dehydrate the tissue. This was carried out by gradually processing the gland through increasing strength alcohol to 100% alcohol then into chloroform as described below in Table 2.4.

Table 2.4

| Step number | Solution | Time (hr) |
|---|---|---|
| 1 | 75 % alcohol (2 ml) | overnight |
| 2 | 75 % alcohol (2 ml) | 2 |
| 3 | 96 % alcohol (2 ml) | 1 |
| 4 | 96 % alcohol (2 ml) | 2 |
| 5 | absolute alcohol (2 ml) | . 1 |
| 6 | absolute alcohol (2 ml) | 1 |
| 7 | chloroform (2 ml) | 1 |
| 8 | chloroform (2 ml) | overnight |

Following the overnight incubation in chloroform the dehydrated gland was added to a bath of melted paraffin wax (melting point 58$^0$C) in an oven maintained at 58-60$^0$C under a vacuum for 5 hours. The gland was then removed from the oven and placed in cold water to allow the wax to solidify before being sectioned into slices, 6-8 microns thick, using a steel blade in a rotary microtome. The paraffin sections adhered to each other, producing a ribbon that was collected and laid out on distilled water to separate the sections. The sections were then placed on a glass slide.

2.11 c iii Staining the sectioned gland

A modified Masson's Trichrome Stain (Masson, 1929) was used to stain the sectioned gland. Sections were placed in xylol for 1-2 minutes, to remove the paraffin wax from the tissue. The slide was then passed through absolute alcohol, followed by 96%, 75% alcohol and distilled water ( approximately 30 seconds was spent in each bath). The slide was incubated for 10 minutes in Celestine Blue (0.5 g of dye was added to 100 ml of 5% iron alum and boiled for 3 minutes, the solution was allowed to cool, and then filtered through Whatman number 1 paper and 14 ml of glycerine was then added). Following the Celestime Blue incubation the slide was rinsed in distilled water and then incubated for 10 minutes in Mayer's Haemalum. This solution was prepared by dissolving 1 g of Haematoxylin dye in 10 ml of absolute alcohol, and then adding to 1 L of distilled water containing 5% potassium alum ( aluminium potassium sulphate) (w/v) and 0.02 % sodium iodate (w/v). Once mixed this solution was allowed to stand overnight. Chloral hydrate (50 g) and citric acid (1 g) were added and boiled for 5 minutes. The solution was cooled and filtered through Whatman number 1 paper and stored at room temperature. Following the Mayers Haemalum incubation the section was then rinsed in running tap water for 5 mins and placed for 3 minutes in Yellow Mordant. The Yellow Mordant solution was prepared by diluting 30 ml of a Yellow Mordant stock solution with 70 ml of 75 % alcohol, (stock solution was prepared by combining Orange G (0.4 g) in 160 ml saturated picric acid with Lissamine Fast Yellow (0.4 g) in 160 ml distilled water, the Yellow Mordant stock solution was then stored at room temperature). The tissue section was then washed with running tap water until the tissue was almost colourless and then stained by incubating with Ponceau Acid Fuchsin for 5 minutes. The Ponceau Acid Fuchsin solution was prepared by dissolving 2 g Ponceau 2 rR (Ponceau de Xylidine) and 1 g Acid Fuchsin in 200 ml of distilled water containing 3 ml of acetic acid. On removal from this staining solution the section was rinsed with tap water contining 1 % phosphomolybdic acid. The section was then incubated for 2 minutes in 1 % Aniline Blue in 1 % acetic

acid followed by a rinse with 1 % acetic acid then 96 % alcohol. The section was dehydrated with absolute alcohol and cleared in Xylol followed by mounting in DePeX. This procedure stained the nuclei black, red blood cells and tubules a red-pink colour and the collagen (connective tissue), carbohydrates and mucopolysaccharides blue.

The sections were then observed under the light microscope (Leitz Dialux 20).

### 2.II d Staining of cultured cells for fluorescence microscopy

Either freshly dispersed rectal gland epithelial cells or cultured rectal gland epithelial cells (grown on collagen coated glass coverslips in 35 mm petri dishes or on Cellagen inserts in 35 mm petri dishs) were viewed by light microscopy to examine their viability. The cultured cells were grown for 4 - 15 days after which the cells were washed with 1 ml of phenol red-free SR which was then replaced with SR containing ethidium bromide (10 μg/ml) / acridine orange (3 μg/ml). Ethidium bromide (50 mg) and acridine orange (15 mg) were dissolved in 1 ml of 95% ethanol, added to 49 ml of distilled water and stored at -20°C in 1 ml aliquots. The working strength solution of ethidium bromide / acridine orange was prepared by adding 1 ml of this stock to 99 ml of phenol red-free SR. The cells were then incubated for 1 minute at 20°C in ethidium bromide / acridine orange SR solution and then washed twice with phenol red-free SR. The glass coverslip or cellagen insert was carefully inverted onto a clean glass slide.

Cells were initially observed using the phase contrast microscope (Leitz Dialux 20) and then the same field was viewed by fluorescence using a 495nm filter. Most intracellular organelles (with the exception of lysosomes) of viable cells stained green (with acridine orange) whilst the nuclei of damaged cells were red (with ethidium bromide). Lysosomes in the cell cytosol were visible as red-yellow vesicles due to the accumulation of acridine orange with in the acidic compartment.

In some cases cells grown in suspension and single cell suspensions prepared by trypsinization of cultured cells were also observed by light microscopy.

### 2.II e Fixing and staining the cells for analysis under the electron microscope

#### 2.II e i Fixation, dehydration and embedding of cells

Cells either in suspension or on collagen coated glass coverslips, were fixed for 30 minutes in SR containing 2.5 % glutaraldehyde. Cells were then washed in SR for 1 minute and incubated in 1% osmium tetroxide in SR for 30 minutes at room temperature. Following fixation, cells were washed in SR for 1 minute and then dehydrated stepwise in 50%, 70%, 80% and 90% ethanol for 10 minutes in each solution. The cells were finally dehydrated in 100% ethanol for two 20 minutes periods. Cells grown in suspension were pelleted by centrifuged at 1000 rpm (200 x g) for 90 s at 4 °C in a MSE Coolspin and then placed in en block stain (0.1 g of uranyl acetate mix in 10 ml of 100% ethanol). Cells grown on coverslips were also then placed in en block stain and both sets of cells incubated overnight in the dark. On removal from the stain the cells were incubated in 100 % propylene oxide for two 20 minutes periods at room temperature. They were then infiltrated in a mixture of 2 parts propylene oxide to 1 part resin (prepared by mixing 19 ml MY753, 21 ml DDSA, 0.6 ml dibutyl phthalate and 1.2 ml BDMA) for 1 hour. The cells were then incubated in a mixture of 1 part propylene oxide to 2 parts resin for 1 hour. Finally they were embedded in the resin for 48 hours and cured at 60 °C in an oven in a fume cupboard.

#### 2.II e ii Sectioning and staining of cells

Once the embedding was complete the cells were sectioned to 50-60 nm thick slices using a diamond knife (RMC ) in a microtome (Ultracut, Reichert ). Once sectioned the slices were mounted on pioloform carbon -coated grids (Agar supplies) which were then placed onto hexagon grids (copper ethene 3.05 nm from Athene supplies) which are necessary for viewing the sections under the electron microscope. Once the sections were mounted they were stained in saturated uranyl acetate in 70% ethanol for 20 minutes then in Reynolds lead citrate for 5 minutes. The cells sections were then observed using a Philips Electron Microscope 301.

## Transfection of Shark Rectal Gland Cells

Although the collagenase perfusion technique greatly increased the cell yield from the rectal gland compared to the yield obtained when using Valentich's method, relatively

small yields of cells were obtained for subsequent culturing. Therefore, experiments which required a significant number of cells, such as RNA extractions for Northern blotting required the use of a large number of fish. Due to the time required to cannulate and perfuse the rectal gland, the number of fish that could be processed at the one time was limited, this therefore limited the number of plates/culture dishes that could be prepared. Problems were also encounter with the supply of fish, especially during the winter months when fish were very difficult to catch. Therefore an immortal cell line would be advantageous since it would result in an homogenous cell population and would also mean that you would not need to spend large amounts of time preparing cultures.

As a result of problems associated with low cell yields, cells were transfected with a transforming vector (pSVori) in an attempt to establish an imortalised cell line.

### 2.II f i Transfection of cells with the transforming plasmid vector pSVori using cationic liposomes

It was previously reported that human thyroid epithelial cells could be immortalised without crisis after transfection with a plasmid containing a depleted SV40 virus genome (Lemoin, *et al,* 1989). Human bronchial epithelial cells have also been immortalised by transfection with plasmids containing the SV40 virus early region genes (Reddel, *et al,* 1988). All cells transformed expressed the SV40 tumour (large T) - antigen suggesting a selective pressure for the continued retention of this phenotype. All of the SV40 transformed cell strains appeared to retain their epithelial nature based on morphological criteria observed in both the light and electron microscope.

Immortalisation of the rectal gland epithelial cell line was attempted by transfection of cells with the plasmid pSVori (gift from Dr A Riches, University of St. Andrews) which contains the SV40 early region genes. Two methods were used to transfect cells with the SV40 containing plasma:- 1. cationic liposome-mediated transfection (lipofection) and 2. streptolysin-O permeablisation. Cationic liposomes can be easily synthesised and have been proven to be a highly effective chemical method for the transfection of DNA into the cytosol of animal and insect cells (O'Reilly, 1997). Following sonication, the cationic liposomes form unilamellar vesicles (liposomes) with positively charged head groups in aqueous solution. These liposomes form stable complexes with negatively charged protein or nucleic acids and can adhere to the cell

surface and by an as yet unknown mechanism, fuse with the cell membrane and release the protein or DNA into the cytoplasm (Fig 2.2) (Biochemica Update 1991).

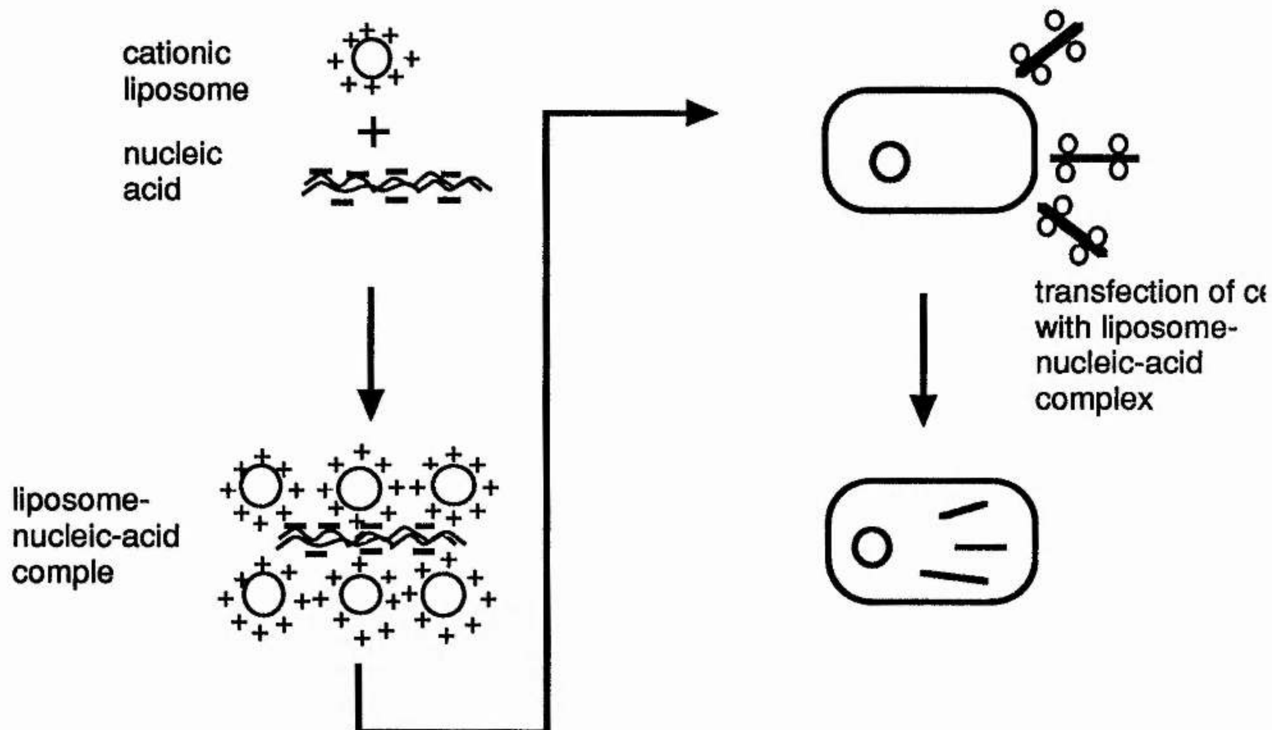



Figure 2.2 Principle of transfection using cationic liposomes.

In order to detect whether the dogfish rectal gland cells may be transfected using a cationic liposome method, the control reporter vector, pSV - β Galactosidase (Promega, cat no. E1081), was used. This reporter vector was of similar size to pSVori, has the same promoter region and its expression can be assessed by monitoring the appearance of coloured reaction products of the transfected β galactosidase in either cell homogenates or in the cell cytosol as viewed by light microscopy. Simultaneously, the same transfection protocol using the pSV - β galactosidase vector was followed using Hela cells, also as a control, as the cationic liposomes have been reported to transfect DNA into Hela cells.

Before initiating the transfection experiments the urea and TMAO which were present in the normal growth medium were gradually reduced in concentrations over a two day period until the cells were maintained in urea and TMAO free medium. This was

necessary because the high concentrations of these denaturants may have inhibited successful transfection and/or expression of the β Galactosidase activator or the immortalisation of the cells. The following table (Table 2.5) shows the procedure used for the removal of urea and TMAO from the cells growth media:-

Table 2.5

| Urea/TMAO concentration | Incubation time |
|---|---|
| 100 % (60 mM urea and 150 mM TMAO) | 2 days |
| 80 % | 3 hours |
| 60 % | 3 hours |
| 40 % | over night. |
| 20 % | 3 hour. |
| 10 % | 3 hours. |
| 5 % | 3 hours. |
| 0 | overnight |

To optimise transfection effeciency the cells should be sub confluentis to allow for subsequent growth following transfection. Therefore four days prior to transfection the cells were seeded into a 12-well plate such that they were approximately 40% confluent on the day of transfection. The transfection mix was made in a glass jar as the cationic lipids bind to plastic. Serum free dogfish growth medium ( 0.5 ml) was added to a sterile glass jar followed by either 0.5, 1, 1.5, 2, 2.5, 3, 4 or 5 μl of DNA (1μg/μl) (reporter plasmid or pSVori) and gently mixed. Next, either 10, 15, 20 or 30 μl of commercialy prepared cationic liposomes (DOTAP (N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N,-trimethylammonium methylsulfate), Promega) or cationic liposomes made in house were added. The mix was incubated at room temperature for 10 -20 minutes to allow attachment of the cationic liposomes to the DNA and then a further 0.5 ml of growth medium was added to make a final volume of approximately 1ml. The growth media was aspirated from the cells and 1 ml of transfection mix added per well. The plate was gently swirled to ensure an even distribution and the cells were incubated for 6 - 16 hour at 20°C. After incubation the transfection mix, was aspirated and the cells gently washed once with urea and TMAO free dogfish growth media (1 ml) and then fresh growth media ± urea / TMAO (1 ml) was then added and the cells incubated for a further 24 hours. When using the reporter vector in transfection, cells were then stained for β-galactosidase expression or cell

homogenates assayed for β-galactosidase activity (refer to sections 2.II f iii and 2.II fiv).

Two types of liposomes were used in the transfection procedure. The commercialy prepared lipid DOTAP, was used in the initial experiments, however as this chemical was very expensive and was rapidly inactivated by oxidation, cationic liposomes were also prepared in the laboratory and used in transfection experiments.

2.II f ii Preparation of cationic liposomes

Dioleoyl L-α-phosphatidyl ethanolamine, (Sigma) (purchased as a solution in chloroform) (1 ml) was dispensed into a 5 ml glass universal vial. Dimethyldioctadecyl ammonium bromide (Sigma) (4 mg) was then added and vortexed until dissolved. The chloroform was evaporated at room temperature using a stream of nitrogen whilst the universal was being rotated to ensure an even film of lipid around the walls. Sterile water (10 ml) was added and the lipids resuspended at room temperature by incubation for 10 - 15 minute in a sonic water bath. After resuspension, the turbid mixture was sonicated repeatedly for 30 -60 s every 2 minutes for up to 1 hour with a soniprobe until the mixture became clear. The liposomes were then aliquoted (1.5 ml) into sterile 2 ml glass tubes and stored at $4^0$C.

**2.II f iii Staining cells for β -Galactosidase expression**

Cells that were successfully transfected with the control reporter vector pSV - β Galactosidase will express β–galactosidase activity due to the presence of the gpt promoter located upstream of the lac Z gene. When cells expressing β–galactosidase activity are incubated in phenol red free SR containing X-gal, the β–galactosidase will cleave X-gal to a coloured reaction product which turns the cells blue. Following liposome transfection and incubation for 24 hours in normal growth medium ± urea / TMAO dogfish rectal gland primary cultured cells were gently washed with urea and TMAO-free SR. A fixative (1% glutaraldehyde in urea-and TMAO-free SR) was then added and the cells were incubated at room temperature for 10 minutes. After fixation the cells were gently washed twice with urea and TMAO-free SR and the staining solution added (urea and TMAO-free SR containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride and 2.5 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Promega). Although blue stained cells began to appear within a few hours when Hela cells were transfected as a control,

the stain was left on all cells overnight for true determination of the number of transfected cells and to see if any rectal gland cells showed any signs of positive staining.

### 2.II f iv Assaying cell homogenates for β-galactosidase activity

Cells that were successfully transfected with the control reporter vector pSV - β Galactosidase will express the β galactosidase enzyme due to the presence of the gpt promoter located upstream of the lac Z gene. β-Galactosidase activity was determined using a commercial assay kit (Promega cat number E200). This kit provides a convenient method for assaying β-galactosidase activity in extracts prepared from cells transfected with pSV-β-Galactosidase Vector. The principle of this assay is that the substrate o-nitrophenyl-β–D–galactopyranoside (ONPG) is hydrolysed to the yellow coloured product o-nitrophenol when incubated with cell homogenates containing β-galactosidase activity . The yellow colour can then be detected and quantified by reading the absorbance at 420 nm using a spectrophotometer (Philips PU962 series).

As a control the extract and assay procedures were carried out on cells transfected by the above method and on non-transfected cells. Before starting the cell extraction procedure 4 volumes of water was added to 1 volume of 5x reporter lysis buffer to produce a 1x working strength solution. Following transfection and subsequent incubation for 24 hours the growth medium (± urea / TMAO) was aspirated and the cells washed twice with urea and TMAO-free SR. As much SR as possible was removed after the final wash. Working strength reporter lysis buffer (450 µl per well in a 12 well plate) was added, and the dish was rocked slowly by hand several times to ensure complete coverage of the cells. The cells were incubated at room temperature for 15 minutes and the dish was rocked by hand halfway through the incubation period. All areas of the plate surface were then scraped using a pipette tip and the cell lysate transferred to a microcentrifuge tube with a pipette. The tube was vortexed for 10-15 seconds then centrifuged at full speed (14926 x $g_{max}$) in a microcentrifuge (Heraeus Sepatech, Biofuge A) for 2 minutes at room temperature. The supernatant was transferred into a fresh tube and stored at -70$^0$C or assayed directly.

The components of the assay system comprising β-galactosidase stock enzyme, 2x assay buffer and 1M sodium carbonate were thawed. The β-galactosidase enzyme and the 2x assay buffer were placed on ice, the 1M sodium carbonate was warmed to 37$^0$C

to dissolve any precipitate formed during storage and was then kept at room temperature. Equal volumes (150 µl) of the cell extract supernatant and 2 x assay buffer were pipetted into labelled tubes and mixed by vortexing. The assay tubes were then incubated at 37 $^{0}$C overnight or until a faint yellow colour had developed. The reaction was stopped by the addition of 500 µl of 1M sodium carbonate and vortexed. The absorbance at 420 nm was determined.

Standard curves

The following dilution series of β-galactosidase activity was prepared using the stock β-galactosidase enzyme provided by the kit and 1x reporter lysis buffer. Stock β-galactosidase enzyme was diluted 1:10,000 with 1x reporter lysis buffer and a series of β-galactosidase standard solutions were prepared as described in table 2.6.

Table 2.6

| β-Galactosidase standard solution (milliunits/µl) | Volume of 1:10,000 stock (µl) | Volume of 1x reporter lysis buffer (µl) |
|---|---|---|
| 0 | 0 | 150 |
| 1 | 10 | 140 |
| 2 | 20 | 130 |
| 3 | 30 | 120 |
| 4 | 40 | 110 |
| 5 | 50 | 100 |
| 6 | 60 | 90 |

The β-galactosidase standard solutions were then assayed for activity as described above in section 2.II f iv.

### 2.II f vii Preparation of pSVori plasmid and β-galactosidase control vectors

To provide enough plasmid, it was first neccessary to transfect the plasmid into bacteria and select colonies following growth on agar plates. Colonies on the agar plates were removed and further grown in Luria broth overnight, it was then neccessary to extract and purify the plasmid of interest from the bacteria. The pSV-β galactosidase control

vector was used as a positive control vector for monitoring transfection efficiencies of cells. The SV40 early promoter and enhancer drive transcription of the bacterial lacZ gene which in turn is translated into the β-galactosidase enzyme, cause cells to appear blue when plated on media containing X-gal. The pSVori plasmid also contains the SV40 early region genes, which is known to induce immortalisation in many different epithelial cell types (Reddel, *et al,* 1988).

A Preparation of agar plates

Nutrient agar (14 g) was added to 500 ml of water in a 1 L flask, autoclaved for 90 minutes and allowed to cool to 48°C before addition of the appropriate antibiotic; (2.5 ml of 10 mg/ml kanamycin for pSVori or 1 ml of 50 mg/ml ampicillan for β-galactosidase control vectors). Approximately 25 ml aliquots were poured onto 90 mm plastic bacterial grade petri dishes and the plates were left to set for 3 hours at room temperature.

B Transformation

From the stock solution of plasmid (50 ng/μl), 1 μl was added to 9 ul water. The competent bacteria (E. coli DH505 α for pSVori and E. coli XL1-blue MR for β-galactosidase vector) were removed from storage in the - 90 °C freezer and defrosted on ice and 2 x 4 μl aliquots dispensed at 4 °C into two 1.5 ml microfuge tubes containing 0.4 μl of β mecaptoethanol (βME) (98% v/v). Plasmid solution (1 μl; 50 ng for pSVori or β-galactosidase vector) was then added to one sample tube and 1μl of water added to the second tube, to act as a control. Both tubes were incubated on ice for 30 minutes. Cells were then heat shocked at 42 °C for 90 seconds and returned to ice for 10 minutes before addition of 1 ml of pre-warmed (37 °C) SOC medium (1 % tryptone, 0.5% yeast extract, 15 mM NaCl, 50 mM glucose and 10 mM $MgSO_4$, pH 7.5 with NaOH and autoclaved for 90 min at 121°C) and incubating for 1 hour on a rotating wheel at 37°C. Samples (100 μl) from both the transfected and the control tubes were then plated and spread on to separate nutrient agar plates containing the appropriate antibiotic and spread. The plates were inverted and incubated at 37°C overnight. After the overnight incubation the control plates showed no sign of growth whereas the transfected bacteria plate had colonies present. Fast growing colonies were isolated using an sterile inoculating loop and placed in 5 ml of Luria (1 % tryptone, 0.5% yeast extract, 15 mM NaCl, pH 7.5 NaOH and autoclaved for 90 min at 121°C) in a sterile universal tube. This was incubated on the rotating wheel overnight

at 37°C. Subsequently 2 ml was used for a plasmid mini preparation and 3 ml used to inoculate 1L of luria broth for a large scale plasmid DNA extraction.

C Plasmid mini preparation

The purpose of this technique is to check that the colonies chosen contains the desired plasmid.

From the 2 ml of the culture that was put aside for the mini preparation 1.5 ml was placed into a microfuge tube and spun for 5 minutes, 14926 x $g_{max}$ at room temperature in a bench top microfuge (Heraeus Sepatech, Biofuge A). The supernatant was aspirated and the pellet resuspended in 100 ml of ice cold solution I (50 mM glucose, 25 mM Tris-Cl and 10 mM EDTA, pH 8.0). Solution II (0.2 N NaOH and 1% SDS) was then added (200 μl) and the tube was mixed by repeated inversion. Ice cold solution III (150 μl of 5 M potassium acetate) was then added and the tube was vortexed, stored on ice for 5 minutes and then centrifuged at room temperature for 5 minutes, at 14926 x $g_{max}$ in a bench top microfuge. The supernatant was then transferred into a fresh microfuge tube and an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added and mixed by vigourous vortexing. The tube was recentrifuged as above and the top aqueous layer transferred into a new microfuge tube and an equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed again by vortexing vigously. The tube was recentrifuged as above and the top aqueous layer transferred into a new microfuge tube and 2 volumes of 96 % ethanol added vortexed and incubated at room temperature for 2 minutes before centrifuging at 15,000 rpm, (23,550 x $g_{max}$) 4°C for 60 minutes using a JA-18.1 rotor in a Beckman centrifuge, model J2-MC. The supernatant was carefully poured of and the tube inverted removing any drops of ethanol. Any drops of fluid on the walls of the tube were carefully removed with tissue paper. The pelleted DNA was washed without resuspension with 1 ml 70 % ethanol at -20 °C and recentrifuged as described above. The supernatant was again discarded and the pellet was allowed to dry at room temperature in a vacuum oven for 10 minutes, before dissolving in 50 μl of TE ( 10 mM Tris, 1mM EDTA, pH 8.0). The plasmid is then digested overnight to linarise the plasmid to enable its size to be determined using gel electrophoresis. For digestion of the pSVori plasmid, 10 μl of extracted plasmid (approximateky 3 μg) was added to 7 μl DEP $H_2O$, 2 μl React 4 buffer (200 mM Tris/HCl, 50 mM $MgCl_2$, 500 mM KCl, pH 7.4) and 1 μl Kpn I enzyme (1.75 units) and incubated overnight at room temperature. For digestion of the β-galactosidase control vector two enzymes were

used to excise the plasmid and hence determine its siz. Reaction comprised of 10 μl of extracted plasmid (approximately 2.5 μg), 6 μl DEP $H_2O$, 2 μl of React 2 buffer (50 mM Tris-HCl, 10 mM $MgCl_2$ and 50 mM NaCl, pH 8.0), 1 μl Hind III (0.625 units) and 1 μl Bam H1 (3.5 units). Again incubations were conducted overnight at room temperature.

E Agarose gel electrophoresis

Gel electrophoresis is a method whereby charged molecules in solution migrate in response to an electrical field through a insoluble material of variable porosity. The rate of migration or mobility of nucleic acids through the electrical field depends on the strength of the field, on the net charge, size, and the shape of the molecules, and also on the chemical composition, pore size and temperature of the agarose gel.

In general, due to the relatively large size or molecular weight of nucleic acids agarose gels are often the material of choice for separation of DNA or RNA molecules. Agarose is a highly purified polysaccharide derived from agar. Agarose, which comes in powder form, dissolves readily when added to boiling aqueous solutions and remains in a liquid state until the temperature is lowered to about 40°C, at which point it gels. The gel is stable and does not melt again until the temperature is raised back to about 100°C. The pore size of the matrix may be predetermined by adjusting the concentration of agarose in the gel: the higher the concentration, the smaller the pore size. The gel concentration used therefore depends on the size of the DNA or RNA fragments to be analysed and the degree of separation required. Most of the DNA or RNA fragments currently used for further manipulations are of such a size that they are usually resolved in agarose gels with concentrations between 0.8 and 1.2 % .

Agarose powder (0.3 g) was dissolved by boiling in 30 ml electrophoresis buffer comprising of 0.04 M Tris-base, 5 mM sodium acetate, 1 mM EDTA, pH 7.6 (1 x TAE). The gel solution was then transferred to a water bath at 45 °C, where it was kept without solidifying. Ethidium bromide (3 μl of 10 mg/ml) was then added to the gel solution and mixed without creating any air bubbles. This is the most convenient dye for detecting DNA fragments in agarose gels, as it intercalates between stacked bases and emits a characteristic orange red fluorescent light on UV illumination.

The molten gel was poured onto a 50 x 75 mm glass plate held in a flat bed gel tray and gel wells cast using a 5 well comb. The gel was allowed to set, at room temperature,

the comb was carefully removed and the glass plate together with the gel was carefully slipped out of the gel bed and placed into the electrophoresis tank. The gel was then covered with electrophoresis buffer (1x TAE) and the samples loaded directly into the wells. The DNA samples consisted of plasmid (2-3 μg of digested or undigested DNA) or DNA standard markers, Hind III digest of λ (2-3 μg), at a final volume of 20 μl, loading buffer (1 %) (bromophenol blue in 10% glycerol) was added to each sample. Gels were run at 100 volts for 10 minutes to allow samples to enter the gel and then electrophoresis continued for a further 3 hours at 60 volts.

The gel was then placed on a transilluminator (UVP products) and the size of the plasmid fragment cut from the extracted plasmid was determined by comparison with the Hind III λ DNA size marker. If the appropriate sized plasmid / fragment was present the large scale plasmid preparation was carried out to isolate sufficient quantities of plasmid for experimental purposes.

F Large scale plasmid harvesting preparation

If the mini plasmid preparation confirmed that the bacteria were carrying the pSVori or β-galactosidase vector a large scale preparation was initiated. The remaining 3 ml of culture was used to innoculate 1 litre of Luria broth containing 25 μg/ml kanamycin for the pSVori plasmid or 50 μg/ml ampicillan for the β-galactosidase plasmid. Cells were grown overnight at 37 $^{0}$C on a shaking platform (MRV Orbital Shaker) and the culture medium was then divided between 4 x 250 ml centrifuge tubes and centrifuged at 2800 x $g_{max}$ 4000 rpm, in a JA10 rotor, Beckman centrifuge model J21 B for 15 minutes, at 4 °C. The supernatant was discarded and the pellet from each 250 ml tube was resuspended in 100 ml STE (0.1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH8.0) using a sterile spinal biopsy needle (1 mm in diameter). The resuspended cells were recentrifuged at 2800 x $g_{max}$ for 15 minutess at 4 °C as above. The supernatant was discarded and the pellet from each 250 ml flask was resuspended in 20 ml of ice cold solution I (50 mM glucose, 25 mM Tris-HCl and 10 mM EDTA, pH 8.0) and 40 ml of freshly prepared solution II (0.2 N NaOH and 1% SDS) was then added. The contents of the tubes were gently mixed and stored at 4°C for 10 minutes. Ice cold solution III (5 M potassium acetate) (15 ml) was then added and the contents mixed and stored at 4 °C for 10 minutes. The tubes were centrifuged at 8500 rpm (12,700 x $g_{max}$), JA10 rotor in a Beckman centrifuge model J21 B at, 4°C for 45 minutes. The supernatant was then filtered through 4 layers of sterile gauze into a fresh tube. The pellet was discarded and 0.6 volumes of iso-propanol at 20°C was added to the supernatant. This

mix was incubated at room temperature for 10 minutes and then centrifuged at 5000 rpm (4416 x $g_{max}$),, rotor JA-10, Beckman centrifuge model J21 B at room temperature, for 15 minutes. The supernatant was discarded and the pellet rinsed without re-suspension with 70% ethanol. The pellet was then resuspended in 5 ml of 70% ethanol and transferred to a 15 ml siliconised sterile Corex tube, the orginal tube was washed twice with 5 ml of 70% ethanol each time transferring the wash to the 15 ml Corex tube. The Corex tube was then centrifuged at 10,000 rpm (15,700 x $g_{max}$), rotor JA-13.1 at 4°C for 15 minutes, the supernatant was discarded and the pellet resuspended in 7.6 ml 1 x T.E. (10 mM Tris, 1 mM EDTA ,pH 8.0). Once the sample was completely resuspended in 1 x T.E., 8.13g of CsCl was added, dissolved, and 240 µl of 10 mg/ml ethidium bromide added. This was mixed and equally split between two 5 ml Beckman Ultra Clear centrifuge tubes, overlaid with paraffin oil to within 2 mm of the top of the tube, and balanced to within ± 100 mg, before centrifugeing at 36, 000 rpm(162,800 x $g_{max}$) in a SW55 Ti rotor, Beckman ultra centrifuge at 20°C for 40 hours.

The plasmid bands which appeared approximately in the middle of the tube were carefully removed from the caesium gradient using a Pasteur pipette and separated into 0.5 ml aliquots in 1.5 ml microfuge tubes. To each tube an equal volume of water saturated butanol was added and vortexed vigorously for 2 minutes. Tubes were centrifuged at full speed (13,000 rpm, 15,000 x $g_{max}$) for 2 minutes on a bench top microfuge centrifuge. The upper layers containing extracted ethidium bromide were removed. This procedure repeated until the butanol layer was free from the ethidium bromide and then the aqueous samples transferred to 15 ml Corex tubes before addition of 2 volumes of DEP water and 2.5 volumes of absolute alcohol at -20 °C. The tubes were mixed and stored at 4 °C overnight to precipitate the DNA. The DNA was recovered by centrifugation at 1,000 rpm (15,700 x $g_{max}$) using JS 13.irotor at 4 °C for 15 minutes in a Becknam J2 MC centrifuge. The supernatant was discarded and the pellets washed with 2 ml of 70% ethanol before recentrifugation as above and the pellets were finally air dried and resuspended in 1 ml TE.

G Quantification of purified plasmid DNA

The sample of the stock plasmid solution (5 µl) was diluted to 1 ml with water and the absorbance at 280 nm and 260 nm measured using a Philips PU9620 series spectrophotometer. The absorbance values were used to calculate the DNA concentration based on the assumption that 1 O.D. unit at 260 nm is equivalent to 50

μg/ml of DNA. The purity of the sample was quantitatively assessed by the 260 nm / 280 nm O.D. with a value of 2.0 equating to a pure DNA sample.

The pSVori and β-galactosidase plasmid prepartions were digested with the appropriate restriction enzymes and the fragments checked for size by running on an agarose gel and comparing to Hind III digested λ DNA markers as described in section 2.IIf E.

## 2.II g Transfections using Streptolysin O

Streptolysin O is a bacterial toxin. It is a 69-kDa protein produced by strains of *Streptococcus pyoenes* and other β-haemolytic group A streptococci (Bryant, 1992). Streptolysin O may be used to induce holes or pores in cells, and hence allow the entry of large molecular weight compounds in the extracellular medium or exit of components in the intracellular medium. Studies with this toxin utilising the release of radio-labelled proteins from loaded erythrocytes have shown that the membrane pores produced by Streptolysin O are in excess of 12 nm in diameter (Bryant, 1992). Evidence points to cholesterol as the adsorption and target site of Streptolysin O (Bryant, 1992). Streptolysin O (RBI, stock solution 40 international units (I.U.) / ml) was used in an attempt to permeabilise the dogfish cells to enable the plasmid pSVori or the β-galactosidase vector to enter the cells. As a control to measure the permability of the cell to low molecular components, the dyes ethidium bromide and acridine orange were used (refer to section 2.IId). These dyes were chosen as entry in to the cell could be monitored by fluoresence microscopy. As acridine orange is lipid soluble entry to the cell will be gained independent of the cell viablity causing cellular organelles to fluoresce green. Ethidium bromide however can only gain entry to the cell when the plasma membrane is disrupted as this reagent is not lipid soluble. Therefore ethidium bromide may be used to monitor the formation of pores created in the cell membrane by streptolysin O as cells fluoresce red due to the binding of this agent to the nucleic acids within the cell nucleus. The cells used were maintained in TMAO, urea and calcium free medium as described in section 2.II f i. The medium was then aspirated from the cells and the cells were incubated in urea, TMAO and calcium free SR containing various concentrations of Streptolysin O ranging from 0.5 to 10 I.U / ml. (manufactures guide recommends 2 I.U. / ml). The incubation times in the streptolysin O solution ranged from 1 minute up to 3 hours. Following aspiration of the streptolysin O solution from the cells, the cells were incubated in phenol red-free SR

containing containing 10 μg/ml ethidium bromide and 3 μg/ml acridine orange for 1 minute. The cells were then viewed under the fluorescent microscope, Leitix Dialux.

### 2.II h Spontaneous Immortalisation

During the normal growth of cells it is possible that spontaneous cell mutations could result in cell immortalisation. Therefore the primary cell cultures were maintained for as long as possible (approximately 36 days) to see if any spontaneous mutations would result in the induction of an immortalised cell line. The primary cultures were maintained under normal conditions for 36 days, however after this length of time the cells entered crisis. Although the cells all appeared to be loosing viability as judged by various morphological characteristics eg rounding of cell membrane and excessive vacuolation, they were carefully maintained by routine changes with fresh medium. However after 45 days all cells had detached from the flask / culture dish and no adherent cell lines were established.

## 2.III RESULTS AND DISCUSSION

### 2.III a The intact gland

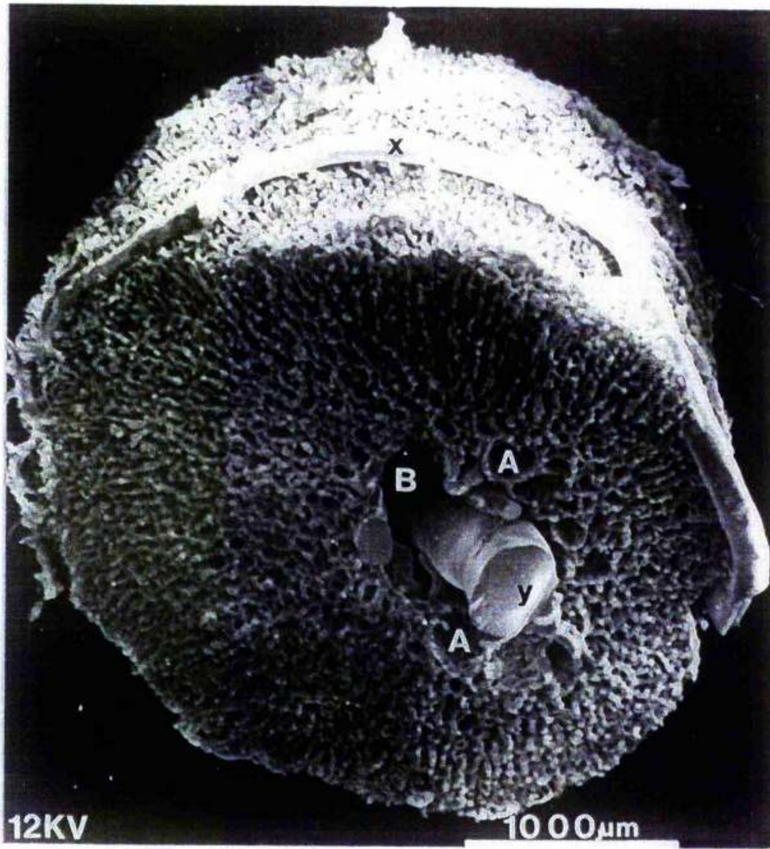
The rectal gland of the dogfish is a highly vascularised organ (figure 2.3) which consists largely of branched, blind-ended secretory tubules that radiate from a central duct (figure 2.3). The central duct presumably serves as a simple excretory conduit and is lined by a stratified epithelium 4 to 6 cells thick. The epithelial cells of the secretary tubules exhibit extensive basolateral plasma membrane interdigitations and are rich in mitochondria. Figure 2.4 shows a transverse histological section of the dogfish (*Scyliorhinus canicula* ) rectal gland, showing the characteristic structures of epithelial cells, tubules and blood capillaries in the intact gland. The tubules appear in various shapes and sizes and contain epithelial cells, connective tissue appears around the periphery of the tubules along with a small capillary network. The reagent used in this study caused the nuclei to stain black, the muscle cells, tubules and red blood cells to stain pink/red and the connective tissue and mucin to stain blue. Figure 2.4 also shows that interstitial connective tissue matrix is sparse, thus allowing the tubules to be dissociated by collagenase perfusion with minimal connective tissue contamination.

### 2.III b Cell isolation techniques and microscopy

Following tissue digestion, the dogfish rectal gland epithelial cells were plated in a culture dish at a density of approximately 33,000 cells/cm$^2$. The cells took approximately 48 hours to become securely attached to the collagen culture substratum, where after the cells rapidly began to grow and divide, resulting in a confluent monolayer some 5 to 7 days later. Figures 2.5 and 2.6 show typical cultured monolayers prepared as described in section 2.II a ii or 2.II a iii by either method I or II. There was no obvious difference in cell morphology when using either method for cell isolation, however method II resulted in a substantial increase in cell yield compared to method I. Although method I was initially used to isolate the cells, due to the low cell yields obtained and the need for multiple plates in the desired experiments, the isolation procedure was altered to that described in method II. Method II was the method of choice as it resulted in a three to four fold increase in cell yield and there was no appreciable change in cell viability. As shown in figures 2.3 and 2.4 the rectal gland is a highly vascularised organ which has limited amounts of extracellular connective tissue between the tubules and blood vessels. Method II has made use of

Figure 2.3

Figure 2.3 Cross section of a vascular corrosion cast of the rectal gland of *Scyliorhinus canicula* viewed under the scanning electron microscope This photograph was kindly supplied by Dr G Anderson. The gland was perfused with an epoxy resin which polymersised and solidified after 5-10 minutes, once hard the cast was submerged in a solution of 10 % KOH overnight to dissolve all tissue. X illustrates a single circumferential artery supplying blood vessels, Y show the large central vein draining blood from the perfused secretory tissue, A demonstrates smaller ducts, running in parallel to, and which will eventually drain into the central duct B. The scale bar represents 1000 µm.



x
B
A
y
A
12KV
1000µm

Figure 2.4

Figure 2.4 Light micrograph of a transverse section of the dogfish rectal gland. Stained with Masson's Trichrome Stain:- nuclei (N), connective tissue (CT), tubular epithelial cell (T) and capillary containing red blood cell (RBC). The scale bar represents 20 μm

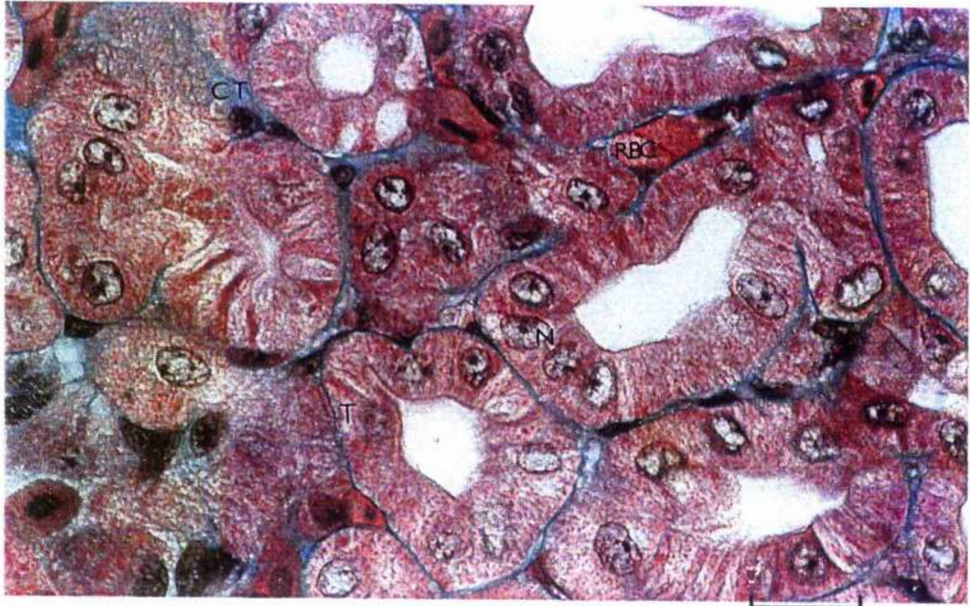
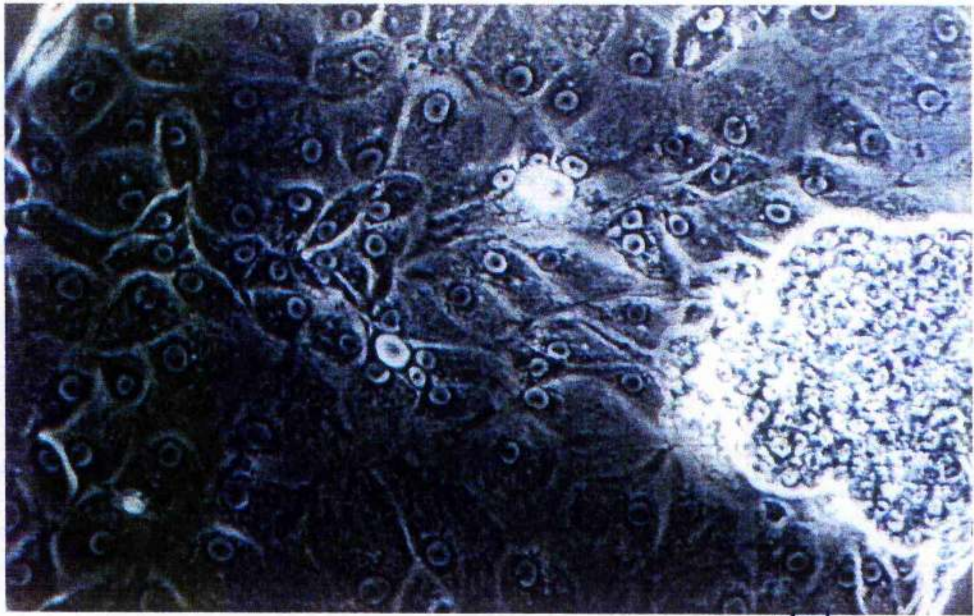
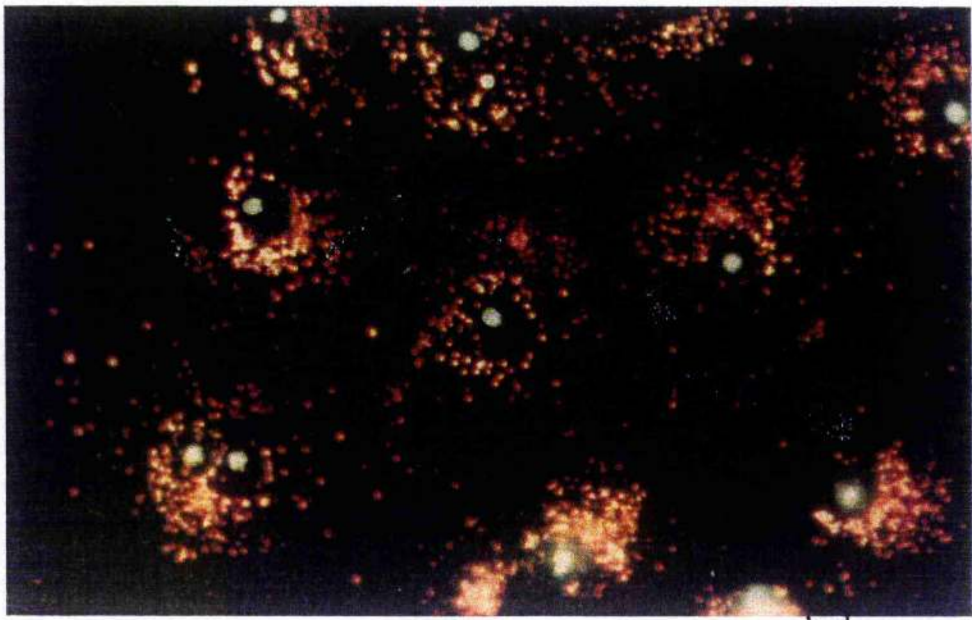

CT
RBC
N
T

Figure 2.5 and Figure 2.6

Figure 2.5 Phase-contrast micrograph of a dogfish rectal gland epithelial cell monolayer, (5-days old). The scale bar represents 20 µm

Figure 2.6 Fluorescence micrograph of a dogfish rectal gland epithelial cell monolayer, (5-days old), stained with ethidium bromide and acridine orange as reporter in section 2.II, nucleus (black), nucleolus (bright green), and lysosomes (orange-red). The scale bar represents 20 µm





the extensive capillary net work to deliver collagenase to all parts of the gland. As a result of the limited amounts of connective tissue only relatively low concentrations of collagenase (2 mg/ml) applied for quite short incubation / perfusion times (approximately 5 minutes) were required to dissociate the epithelial cells (which constitute the majority of cells and also tissue mass of the gland). The collagenase perfusion technique (method II) also eliminated the need to chop up the gland with scalpel blades which undoubtedly resulted in the loss of cells due to physical damage. Cells prepared by method II grew rapidly when either attached to collagen treated tissue culture vessels or when grown in suspension.

Transmission electron microscopy was used to further investigate the morphology of dogfish rectal gland epithelial cell cultures. Epithelial cell cultures of the dogfish rectal gland isolated from *Squalus acanthias* are described by Valentich *et al,* (1996) as having extensive basolateral plasma membrane foldings and rich populations of mitochondria. Transmission electron microscopy was used to investigate whether the cell cultures isolated from the *Scyliorhinus canicula* dogfish rectal gland exhibited similar characteristics. The cells (either cultured monolayers or cells grown in suspension) were fixed using 2.5 % w/v gluteraldehyde in SR, embedded in resin and sectioned as described in section 2.II e, before analysis under the electron microscope.

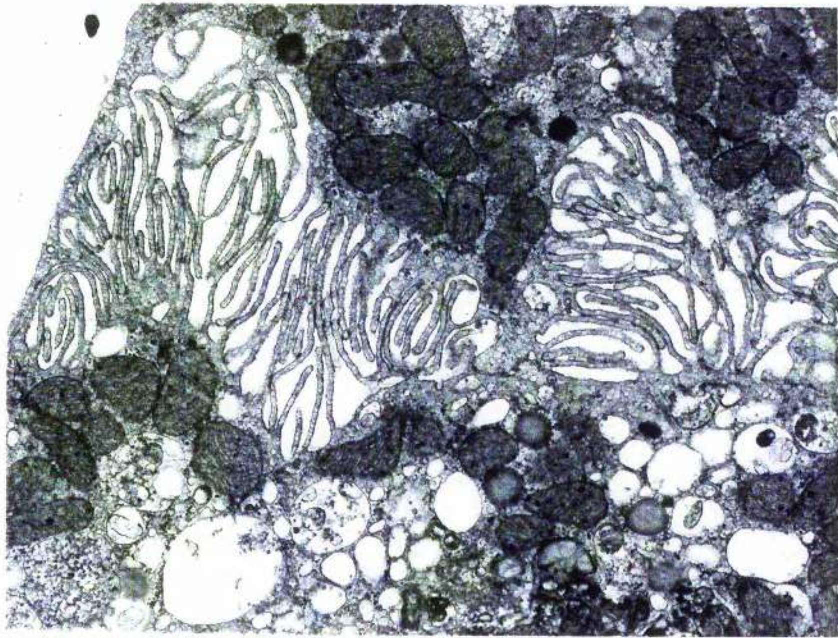
Rectal gland cells isolated from *Scyliorhinus canicula* exhibit extensive plasma membrane foldings and are rich in mitochondria as shown in figure 2.7. This micrograph also shows the presence of numeous densely stained small particles residing within the cystosol and the mitochondria. Further investigation at higher magnification (figure 2.8) identified the larger cytosolic components (A) as lipid droplets. The densely stained particles in the mitochondria (B) could represent sites of accumulation of divalent cations in a non ionised form (Cross and Mercer, 1993). Mitochondria may sequester calcium in this form and contribute to the extremely low cytosolic concentration of this ion (Cross and Mercer, 1993).

When the epithelial cells attach to the collagen-coated culture substrata they rapidly adopt a very flattened morphology over the entire culture dish, hence covering a large surface area with respect to cell volume. Although many of the cells are multi-vesiculated (figure 2,12), a significant number contain very large vacuoles which can account for as much as 90 % of the cell volume (figure 2.9). Therefore, in some cells the cytoplasm accounts for less than 10 % of the calculated cell volume. Figure 2.10

Figure 2.7 and Figure 2.8

Figure 2.7 Transmission electron micrograph of a dogfish rectal gland epithelial cell monolayer grown on a collagen coated coverslip. Magnification x 11,000. Showing the numerus plasma membrane interdigitations between adjacent cells.

Figure 2.8 Transmission electron micrograph of dogfish rectal gland epithelial cell monolayer grown on collagen coated coverslip. Magnification x 19,000.



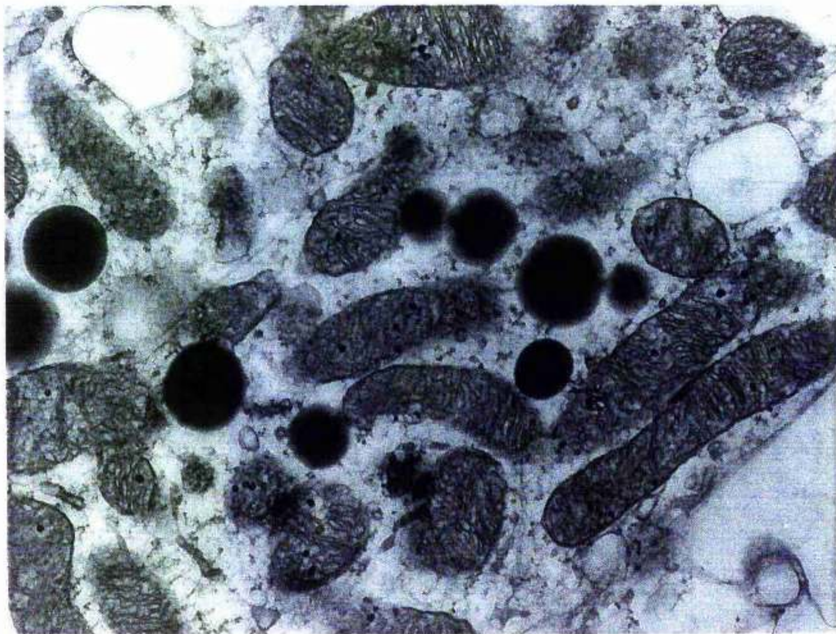
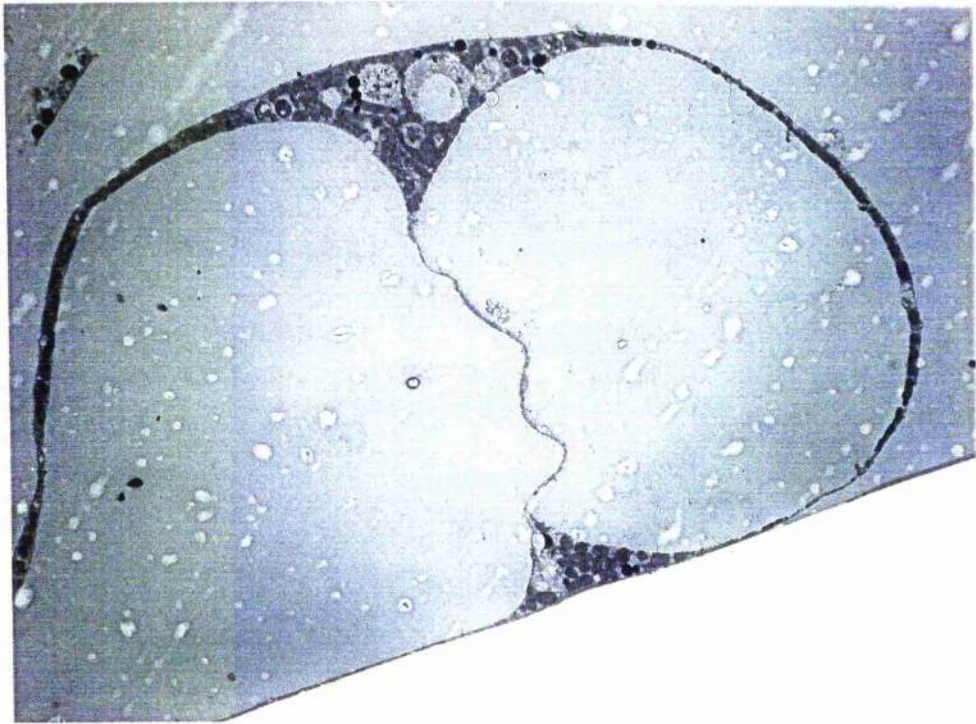
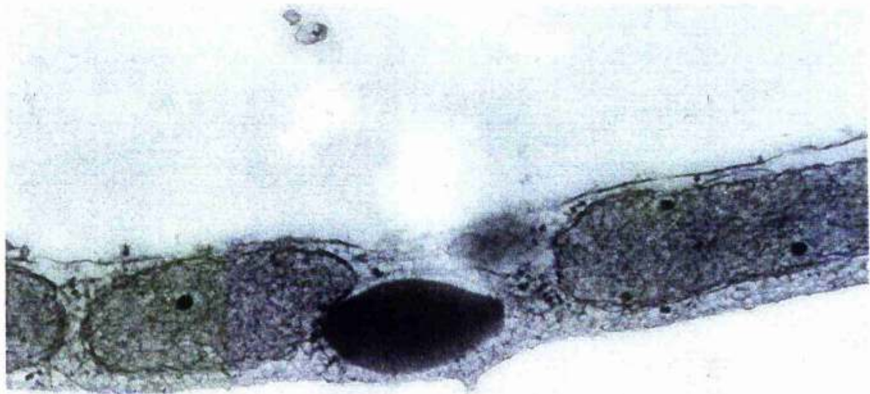


Figure 2.9 and Figure 2.10

Figure 2.9 Transmission electron micrograph of a transverse section of a dogfish rectal gland epithelial cell grown on collagen coated coverslip. Indicating the "flattened" morphology of the cells and the large intracelular vacuoles associated with a number of cells. Magnification x 2,500

Figure 2.10 Transmission electron micrograph of a transverse section of a dogfish rectal gland epithelial cell monolayer grown on a collagen coated coverslip. This figure shows a higher magnification of figure 2.9 showing that much of the cytoplasm surrounding the central vacuole is associated with mitochondria and lipid droplets. Magnification x 34,000.





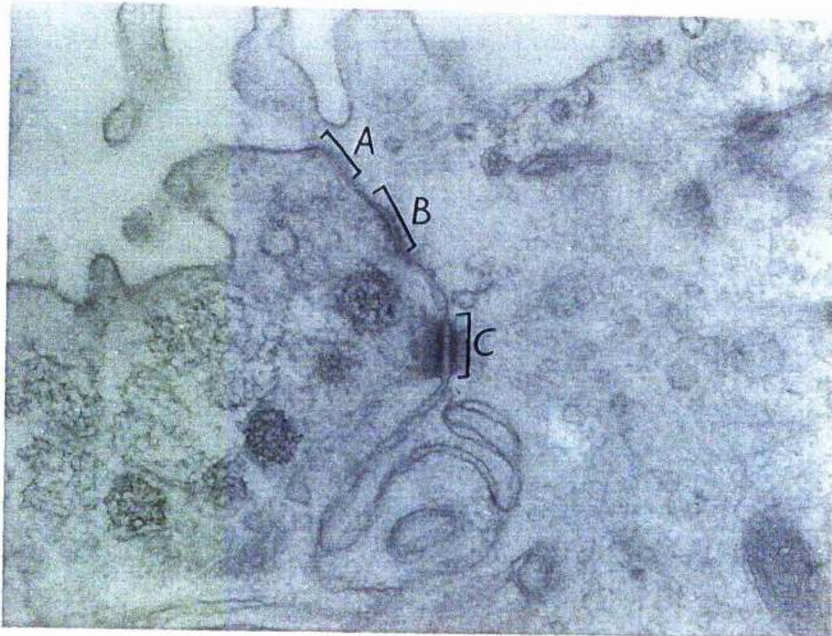
demonstrates that the area of cytoplasm present in the cell is further reduced due to the large number of mitochondria present.

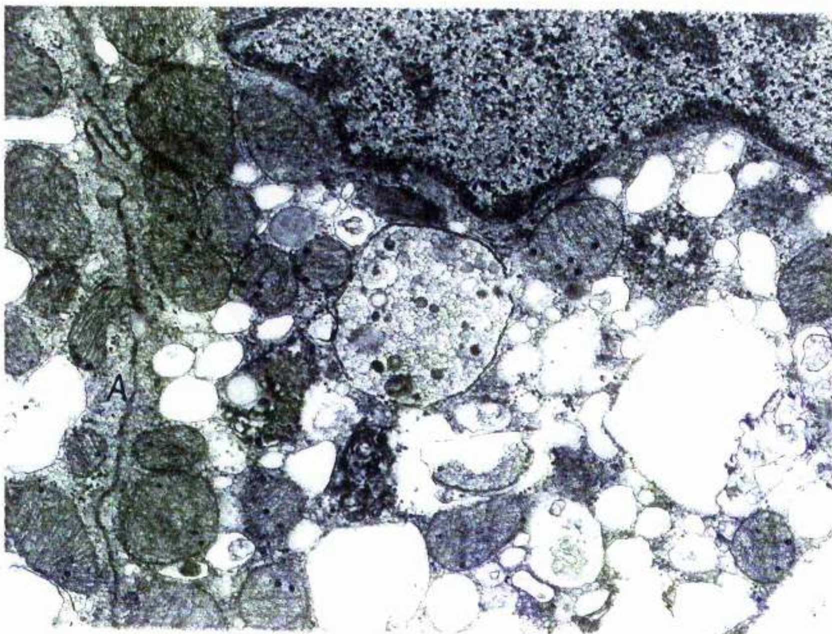
To allow transcellular transport, epithelial cells form different types of junctions (Madara *et al.* 1992). At low magnifications these complexes (e.g. zonula occludens, tight junctions etc) appear as dense strips at adjacent boundary cells. At higher magnification (eg x 34,000) as shown in (figure 2.11), it is obvious that a single complex is composed of individual junctions that have a distinct order with respect to the luminal surface: the zonula occludens, or tight junction (A), occupy the most apical position, the zonula adherens (B) in the central region, and the macula adherens, or desmosome (C) occupies the more basal junctional complex (figure 2.11). Each junction has a unique role in co-ordinating the function of the epithelial monolayer. The zonula occludens are the regions where the membranes of adjoining cells visibly fuse. Here the membranes of adjacent cells come together at regular intervals fusing adjacent two cells together effectively separating the extracellular fluid in the apical and basolateral compartments. The areas of contact are anatomising strips that continue around the entire circumference of the cell effectively preventing the movement of substances between cells. Paracellular movement of macromolecules and polar molecules is essentially prevented here and movement of ions and small non polar molecules are restricted to varying degrees (Madara *et al.* 1992). This paracellular transport through zonula occludens can vary from very leaky (electrical resistance as low as 5 $\Omega.cm^2$) to tight (2000 $\Omega.cm^2$). The degree of resistance has been correlated with the number of fusion areas that encircle the cell parallel to the surface (Schneeberger and Lynch, 1992). The zonula adherens, like the zonula occludens, is a belt-like junction that surrounds the entire cell. Actin filaments associated with this junction are part of an extensive network concentrated in the microvilli and apical cytoplasm (Tsukita *et al.* 1992). Unlike the zonula occludens and the zonula adherens the macula adherens does not surround the entire cell. This junction is only found in occasional areas of cell junctions, however associated intermediate filaments (deratin tono filaments) extend from one macula adheren to another on the cell surface (Stauffer and Unwin, 1992). Stress applied to any one macula adheren is rapidly distributed to the others by the intermediate filaments. In contrast to the tight association of cell membranes in the zonula occludens, adjacent cell membranes of adherens junctions (zonula adherens and macula adherens) appear separated by a relatively wide extracellular space (refer to Fig 2.11).

Figure 2.11 and Figure 2.12

Figure 2.11 Transmission electron micrograph of a dogfish rectal gland epithelial cell monolayer grown on collagen coated coverslip, illustrating the zonula occludens (A), zonula adherens (B) and macula adherens (C). Magnification x 34,000.

Figure 2.12 Transmission micrograph of a dogfish rectal gland epithelial cell monolayer illustrating many vacuoles throughout the cytosol and a gap junction (A). Magnification x 15,000


A
B
C


A

Cells that are in close contact, such as epithelial cells, frequently communicate with one another via gap junctions. At low magnification, as in figure 2.12, gap junctions appear as electron-dense strips between adjacent cells. The intercellular space seems occluded at this magnification, but in fact there is 2-nm gap between opposing cells. The increased electron density is an accumulation of integral membrane proteins specialised as channels between these two cells (Warner, 1992).

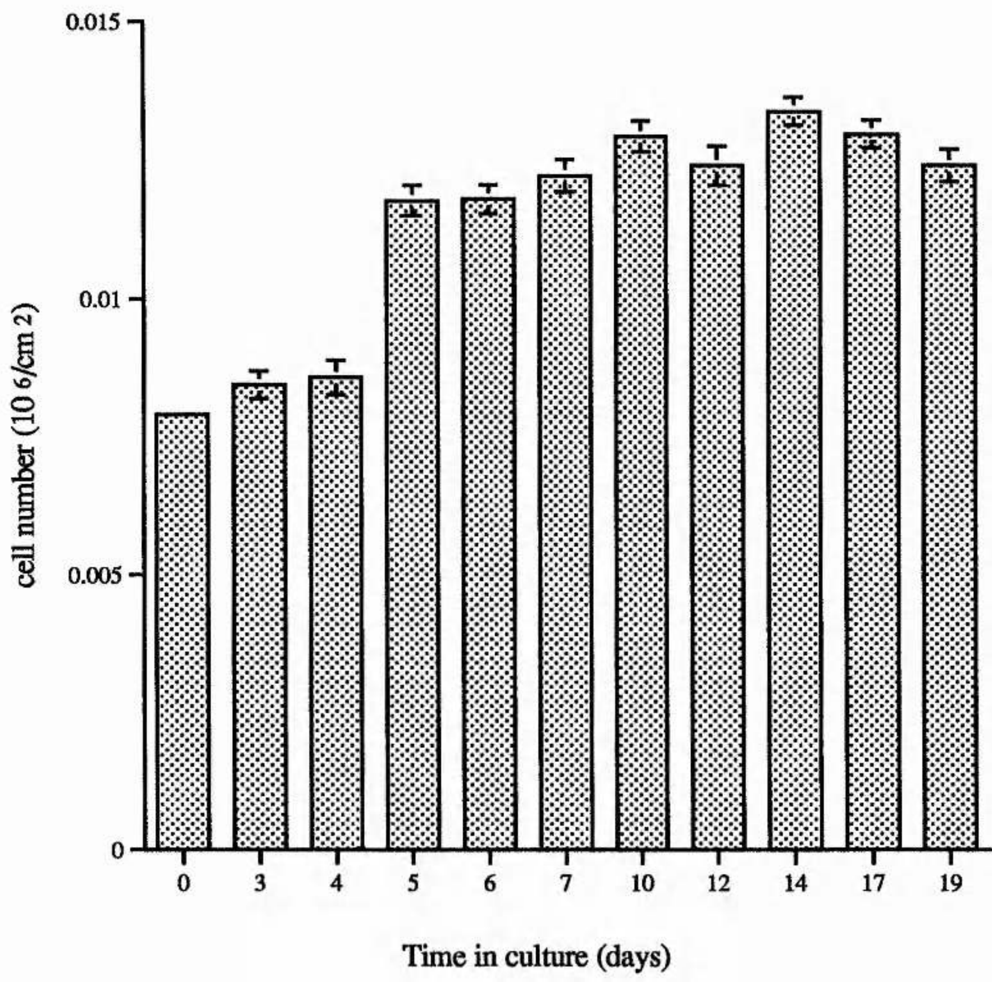
In conclusion the primary cultures established by collagenase digestion of the dogfish rectal gland exhibit the classical morphology of epithelial cells. These cells have been shown to form a complete monolayer approximately 5 days after plating (33,000 cells/$cm^2$), and on closer inspection using an electron microscope they were shown to exhibit extensive membrane folding and to be rich mitochondria in (figure 2.7) which is characteristic of ion transporting cells. Epithelial cell monolayers require specialised junctions between cells to enable them to coordinate ion transport and several of such junctions (figures 2.11 and 2.12) have also been identified in the dogfish rectal gland primary cultures. Dome/blister formations were also observed under the light microscope, these however have not been discussed in this section but have been investigated in detail in section 2.III d. It was concluded from the results of microscopic analysis that the cultured cells arising from the original cells isolated from the dogfish rectal gland were predominantly epithelial cells and were therefore suitable for use in experiments to characterise ion transport.

### 2.III c Growth charactersitics of rectal gland epithelial cells in culture

Following microscopic analysis of the primary cell cultures an experiment was carried out to investigate the growth rate of the cells. This experiment involved trypsinizing the cell monolayers at different periods and recording the cell number and cell volume as determined using a Coulter counter and channelyser. The cells were plated onto 35 mm petri dishes (33,000 cells/$cm^2$) and maintained for various periods at 18 °C before dispersion by trypsinization as described in section 2.II b. As demonstrated by figure 2.13 the cells are slow growing and do undergo a vast amount of division, cell density reaches a steady state after about 5 days in culture and cell numbers are maintained for at least 19 days. It was more difficult to estimate an average cell volume using the Coulter counter because the cells in the trypsinised suspensions ranged from 4 µm to 30 µm in diameter as determined by the channelyzer. Analysis using the light microscope showed that this was due to the cells containing various sizes of vaculoes

Figure 2.13

Figure 2.13 Effect of age of monolayer on cell density. (n=3)


0.015
0.01
0.005
0
cell number (10 6/cm 2)
0
3
4
5
6
7
10
12
14
17
19
Time in culture (days)

and some cells were incompletly digested, therefore small groups of cells were counted.

Following trypsinzation the cells were used to investigate the possibility of passaging and hence establishing a second culture. The dispersed cells were resuspended in growth medium (300,000 cells/ml) and plated onto a collagen coated substrata (33,000 cells/$cm^{2}$). However although they retained their ability to grow and divide, they did so only in suspension and cells failed to reattach to the collagen coated culture dishes. Therefore the ability of the cells to adhere to collagen coated culture substrata was lost after trypsinization. This might be due to the effect of the trypsin or might have occurred as a result of the cells being removed from their natural environment for a prolonged period resulting in the loss of this particular characteristic. Passaging of cells for reattachment was therefore not possible.

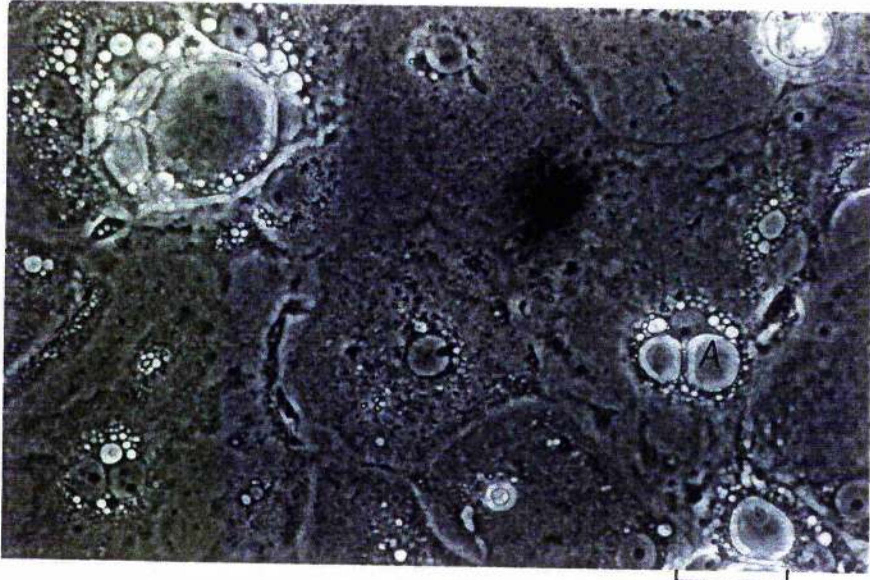
**2.III d Vacuoles and dome formations.**

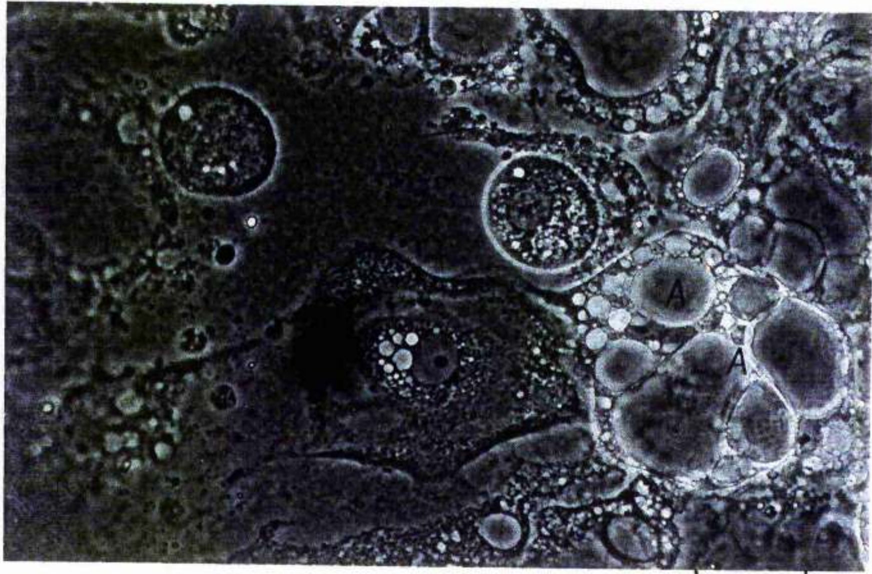
Dogfish rectal gland epithelial cells viewed under the light microscope exhibited numerous dome and blister like vacuoles which were apparent in both sub-confluent and confluent monolayers (figure 2.14). This is characteristic of many epithelial cell lines, i.e. blisters were also observed in Madin Dorby Canine Kidney (MDCK) cell cultures (Taub, 1985). Blister formation in MDCK cells is due to the lifting of the cell monolayer resulting from active transport of ions and water across the cell monolayer in an apical to basolateral direction. The basolateral surface of the cells detaches from the substrate and this results in a trapped bubble of fluid between the cell layer and the culture dish. If the fluid-filled vacuoles or domes observed with the dogfish rectal gland epithelial cell cultures were produced in the same manner as those observed in MDCK cell cultures they would be transporting in the reverse direction of in vivo ion transport in the dogfish rectal gland. Also these vacuoles would not form when the cells were grown on collagen-coated cellagen (ICN) filter inserts since fluid transported to the basolateral side of the monolayer would have be free to permeate the filter material of the insert to the basolateral fluid. However cells grown on these permeable insert supports still exhibited substantial dome/vacuole formation ( figure 2.15). It was therfore assumped that the dome formations was not due to reversed in vivo ion transport, and that they were possibly intracellular vacuoles and components of the cells. If this was indeed the case then the dome/vacuoles formations would also appear in cells which were grown in suspension.

Figure 2.14 and Figure 2.15

Figure 2.14 Phase-contrast micrograph of a dogfish rectal gland epithelial cell monolayer, (6-days old) illustrating the dome/blister formations (A). The scale bar represents 20 μm

Figure 2.15 Phase-contrast micrograph of a dogfish rectal gland epithelial cell monolayer, (5-days old) illustrating the dome/blister formations (A) when cells were grown on Cellagen supports. The scale bar represents 20 μm


A


A
A

When shark rectal gland cells were cultured in flasks or plates which were non-collagen coated, the cells did not attach to the glass or plastic but tended to adhere to each other. Lack of attachment to the substrata did not prevent cell growth and division and in fact cells in suspension were found to grow and divide rapidly forming large groups or "clumps" of cells throughout the culture medium. Figure 2.16 shows cells grown in suspension. The cells grown on collagen treated supports quite clearly demonstrated that these cells exhibited what appeared to be large vacuoles within the cytosol. The vacuoles appeared somewhat similar to the intracellular vacuoles/ domes which were associated with the cells grown on collagen coated coverslips or those grown on Cellagen inserts. Figure 2.17 shows a transverse section of a cell grown across the Cellagen membrane (this cell was fixed and sectioned using the method described in section 2.II e). The transverse section of the fixed cell indicates that certain cells contain a number of large vacuoles and in certain cases, vacuoles comprise more than 90 % of the total cell volume. These results again support the case that the vacuoles observed under normal phase contrast microscope are not due to MDCK "like" fluid filled compartments but are in fact vacuoles that exist within the cell.

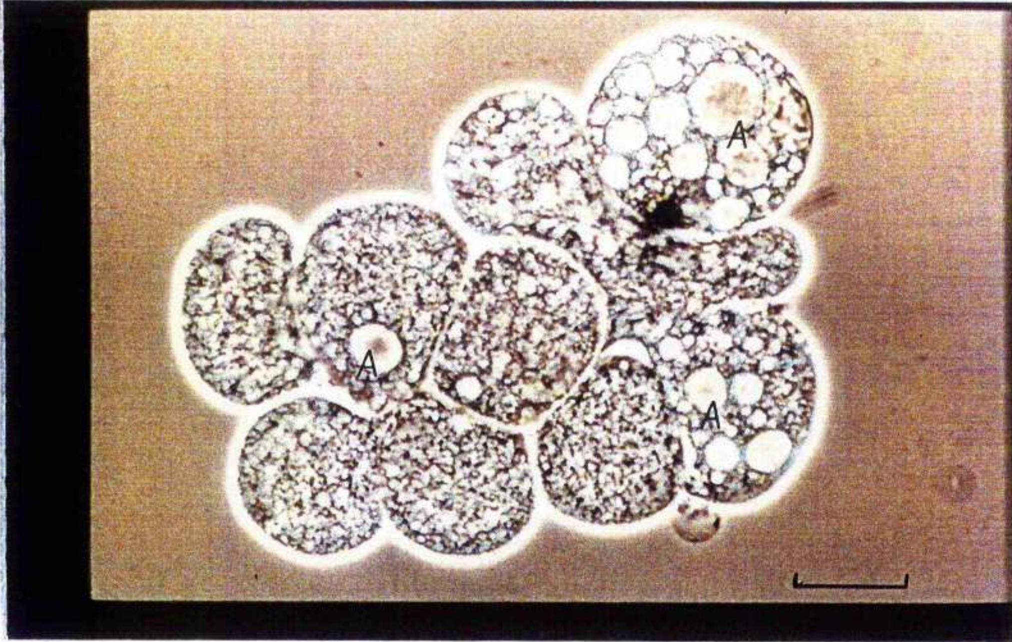
As exhibited under the light microscope, the electron microscope shows the extensive vacuolation of shark rectal gland cells grown in suspension cultures (figure 2.18) and in monolayer cultures (figure 2.19). These vacuoles are within the cells, the size of the vacuoles vary greatly and can frequently account for more than 50 % of the total cell volume. Numerous multicellular vacuoles therefore exist in cells grown either in suspension or as attached monolayers, concluding that the vacuole formations observed in dogfish rectal gland cell cultures are intracellular rather than extracellular, as seen in the dome/blister formations found in other epithelial cell monolayers.

As the observed vacuole formations were not attributed to fluid accumulation between cells caused by transcellular fluid transport, but were concluded to be intracellular vacuoles, it was therefore reasonable to presume that these vacuoles would remained intact after trypsinization of the cell monolayer. If this was in fact the case the presence of the vacuoles in some cells would explain the large variance in cell volume observed in trypsinised cell suspensions as described in section 2.IIIc. Cells were plated on to 35 mm collagen-coated petri dishes (as described in section 2.II a iii) and trypsinized to form a cell suspension (as described in section 2.II b). Examination of cell morphology under the light microscope (figure 2.20) and the electron microscope (figure 2.21) revealed that the vacuoles remained present after treatment with trypsin. This together with the fact that some cells remained as clumps or groups of up to 5 to 6

Figure 2.16 and Figure 2.17

Figure 2.16 Phase-contrast micrograph of dogfish rectal gland epithelial cells grown in suspension for 6 days. Numerous vacuoles (A) were oberved within many of the cells which grew in groups or clumps. The scale bar represents 20 μm

Figure 2.17 Micrograph of a transverse section of a dogfish rectal gland epithelial cell, in a 7 days old monolayer culture on a collagen-coated cellagen filter support. The larger intracellular vacuole is evident. The scale bar represents 20 μm


A
A
A

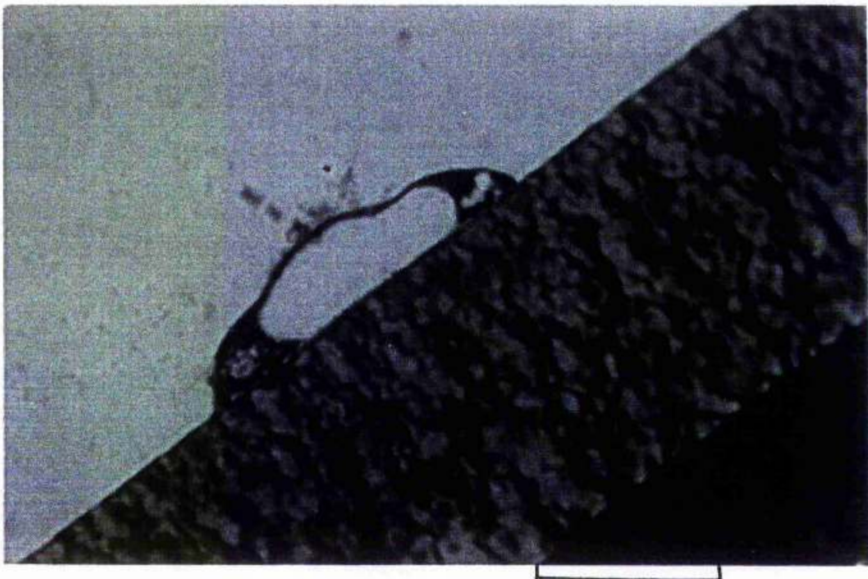
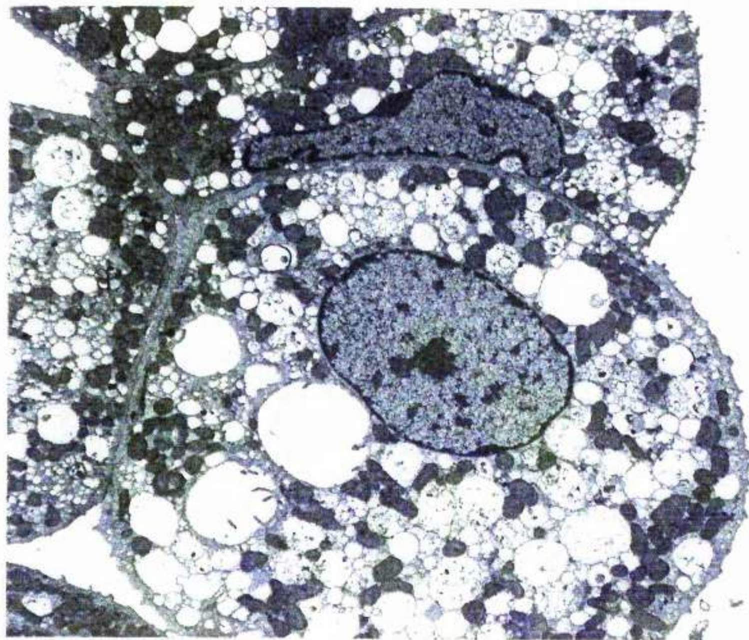


Figure 2.18 and Figure 2.19

figure 2.18 Transmission electron micrograph of 6 day old dogfish rectal gland epithelial cells grown in suspension. Magnification x 3,400

Figure 2.19 Transmission electron micrograph of a dogfish rectal gland epithelial cell monolayer grown on a collagen coated cover slip. This is a longitudial section along the plane, immediatel above and paralled to the coverslip. Magnification x 2,500.



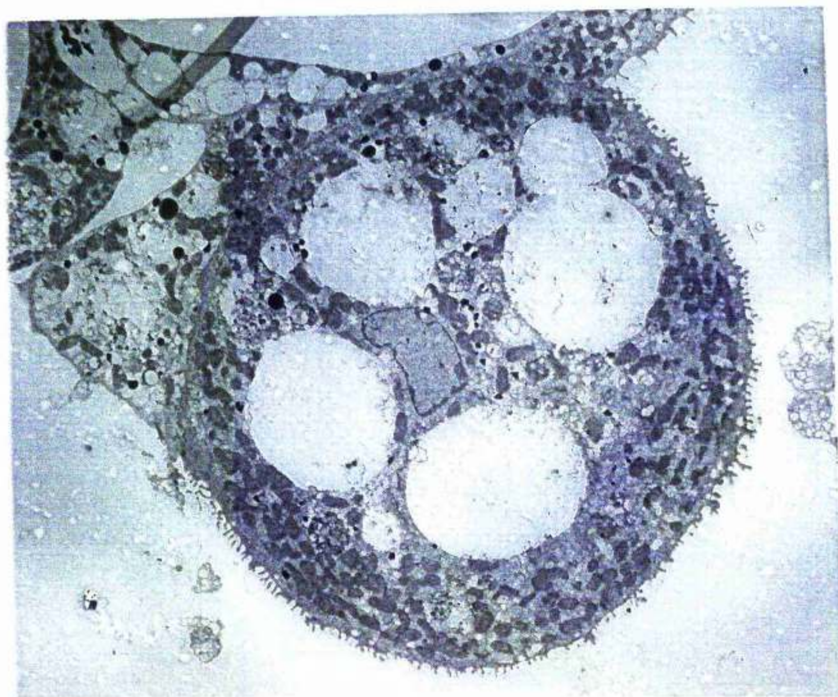
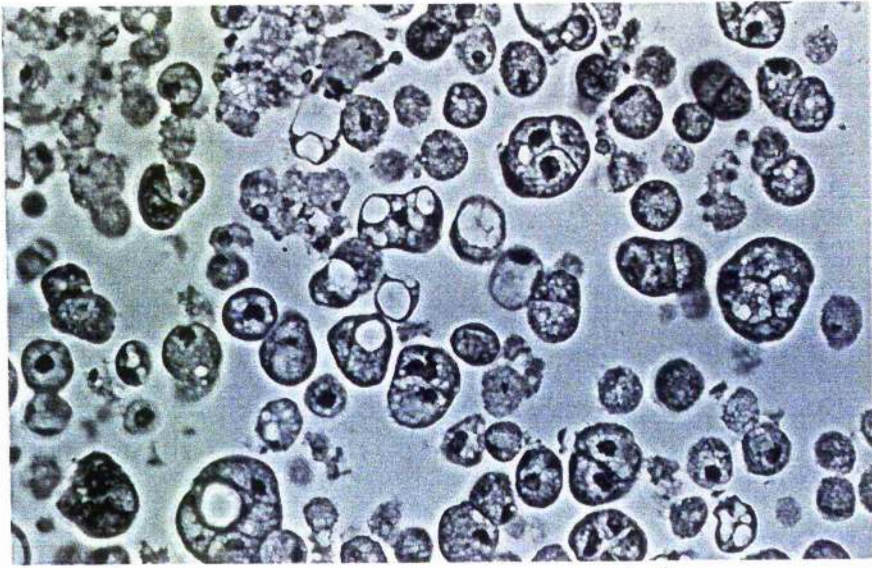
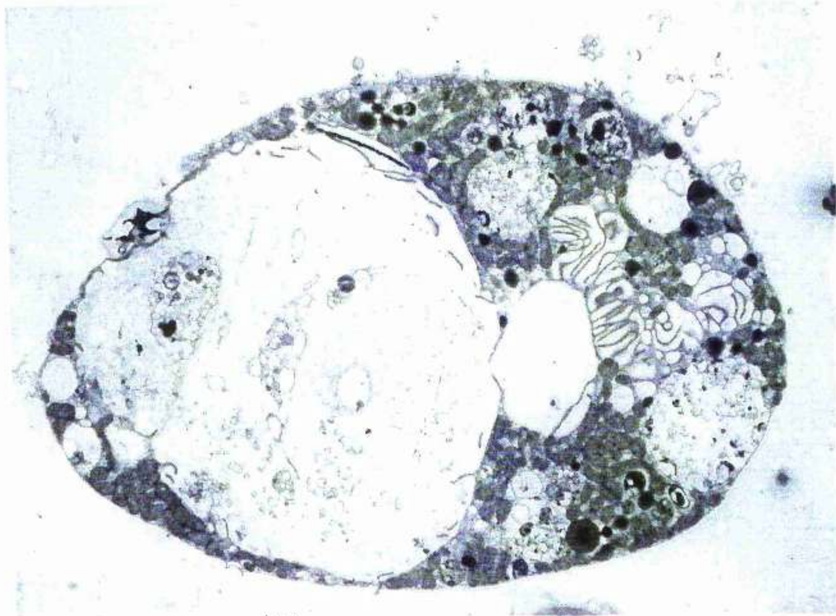


Figure 2.20 and Figure 2.21

Figure 2.20 Phase contrast micrograph of dogfish rectal gland epithelial cells in suspension after trypsinzation of a 7 day old monolayer. Size bar represents 20 μm.

Figure 2.21 Transmission electron micrograph of dogfish rectal gland epithelial cells in suspension following trypsinization of a 5 day old monolayer. Magnification x 3,400.





cells may therefore explain the large variance in apparent cell size as assessed by the couldter channelyser.

### 2.III e Cell immortalisation

An established immortalised cell line is one that has the apparent potential to be sub-cultured indefinitely. Immortalisation of primary cultures of rectal gland epithelial cells would not only eliminate the problem of fish supply, but would hopefully result in better reproducibility of cell cultures and therefore reduce experimental variability. An homogenous cell population would exist without contamination from other cells present in the rectal gland which is currently a problem with the primary cell cultures. If successful immortalisation of the dogfish rectal gland epithelial cells was achieved the routine subculturing of cells would become much simpler and large scale experiments could be carried out. However there are disadvantages involved with the use of immortal cell lines as the cells may lose some of their specialised characteristic which are retained in primary cultures. Abnormal chromosome number and loss of contact inhibition are also common occurrences due to cell immortalisation. In this study the particular concern with immortalised cell lines would be the fear that they might lose there normal ion transporting capacity and possibly lose the normal hormone / ionic regulation of transporter activity.

Some primary cell cultures, if kept for a prolonged period of time, can spontaneously form immortalised cell lines due to a mutation in one or several genes. However although dogfish epithelial cells were cultured for as long as 34 days and carefully nursed when they reached crisis no spontaneous immortalisation resulted. Therefore it was necessary to try and induce immortalisation by some other mechanism.

Establishment of an immortal epithelial cell line from the rectal gland primary cell cultures was attempted by a number of techniques including transformation using the plasmid pSVori. The plasmid pSVori contains the SV40 early region genes which have been previously reported to immortalise bronchial epithelial cells (Reddel *et al.* 1988). SV40 early region genes cause immortalisation of cell lines by causing them to express the SV40 tumour large T- antigen, a large multi function protein (90,000 daltons), the exact mechanism by which the immortalisation occurs is unknown (Reddel *et al.*1988). Cationic liposomes and poration of the cell membrane using Streptolysin O were both methods used in attempts to trasfect cells with the pSVori plasmid. In order to detect if the dogfish rectal gland cells were transfected using the

cationic liposome method, the control vector, pSV-β-Galactosidase, was used. This control vector was used as it is of similar size to the pSVori vector and has the same promoter region. Its expression was assessed by monitoring the appearance of colour reaction products either following enzyme assay of cell homogenates or by viewing coloured products generated in cell cultures by light microscopy. The same transfection protocol was followed using the human epithelial tumour cell line Hela. Despite using a number of conditions (0.5 to 5 μg of DNA, 10 - 30 μg of cationic liposomes and incubations of 6 - 16 hours) functional ß-galactosidase enzyme activity was not detected in the shark rectal gland cells following the cationic liposome transfection technique (figure 2.22). If a successful transfection had occurred the cells would have appeared blue after staining with X-gal (5- bromo-4-chloro-3-indolyl-ß-galactosidase), as cells expressing ß-galactosidase activitity, cleave X-gal at the indolyl residue causing it to turn blue. Light microscopy clearly showed that a small percentage (approximately 6%) of the Hela cells had been successfully transfected with pSV-ß-galactosidase as a number of cells stained blue due ß-galactosidase cleavage of X-gal (figure 2.23).

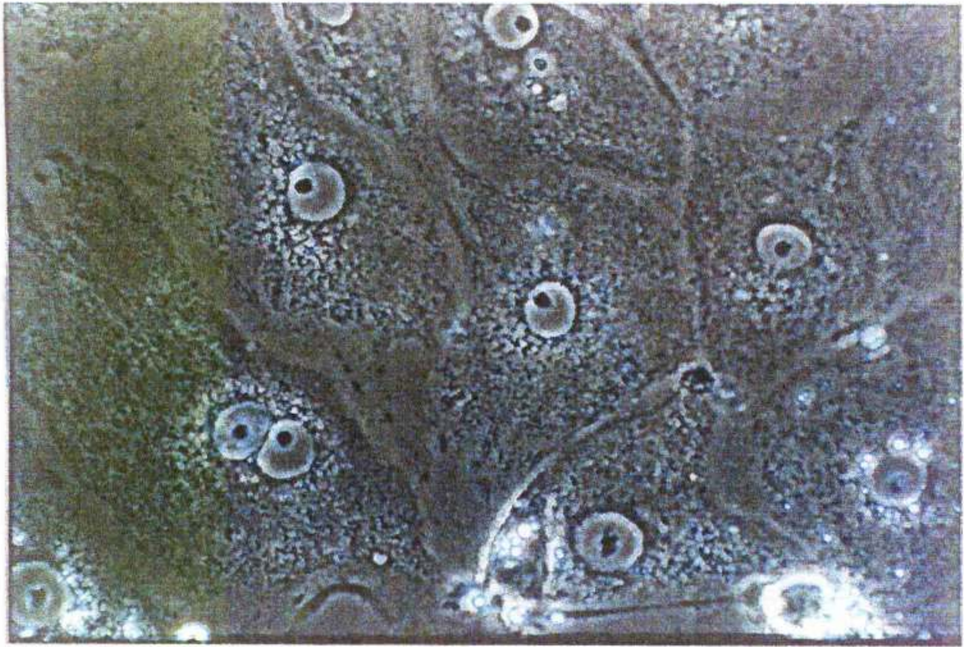
Streptolysin O was also used to permeablise the cells for the delivery of the pSVori vector. This method of DNA delivery however was also unsuccessful as at low concentration Streptolysin O (0.5-2 I.U./ml) (figure 2.24) had no effect on cell membrane permeability as judged by the exclusion of the stain ethidium bromide, whereas at higher concentrations (2.5 I.U./ml) cell integrity was disrupted to such an extent that cells could not recover from the damage on Streptolysin O removal (figure 2.25). The extent of cell damage due to Streptolysin O permeablisation was assessed by monitoring cell fluorescence with ethidium bromide / acridine orange stain. Due to the lack of cell survial following treatment with Streptolysin O, transfection with pSVori or β galactosidase was not attempted using this method.

In conclusion, attempts to immortalise the dogfish rectal gland epithelial primary cell cultures by transfection with the pSVori vector was unsuccessful. The reasons for this are unknown, however since no successful transfection was obtained with the β galactosidase vector it is possible neither of the vectors gained access to the cell cytosol. Alternatively it is possible that one or both vectors did successfully gain entry into the cell, however the transfected vectors were not transcribed/translated into functional proteins in the elasmobranch cells. Since attempts to immortalise the cell cultures established from the dogfish rectal gland failed, only primary cell cultures

Figure 2.22 and Figure 2.23

Figure 2.22 Phase contrast micrograph of dogfish rectal gland epithelial cells transfected with β-galactosidase vector using cationic liposomes for DNA delivery. Cells were treated with X-gal as outlined in section 2.II f. Presence of blue cells indicates successful transfection of the plasmid. Scale bar represents 20 μm

Figure 2.23 Phase contrast micrograph of Hela cells transfected with β-galactosidase vector using cationic liposomes for DNA delivery. Cells were treated with X-gal as outlined in section 2.II f. Presence of blue cells indicates successful transfection of the plasmid. Scale bar represents 20 μm.



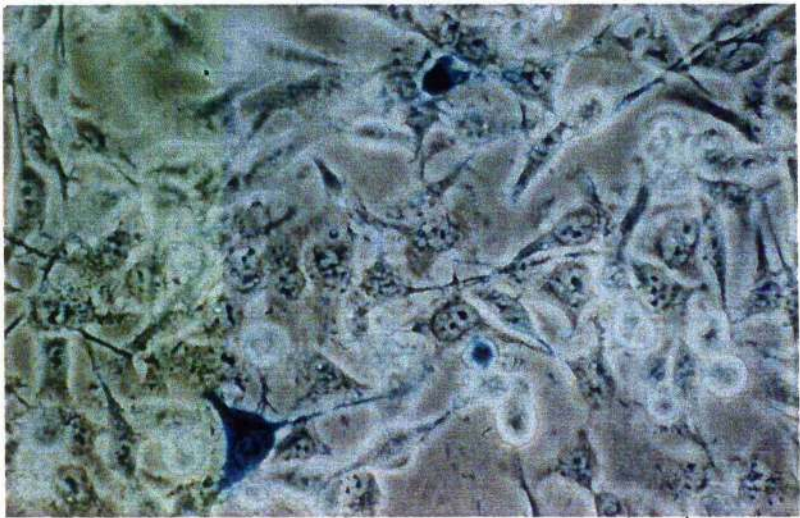
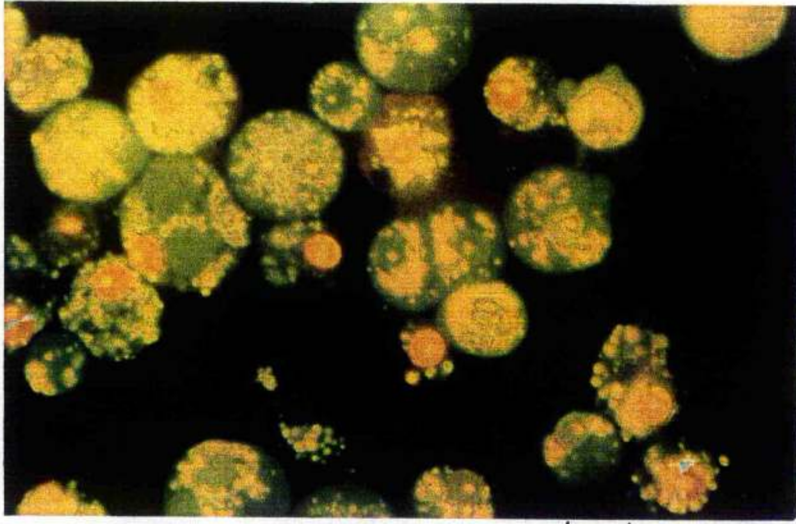
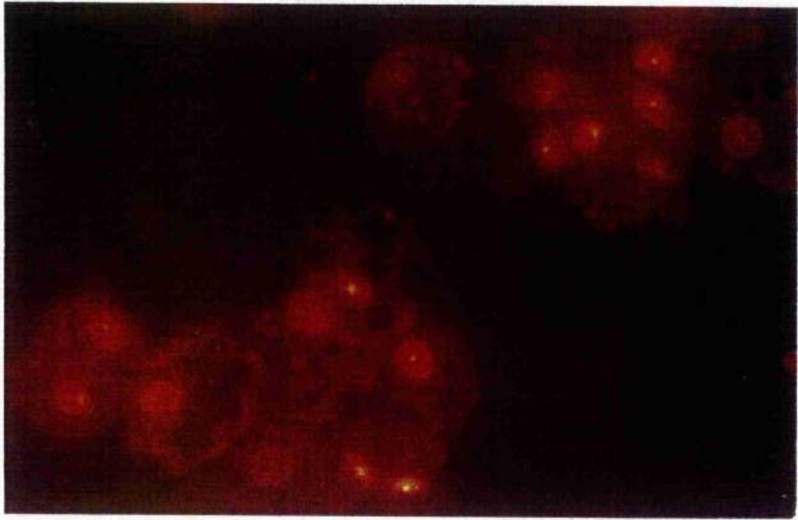


Figure 2.24 and Figure 2.25

Figure 2.24 Fluorescence micrograph of dogfish rectal gland epithelial cells following a 3 hour incubation in Streptolysin O (2 I.U./ml), and a 1 minute incubation in shark ringer containing ethidium bromide/acridine orange as described in section 2.II g.

Figure 2.25 Fluorescence micrograph of dogfish rectal gland epithelial cells following a 1 minute incubation in Streptolysin O (2.5 I.U./ml) and a 1 minute incubation in shark ringer containing ethidium bromide/acridine orange as described in section 2.II g.





could be used to investigate the effects of extracellular sodium chloride on Na,K-ATPase activity and to assess second messenger responses to hormones.

# Na,K-ATPase

## 3.I INTRODUCTION

The function of the Na,K-ATPase in eukaryotic cells is to extrude intracellular sodium ions and to import extracellular potassium ions, thus creating the desired ionic composition for optimal activity of a number of intracellular enzymes and proteins and to establish membrane electrical and ionic gradients required for secondary transport of other ions and metabolites into and out of the cell (Bertorello *et al*, 1993). The Na,K-ATPase is a highly conserved membrane enzyme essential for homeostasis of a number of intracellular, and hence body ions and metabolites. As a result of its role in the regulation of ion homeostasis the Na,K-ATPase is also central to the control of cellular volume (Rossier *et al*, 1987). In certain tissues, the Na,K-ATPase is also involved in additional roles. In particular, active coupled Na,K transport is essential for the removal of excess potassium from the extracellular space, a function essential to maintain normal excitability of muscle and nerve (Ewart *et al*, 1995). Thus release of potassium from muscle during exercise can increase the plasma levels of potassium by as much as 3 mM within minutes, and it is the concomitant increase in $[Na]_i$ and activation of Na,K-ATPase in muscle that returns plasma potassium to resting values (Ewart *et al*, 1995). In addition, the Na,K-ATPase has a major function in adjusting the cells basal membrane potential and ionic gradients, thus allowing the cell to maintain homeostasis in the face of cellular processes that utilise the sodium gradient to transport substrates and other ions. Processes that utilise the sodium gradient include uptake of most neutral amino acids, Na/Ca counter transport and Na/H exchange (Ewart *et al*, 1995). The importance of the Na,K-ATPase is further highlighted when it is considered that it is the only route for sodium extrusion available to most mammalian cells under normal physiological conditions (Ewart *et al*, 1995).

In order to drive sodium and potassium ions against their ionic gradients the Na,K-ATPase utilises the energy retained in the cells universal energy store, cytosolic ATP. Na,K-ATPase is one of the single major users of this cellular energy source; responsible for up to 40% of the steady state energy consumption of certain cells (Ewart *et al*, 1995). This expenditure fuels the extrusion of 3 sodium ions from the cell in exchange for 2 potassium ions per molecule of ATP hydrolysed. The transport ratio of 3 Na : 2 K contributes to the membrane potential, positive on the outside (Skou *et al*, 1992).

## 3.I. a. Biochemical characteristics of the Na,K-ATPase subunits

The functional Na,K-ATPase enzyme is a complex of two polypeptides, the catalytic $\alpha$ subunit and the glycosylated $\beta$ subunit, and a number of lipid molecules which are associated with the core enzyme which is embedded in the lipid bilayer of the plasma membrane. The $\alpha$-subunit is an integral membrane protein that transverses the plasma membrane from 6 to 10 times, according to different models (Ewart *et al*, 1995) (Fig 3.1). The membrane spanning segments of the $\alpha$-subunit are of $\alpha$-helical structure and the amino terminus of the $\alpha$-subunit is located on the intracellular side of the membrane (Ewart *et al*, 1995). Recent studies suggest that the C-terminus is also on the intracellular side (Skou *et al*, 1992). The $\alpha$-subunit contains the binding sites for $Na^+$, $K^+$, $Mg^{2+}$, ATP and $PO_4^{2-}$ and for the specific cardiac glycoside inhibitor ouabain (Yamamoto *et al*, 1996). The $\alpha$-subunit also has several potential phosphorylation sites (e.g. Asp 369, refer to Fig 3.1) on the cytoplasmic side which may be sites of regulation of activity by various hormones (Yamamoto *et al*, 1996). Phosphorylation of Na,K-ATPase is discussed in more detail in section 1.III c iv. The ouabain binding site is associated with the first two membrane spanning regions termed H1 and H2 and recent sequence analysis and site-directed mutagenesis have also indicated that Cys 106 in the human $\alpha$-subunit or at Cys 104 in the sheep $\alpha$-subunit plays a critical role in determining the affinity for ouabain (Yamamoto *et al*, 1996). Although the exact cation binding sites have not yet been identified, it has been suggested that the ouabain binding site is allosterically linked to a site involved in potassium binding or transport (Yamamoto *et al*, 1996).

The first mammalian $\alpha$-subunit to be sequenced was a cDNA isolated from sheep kidney by Shull in 1985. This $\alpha$-subunit contained 1012 amino acids (Shull *et al*, 1985). Since then cDNAs for the $\alpha$-subunit have been isolated and sequenced from a number of other species. In addition cDNAs encoding two additional isoforms of $\alpha$, designated $\alpha_2$, and $\alpha_3$ have been cloned from a number of species (Pressley, 1992). Significant differences in nucleic acid sequence, codon usage and patterns of genomic DNA hybridisation indicate that the $\alpha$-subunits are the products of different genes rather than the result of alternative mRNA splicing. The $\alpha_1$-transcript is typically 3.7 Kb, although an alternatively spliced product was detected in mammalian smooth muscle, which results in a truncated protein measuring 4.1 KDa in size (Medford *et al*, 1991). In rat brain, skeletal muscle, and vascular smooth muscle there are two

transcripts of $\alpha_2$ measuring 5.3 Kb and 3.4 Kb in size which arise from differential polyadenylation sites (Ohara *et al,* 1991; Young and Lingrel, 1987) and in the fetal heart the $\alpha_2$ transcript is 4.5 kb (Herrera *et al,* 1987). The rat $\alpha_3$ gene yields a transcript of 4.5 Kb in most tissues and 6 Kb in fetal brain and adult brain and heart (Medford *et al,* 1991).

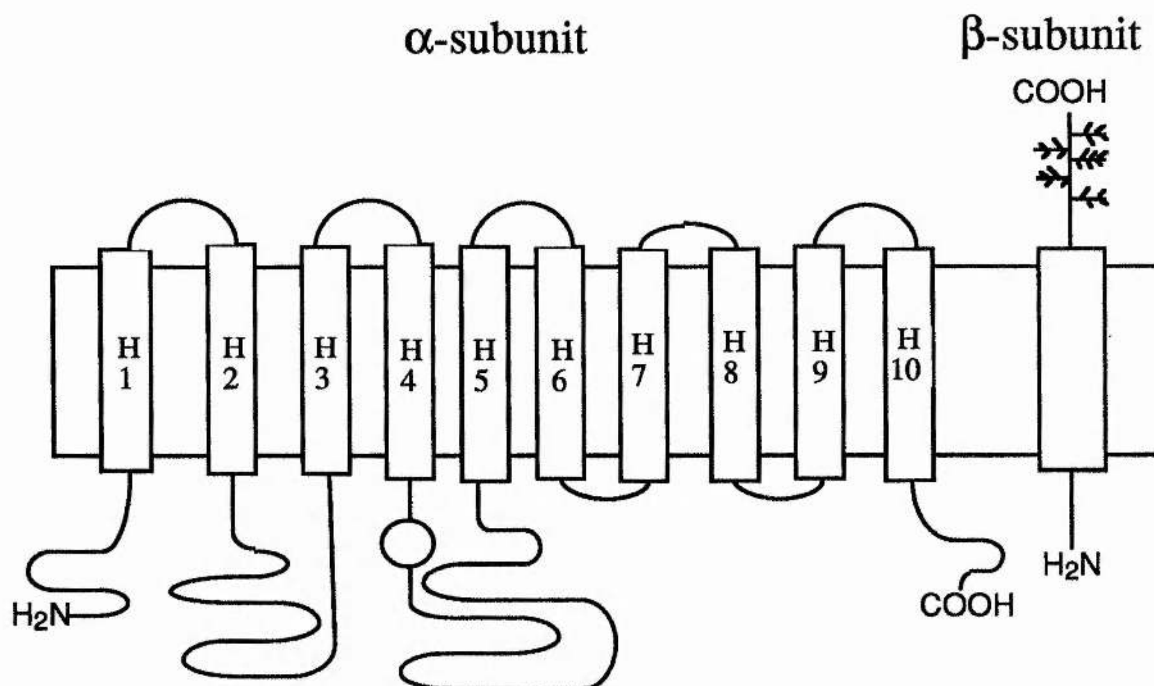



Figure 3.1 Schematic diagram of the α- and β-subunits of the Na,K-ATPase. The rectangles represent putative transmembrane sequences labelled H1-H10. The circle represents Asp 369 on the α-subunit and the branched lines on the β-subunit represent glycosylated amino acids.

The existence of different isoforms of the α-subunit has suggested that they may possess distinct kinetic properties or that they may be differential regulated. In rodents, but not in primates, the $\alpha_1$ isoform has a much lower sensitivity to cardiac glycosides such as ouabain than either the $\alpha_2$ or $\alpha_3$ isoforms (Ewart *et al,* 1995). There is also a variation between subunit isoforms in the specific affinities they have for sodium and potassium ions. The $\alpha_1$ and $\alpha_2$ subunits have a similar affinity for extracellular potassium which is 2.5 times lower than that determined for $\alpha_3$ (Ewart *et al,* 1995). The $\alpha_1$ and $\alpha_2$ subunits also have a similar affinities for intracellular sodium which are about three times higher than that of $\alpha_3$. These different kinetic properties of the α

isoforms suggest that the expression of more than one α-subunit isoform within a given cell, as occurs in muscle, heart and nerve cells, is not redundant and may provide a basis for fine tuning of pump activity in these tissues (Ewart *et al,* 1995).

The β-subunit has one membrane-spanning segment near the intracellular N-terminal of the protein, with the C-terminus, which contains a highly glycosylated domain, located on the extracellular side of the membrane (Fig 3.1). The degree of glycosylation of the β-subunit is tissue and species specific. The apparent molecular mass of the mature fully glycosylated β-subunit, calculated from its mobility following sodium dodecyl sulphate polyacrylamide gel electrophoresis, ranges from 42 to 55 KDa between species, with the protein core corresponding to only 32 KDa (Martin-Vassallo *et al,* 1989). Three isoforms of the β-subunit, designated $\beta_1$, $\beta_2$ and $\beta_3$, have been identified (Lescale-Matys *et al,* 1993). The $\beta_1$-subunit is widely distributed, occurring in most cell types (McDonough *et al,* 1990). The $\beta_2$ and $\beta_3$ subunits have also been cloned from many species (McDonough *et al,* 1990) including rat (Gloor *et al,* 1990) and mouse (Martin-Vassallo *et al,* 1989) brain and a $\beta_3$ isoform has been cloned from the amphibian nervous system (Good *et al,* 1990). Interactions between the β-subunit and the α-subunit appear to include the extracellular domain of the β polypeptide and the extracellular loop between the putative transmembrane domains H7 and H8 of the α-subunit and that this association is necessary for efficient assembly of functional pumps in the cell membrane (Fambrough *et al,* 1994; Rossier *et al,* 1987; McDonough *et al,* 1990). Several studies have provided evidence that by associating with newly synthesised α-subunits, the β-subunit facilitates the formation of the active αβ complex and regulates the amount of functional enzyme transported to the plasma membrane (Geering, 1990: Jainin *et al,* 1993; Noguchi *et al,* 1990). A role in the stability of the complex and in potassium binding has also been proposed for the β-subunit (Eakle *et al,* 1992).

## 3.I b Kinetic studies with the Na,K-ATPase

It is characteristic for the Na,K-ATPase to have two major conformations, denoted the $E_1$ and $E_2$ forms (Fig 3.2). Sodium with a $K_{0.5}$ of about 5 mM binds to the Na,K-ATPase when it adopts the $E_1$-form, $NaE_1$. It is assumed that three sodium ions bind to the Na,K-ATPase in this conformation as three sodium ions are transported. This effect is not specific for sodium, as certain organic cations such as tris and histidine can also bind and induce the $E_1$ conformation. Potassium ions bind to the Na,K-ATPase

when it adopts its $E_2$ conformation, it is assumed that two potassium ions bind to the Na,K-ATPase in the $E_2$-form, $KE_2$, as two potassium ions are transported. When potassium ions bind to Na,K-ATPase in the $E_2$ conformation they are occluded (Skou, *et al,* 1992). In this form potassium has a very low rate of exchange with potassium from the surrounding medium. The rate of transition from $E_1$ conformation state (where sodium is bound to the enzyme) to $E_2$ conformation state (where potassium is bound to the enzyme) is fast (300 $s^{-1}$), at 22°C, while the rate of transition from $E_2$ conformation state (where potassium is bound to the enzyme) to $E_1$ conformation state (where sodium is bound to the enzyme) is slow (0.3 $s^{-1}$), however both rates are influenced by the concentration of cations in both the intra and extracellular compartments (Skou *et al,* 1992). In the transfer to the $E_1$ conformation state the protein exchanges bound potassium for cytoplasmic sodium and this process is accelerated by the binding of ATP (Fig 3.2). Once the sodium ions have bound to stabilise the $E_1$ conformation the Na,K-ATPase is phosphorylated following the binding of magnesium ATP. Following phosphorylation the protein adopts the $E_2$ conformation state and once in this form the protein exchanges the bound sodium ions for potassium ions in the extracellular medium (Fig 3.2) (Jorgensen, 1981). During this transition stage the enzyme is dephosphorylated. Once the potassium ions are bound, they are occluded and the subsequent binding of ATP then causes the protein to change to the $E_1$-form which accelerates the release of potassium into the cell cytoplasm in exchange for sodium ions. ATP then phosphorylates the protein and the cycle continues as described above (Fig 3.2). Magnesium ions are required for phosphorylation, conversion between the phosphorylated $E_1$ conformation state and the phosphorylated $E_2$ conformation state, and for dephosphorylation (Jorgensen, 1981).

The cardiac glycosides including ouabain are specific inhibitors of the Na,K-ATPase (Skou *et al,* 1992). They inhibit Na,K-ATPase activity by binding to the enzyme on the extracellular side of the membrane. The rate of inhibition is increased by the phosphorylation of the enzyme. When the phosphoenzyme is formed in the presence of Mg, Na and ATP, addition of potassium protects against inhibition of cardiac glycosides, probably due to the dephosphorylating effect of potassium and the competion that exits between potassium and ouabain for the binding site.

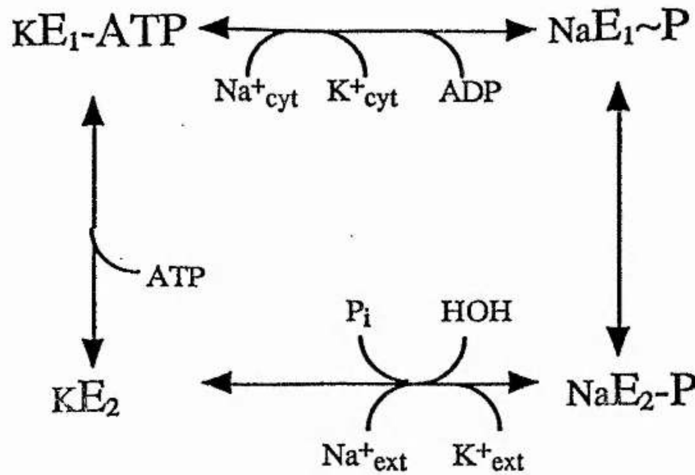



Figure 3.2. Ilustration of the relationship and the principal conformations of the Na,K-ATPase to the reaction states identified in kinetic studies (Jorgensen, 1982). Where K represents potassium ions, Na represents sodium ions, ext represents extracellular and cyt represents cytoplasmic.

## 3.I c Regulation of Na,K-ATPase activity

The activity of the Na,K-ATPase is not constant but is exquisitely malleable, responding to moment-to-moment changes in the ionic environment as well as to situations where a more sustained change in its activity is required. This is achieved by complex regulatory processes which involve biochemical input at various levels, including changes in gene expression and subcellular distribution of Na,K-ATPase.

### 3.I c i Short term hormonal regulation of Na,K-ATPase activity

Short-term modulation of Na,K-ATPase activity is effected by numerous hormones, autocoids, and other compounds that affect sodium and potassium excretion (Bertorello *et al.* 1993). Acute alterations in Na,K-ATPase activity can occur by various cellular mechanisms and multiple regulatory pathways. Hormones known to effect Na,K-ATPase activity include aldosterone, insulin, thyroid hormone and the catecholamines, however the precise molecular mechanisms by which they alter Na,K-ATPase activity

are not understood. To generate their regulatory responses, hormones often elicit both rapid and sustained changes in Na,K-ATPase activity. A need for rapid and sustained switches in activity arises in response to variable salt intake, stress, or exercise. Accordingly, hormones such as aldosterone, insulin and adrenaline exert rapid and long term actions on pump activity in their target tissues.

Rapid changes in Na,K-ATPase activity can be brought about by either sudden variations in substrate concentrations or by changes in the kinetic properties of the pump through modifications initiated by a signalling cascade (Bertorello *et al,* 1993). Under normal steady-state conditions, the Na,K-ATPase is not saturated by the prevailing levels of intracellular sodium (Horowitz *et al,* 1990). This implies that a gain in intracellular Na can drive its own extrusion simply by providing more substrate for the unsaturated pump. The change in Na,K-ATPase activity in response to variations in intracellular Na concentration is not strictly speaking, a regulatory response (Ehrenfeld *et al,* 1992). However changes in intracellular sodium can affect total Na,K-ATPase activity through a regulatory mechanism, in addition to its mass action (Stanton *et al,* 1989). This mechanism may be utilised by several hormones that regulate pump activity. True regulatory changes in Na,K-ATPase activity are induced by signalling cascades linking the event of hormone receptor binding to Na,K-ATPase expression or to modifications in Na,K-ATPase activity. However whether these signalling cascaded act by directly phosphorylating the Na,K-ATPase or by acting on an other component that then affect the enzyme action is still open to debate. Whatever the mechanism, short-term regulation of the Na,K-ATPase by hormones is presumed to involve a change in its turnover number (i.e. the rate of synthesis or degradation of Na,K-ATPase), its affinity for substrates or its abundance at the cell surface as a result of translocation of pre-formed units from an intracellular storage compartment or activation of already membrane bound protein (Bertorello *et al,* 1993).

Increases in intracellular sodium concentration induces recruitment of pre-formed Na,K-ATPase units from intracellular stores in to the cell membrane in rabbit cortical collecting tubules. This process is aldosterone dependent (Blot-Chabaud *et al,* 1990). Aldosterone also elicits a direct and rapid (within 15 minutes) stimulation of Na,K-ATPase activity in rat cortical collecting renal tubules, an effect which is accompanied by a rise in intracellular sodium (Shahedi *et al,* 1993). This effect was blocked by amiloride, suggesting sodium entry through either sodium channels or the Na/H exchanger is directly associated with the increase in Na,K-ATPase activity. The effect of aldosterone was independent of new protein synthesis but could be blocked by

colchicine, a compound that disrupts the microtubular transport system (Shahedi *et al,* 1993). It was concluded that the acute activation of the Na,K-ATPase by aldosterone is a sodium dependent process involving recruitment of previously inactive pump units to the plasma membrane, perhaps facilitated by the microtubular network (Shahedi *et al,* 1993).

Catecholamines have been reported to inhibit Na,K-ATPase activity by reversible phosphorylation of its catalytic subunit by protein kinase A (PKA) (Podevin and Parinin, 1989). The catecholamines activate adenylate cyclase increasing cAMP concentrations, hence activating intracellular PKA (Podevin and Parinin, 1988). Increases in intracellular cAMP concentration, depending on the tissue examined, can either inhibit or stimulate activity of the Na,K-ATPase (McDonough and Farley, 1993), presumably by acting on different intracellular proteins present in the tissue type. Protein kinase A can phosphorylate the $\alpha$-subunit of Na,K-ATPase at an unique, highly conserved serine residue located in the cytoplasmic loop between the putative transmembrane segments H8 and H9 (Beguin *et al,* 1996). Increasing intracellular cAMP levels by means of forskolin, 3-isobutyl-1-methylxanthine (IBMX) or theophylline treatment inhibits Na,K-ATPase activity in adrenal chromaffin cells (Fisone *et al,* 1994) and in addition treatment of isolated proximal tubes with an inhibitor of PKA (PKI-5-24-amide) stimulates the activity of the Na,K-ATPase (Chibalin *et al,* 1992). However in contrast to these findings cAMP is thought to stimulate Na,K-ATPase activity in the shark rectal gland of the *Squalus acanthias* as incubation of cells in the presence of dibutyryl cAMP and the phosphodiesterase inhibitor theophylline reduces cell sodium content and cAMP stimulates both ouabain-sensitive oxygen consumption and $Rb^+$ uptake (Lear *et al,* 1990).

In some tissues, insulin stimulates Na,K-ATPase activity by a mechanism secondary to the elevation of intracellular sodium. It could be argued that this may not be strictly a regulatory response, but rather one elicited by the increase in substrate. This insulin effect has been detected in BC3H1 cells (Rosie *et al,* 1985), as well as in rat hepatocytes (Fehlmann and Freychet, 1981). In these two cell types, the stimulation of the Na,K-ATPase by insulin was the result of a doubling of intracellular sodium due to prior stimulation of the Na/H exchanger by the hormone. How insulin activates the Na/H exchanger is unknown however stimulation of Na,K-ATPase is blocked by amiloride (Rosie *et al,* 1985). The increase in intracellular sodium is also the basis for the stimulation of the sodium pump by insulin in 3T3-F422 adipocytes (Brodsky, 1990). However the Na,H exchanger inhibitor, amiloride does not block the

stimulatory effect of insulin on the pump in these cells as an insulin-sensitive sodium channel is responsible for the increase in intracellular sodium (Brodsky, 1990). In 3T3-L1 adipocytes, the stimulation of ouabain-sensitive rubidium (potassium) uptake by insulin can be prevented by bumetanide (Sargent *et al,* 1995), an inhibitor of the Na, K, Cl- cotransporter. This suggests that sodium uptake through cotransporter activity is required for the activation of the pump. This idea was reinforced by the findings that insulin increased bumetanide-sensitive rubidium uptake (Sargent *et al,* 1995). The expected net result of the activation of the Na, K, Cl-cotransporter and the Na, K-ATPase would be a gain in intracellular KCl with potassium entering through both of the pathways, chloride entering through the cotransporter, and sodium leaving the cytoplasm through the Na,K-ATPase. The gain in intracellular chloride concentration may be of significance, since it has been postulated that this ion can act as a "second messenger" in the activation of other channels (Sargent *et al,* 1995) (section 1.IV a). The gain in KCl would lead to an increase in cell volume, and changes in cell volume in response to insulin have been implicated, although the exact mechanism remains to be established (Sargent *et al,* 1995).

Activation of the Na,K-ATPase in response to insulin-mediated increases in intracellular sodium may not be the only response seen to insulin as direct regulatory mechanisms have also been described for the stimulation of the enzyme. In rat adipocytes which express substantial amounts of the $\alpha_2$-isoform in addition to $\alpha_1$ (Resh *et al,* 1980; Lytton, 1985), the fraction of Na, K-ATPase with high sensitivity to ouabain ($\alpha_2$-isoform) is low, so that the $\alpha_1$-isoform is responsible for the majority of the basal pump activity. Administration of insulin was found to preferentially activate the component of Rb (K) uptake ascribed to $\alpha_2$, but evidence has shown that the activity of the $\alpha_1$-subunit is also stimulated by insulin to a lesser extent (Lytton, 1985). The mechanism underlying the activation of the pump by insulin in rat adipocytes is still unknown, but insulin did not alter the entry of sodium therefore decreasing concentrations of intracellular sodium were found following Na,K-ATPase activation. The insulin-mediated stimulation has been attributed to an increase in the affinity of both the $\alpha_1$ and $\alpha_2$-isoforms for sodium as well as to an increase in the $V_{max}$ of the $\alpha_2$-subunit (McGill *et al,* 1991). In conclusion the mechanism of the short term up-regulation of Na, K-ATPase activity by insulin is tissue dependent and has been reported to be due to either increases in the concentration of intracellular sodium associated with insulin-induced, bumetanide-sensitive or amiloride-sensitive sodium uptake (Bertorello *et al,* 1993), and/or rat adipocytes, to an an unknown direct action

which is not dependent on increased intracellular sodium concentration (Resh *et al,* 1980; Lytton, 1885).

In conclusion hormones that control short term regulation of Na,K-ATPase activity can elicit their response by either directly changing the catalytic activity of functional units possibly by phosphorylation, by affecting the distribution of pump units between the cell surface and some intracellular compartment or by increasing the availability of the limiting substrate, sodium, by increasing influx into the cell by other transporters. The possible mechanisms associated with pump phosphorylation/dephosphorylation are uncertain and this topic of Na,K-ATPase regulation is further disscussed in section 1.III c iv.

### 3.I c ii Short term regulation of Na,K-ATPase activity associated with the cell cytoskeleton.

Actin is a major constituent of the cytoplasm, particularly in the microenviroment immediately next to the plasma membrane (Cantiello *et al*, 1991). The ability of monomeric actin to assemble and polymerise, in a reversible fashion, into filaments and other complex structures has been associated with agonist induced changes in cell shape and motility (Cantiello *et al*, 1991). In addition to actin, several cytoskeletal proteins are responsible for guiding and providing spatial orientation between molecules within the cytoplasm and the plasma membrane. For example the cytoskeletal protein spectrin, is known to bind to certain ion transport proteins including epithelial sodium channels (Smith *et al*, 1991). In MDCK cells, Na,K-ATPase is co-localised with two cytoskeletal actin binding proteins, ankyrin and fodrin (Morrow *et al*, 1988). In addition to its structural linkage to the Na,K-ATPase, the actin based cytoskeleton appears to be functionally associated with it as well. Enzyme activity may be regulated in the short term by actin (Morrow *et al*, 1988). Incubation of purified Na,K-ATPase with G actin results in a time and dose dependent stimulation of enzyme activity (Morrow *et al*, 1988). As the Na,K-ATPase assay medium favours actin polymerisation, the following experimental observations suggest a role for short actin filaments in Na,K-ATPase regulation:- 1) stimulation of Na,K-ATPase activity by actin is prevented by deoxyribonuclease (DNase) I which inhibits actin polymerisation whereas activation is potentiated by cytochalasin D, which favours polymerisation but prevents formation of long polymers, 2) stimulation does not occur in the presence of long actin polymers (F actin) indicating that short actin filaments are probably responsible for this effect, whereas filamentous (F) actin is not (Bertorello *et al*, 1993).

Because monomeric actin binds to the enzyme but does not alter its activity, it is likely that in intact cells the subsequent elongation into short polymers is responsible for the stimulation of Na,K-ATPase activity. It remains unclear whether monomeric actin binds to the $\alpha$ or $\beta$ subunits or both. Although it appears more likely that the binding site is on the $\alpha$-subunit with the peptide domain between amino acids 646-665 as a strong candidate as this range of amino acids bear a strong similarity to the actin binding domain of several actin binding proteins such as gelsolin and villin (Way *et al*, 1992). The effect of actin on Na,K-ATPase activity does not require interaction with other cytoskeletal proteins because it occurs with the purified enzyme (detergent solubilised), which is devoid of other proteins such as ankyrin and fodrin. Stimulation of Na,K-ATPase activity by actin is associated with an increase in its affinity for sodium by approximately 38% but not for K, Mg or ATP suggesting that actin induces a change in the enzyme conformation to the high affinity sodium form ($E_1$ conformation) (Bertorello and Cantiello, 1992).

The ability of monomeric actin to polymerise is controlled either by actin binding proteins which act via intracellular messengers such as cAMP (Pollard and Cooper, 1986) or directly by covalent modification of the actin molecule through the action of protein kinases (Ohta *et al.* 1987). Phosphorylation of the actin molecule by PKA modulates its ability to polymerise and its ability to regulate Na,K-ATPase activity (Ohta *et al.* 1987). As PKA can be activated by increases in cellular cAMP, hormones that induce increases in cellular cAMP concentration can potentially increase Na,K-ATPase activity via interactions of the enzyme with the actin-based cytoskeleton (Ohta *et al.* 1987).

### 3.I c iii Long term hormonal regulation of Na,K-ATPase activity

Hormones that act to cause sustained changes in Na,K-ATPase activity generally do so by increasing the total number of available enzyme units on the cell surface. Regulation of the overall abundance of Na,K-ATPase subunits is controlled at the levels of transcription, transcript stability, translation rate, rates of protein turnover and movement to and from intracellular compartments (Bertorello *et al*, 1993). Increases in the rate of transcription and or stability of mRNA of the $\beta$-subunit can lead to increased levels of $\alpha\beta$-complexes, due to stabilisation of $\alpha$-proteins that otherwise would be degraded if not associated with the $\beta$-subunit (McDonough *et al*, 1990). This conclusion was reached after excess $\alpha_1$-mRNA was generated by genetic manipulation

of frog ooyctes and β-subunit-mRNA was not increased. In this study an increase in $\alpha_1$-mRNA alone did not result in an increase in Na,K-ATPase activity (Noguchi *et al*, 1990). This study suggests that transcriptional regulation of a single subunit gene cannot alter the net abundance of functional Na,K-ATPase.

Thyroid hormones ($T_3$ and $T_4$) stimulate Na,K-ATPase activity by increasing the number of Na,K-ATPase molecules expressed in the plasma membrane. Indeed, in hypothyroidism, there is a decrease in the Na,K-ATPase activity in several tissues (Lingrel *et al*, 1990). The stimulating input from thyroid hormone occurs at various levels including gene transcription of α and β subunit genes, the time of transport of transcripts from the nucleus to the cytoplasm and the level of mRNA stability (Lingrel *et al*, 1990). The regulation of Na,K-ATPase by thyroid hormone is isoform specific and varies between tissues and species. Hyperthyroid rats show no change in skeletal muscle of $\alpha_1$ and $\beta_1$ mRNA or protein whereas $\alpha_2$ isoform mRNA and protein are both increased by 5 fold (Haber *et al*, 1988; Aauma *et al*, 1993). In contrast, in the kidney $\alpha_1$ and $\beta_1$ subunit mRNAs and their respective proteins are coordinately elevated by 1.6 fold in hyperthyroid rats (McDonough *et al*. 1988; Aauma *et al*, 1993).

Aldosterone increases long term Na,K-ATPase activity by inducing the synthesis of new α and β subunits. This has been observed in diverse tissues including toad bladder, rabbit kidney and colon and rat kidney, heart and skeletal muscle (Ewart, *et al*, 1995). This response involves interaction of the aldosterone receptor complex with specific hormone responsive elements in the promoter regions for the $\alpha_1$ and / or $\beta_2$ subunit genes. The effect of aldosterone on Na,K-ATPase expression is confined to the $\alpha_1$ and $\beta_2$ subunits. Treatment of rabbits with aldosterone for several days via a minipump implant, markedly induced the expression of the $\alpha_1$ subunit in cortical collecting tubules (Ewart, *et al*, 1995).

The long term actions of insulin on Na,K-ATPase activity have been studied to a lesser extent than the short term actions of this hormone. It has been shown that exposure of rat aortic vascular smooth muscle cells to insulin for up to 6 hours resulted in increases in the level of $\alpha_2$ mRNA by up to 30% with no equivalent changes in $\alpha_1$ mRNA (Harinder *et al*, 1991; Sampson *et al*, 1994). It has also been shown that when 3T3-L1 fibroblasts cell cultures are exposed to insulin to attain their differentiated phenotype, removal of insulin during cell differentiation causes the lowering of $\alpha_1$ mRNA and the increased of $\alpha_2$ mRNA with no effect on β-mRNA (Russo and Sweadner, 1993). These results suggest that insulin might maintain a state of high $\alpha_1/\alpha_2$ ratio in

differentiated adipocytes. Like the actions of the thyroid hormones the long term actions of insulin on Na,K-ATPase appear to be species and tissue specific.

Cortisol and growth hormone have both been shown to increase Na,K-ATPase activity in teleost chloride cells following migration from fresh water into sea water (Hourdry, 1995). This increase in activity by cortisol is attributed to increased expression of the $\beta$ 1 subunit in *salmoniforms* (Richman and Zaugg 1987) and increased expression of both $\alpha$ and $\beta$ subunits in *anguilla anguilla* (Suzuke and Hirano, 1991). The mechanism of growth hormone action has not yet been defined. Specific receptors, with a high affinity for the hormone have been discovered in the gill chloride cells and suggest direct growth hormone action on these cells, however growth hormone can also upregulate the enzymatic deiondination of T4 into T3 which in turn facilitate ACTH activation of the interrenal gland and accelerate cortisol secretion (Hourdry, 1995).

In conclusion thyroid hormone increases the total amount of Na,K-ATPase units available and this effect may also be elicited by sustained exposure to aldosterone, cortisol, growth hormone and in certain cases by insulin. The effects of all hormones are manifested, depending on tissue, on all isoforms of both $\alpha$ and $\beta$-subunits. The exact mechanisms underlying these actions are not known.

## 3.I d Na,K-ATPase in the dogfish rectal gland

The Na,K-ATPase is located predominately in the basolateral membrane of the dogfish rectal gland epithelial cells (Eveloff *et al*, 1979). It is often juxtaposed to mitochondria which accumulate near the basolateral surfaces of the cell. The Na,K-ATPase in the dogfish rectal gland is indirectly involved in the transport of sodium and chloride into the tubular lumen of the gland from the basolateral serosal side of the cell. The luminal side of the cell is negatively charged during sodium chloride transport, as chloride exits the apical surface of the cell down its chemical gradient via the CFTR-like chloride channel (Riordan *et al*, 1994). The resulting transient reduction in the intracellular chloride concentration allows chloride to enter the cell via the basolateral $Na^+$,$K^+$,$Cl^-$ co-transporter (Riordan *et al*, 1994). This results in the inward flux of all three ions from the blood side on the driving force provided by sodium movement down its large electrochemical gradient. The immediate energy supply for the overall process is maintained by the active transport of sodium back out of the cell across the basolateral membrane by the Na,K-ATPase. The excess potassium that has entered the cell via the co-transporter exits on the basolateral side through potassium channels (Riordan *et al.*

1994). The chloride which has been transported in by the co-transporter restores the intracellular chloride level to above its electrochemical equilibrium with respect to the luminal surface so that exit through the CFTR channel can continue if its activation persists. This transcellular chloride movement contributes substantially to the lumen-negative transepithelial potential that provides the driving force to pull sodium through a paracellular pathway from blood to lumen (Silva *et al*, 1983).

Various studies of Na,K-ATPase using either *Squalus acanthias* (Eveloff *et al*, 1979, Silva *et al*, 1977, Silva *et al*, 1979, Silva *et al*, 1983, Dubinski and Monti, 1986, Marver *et al*, 1986) or *Scyliorhinus canicula* (Shuttleworth and Thompson, 1980a) have reported that enzyme activities in rectal gland homogenates range from 0.05 to 100 µmol $P_i$ / mg protein / hour. The wide range of activities measured seem more dependent on the method of sample preparation used than the species studied. However it is known that Na,K-ATPase activity is lower in the rectal glands of rays and skates than that found in elasmobranchii (Marver *et al*, 1986). The Na,K-ATPase activities reported in the above mentioned studies were measured by assaying homogenised samples of the rectal gland (Silva *et al*. 1977, Silva *et al*. 1979) and incubation of rectal gland slices with various solutions including bumetanide (Shuttleworth and Thompson, 1980a). In addition, purified rectal gland membrane preparations have also been used (Dubinski and Monti, 1986). The difference in sample preparation undoubtedly will account for some of the variation seen in Na,K-ATPase activities, however even in studies that use the same sample preparation method a wide range of activities have been reported. It is therefore difficult to compare results of specific Na,K-ATPase activities between studies.

It has been suggested that there is a sodium-dependent, cAMP-mediated chloride transport system in the dogfish rectal gland (Stoff *et al*, 1977, Silva *et al*, 1977). Shuttleworth and Thompson (1978) demonstrated that incubating European dogfish rectal gland tissue slices with 0.05 mM cAMP and 0.25 mM theophylline resulted in an 86% increase in maximal ouabain binding, suggesting that cAMP increased the number of active Na,K-ATPase sites in the plasma membrane. Shuttleworth (1983), suggested that the cAMP-stimulated increases in Na,K-ATPase activity is an indirect effect, and is secondary to enhance sodium entry into the cell via a furosemide-sensitive chloride coupled transport system. Shuttleworth reached this conclusion as he had found that cAMP-stimulated increases in Na,K-ATPase activity are blocked by furosemide or by the absence of extracellular chloride (Shuttleworth and Thompson, 1980). He proposed that cAMP had its effect by increasing the rate of furosemide-sensitive,

chloride-coupled sodium entry into the cell, which in turn induced an activation of previously latent pump sites in the membrane and therefore an enhanced sodium efflux. Shuttleworth also reported that cAMP stimulation of the Na,K-ATPase is blocked by verapamil a calcium channel blocker, however calcium alone is not able to induce stimulation of the enzyme as in the absence of any changes in cAMP concentration the calcium ionophore A24187 failed to have any effect. The precise mechanism whereby increases in intracellular cAMP lead to stimulation of the Na,K,Cl-cotransport system is not fully understood, although the results obtained from Shuttleworth's studies suggest that cAMP stimulates a verapamil- and furosemide-sensitive uptake of sodium and chloride into the rectal gland cell and the resulting increase in intracellular sodium concentration subsequently stimulates the Na,K-ATPase. In further support of this hypothesis, Greger and Schlatter(1984), reported that intracellular sodium concentrations measured in single isolated perfused rectal gland tubules increased between 2 and 4 minutes following cAMP stimulation.

However there is much controversy surrounding these studies, as conflicting evidence in the literature reports that there is no prior increase in intracellular sodium or chloride concentrations in association with the cAMP stimulation of the Na,K-ATPase in the dogfish rectal gland (Lear *et al,* 1992; Silva *et al,* 1979). By using classical chemical methods to calculate the intracellular electrolyte content of the whole tissue, Silva *et al,* 1979, reported a fall in intracellular sodium and chloride in slices of isolated perfused rectal glands harvested 30 minutes after stimulation with 1 mM cAMP and 0.5 mM theophylline. Later this was confirmed using electron probe analysis, a technique that permits the measurement of ion content in individual cells (Lear *et al,* 1992). Lear *et al,* (1992), reported that when primary epithelial cell cultures from the *Squalus acanthias* rectal gland (compared to the *scyliorhinus canicula* used in Shuttleworths studies) are treated with 1 mM cAMP and 0.5 mM theophylline a 30% decrease in intracellular sodium and an 11% decrease in intracellular chloride is observed after eight minutes. They also reported that ouabain-sensitive oxygen consumption of the cultured rectal gland cells increased within 3 minutes when the cells were treated with 1 mM cAMP and 0.5 mM theophylline, therfore concluding that Na,K-ATPase activity has increased. It was assumed from these results that the increase in Na,K-ATPase activity observed was not driven by high levels of internal sodium or chloride, suggesting that cAMP or protein kinase A has a direct stimulatory action on the Na,K-ATPase independent of intracellular sodium concentration. Several possibilities could account for the difference in the findings between Lear *et al* and Greger and Schlatter. First the measurements of intracellular sodium content made by Lear *et al* began at eight minutes

after stimulation whereas those of Greger and Schlatter were made within the first two minutes, therefore there might be a transient increase in intracellular sodium concentration (missed by Lear and coworkers) which would in some way signal the increase in Na,K-ATPase activity. Or it might be possible that the difference in the preparation used is responsible (cultured cells versus dissected tubules) for the difference. However further evidence for a sodium independent effect of cAMP stimulation on Na,K-ATPase is found in ouabain binding studies. Ouabain binding to a high-affinity site is increased in rectal glands slices (Silva *et al*, 1983) and dispersed cells (Marver *et al*, 1986) after treatment with cAMP and theophylline. The increase in ouabain binding is demonstrable even in the presence of furosemide and after the removal of sodium or chloride from ambient solutions (Marver *et al*, 1986). These experiments support the possibility that cAMP action is mediated by Na,K-ATPase phosphorylation by protein kinase A (PKA).

The above conflicting results suggest that cAMP may simultaneously activate a number of separate membrane transporters in rectal gland cells that operate together to produce active secretion of sodium chloride. The activity of the Na,K-ATPase appears to be enhanced by cAMP stimulation which acts by a mechanism independent of intracellular sodium concentration (Lear *et al*, 1992) or via enhanced intracellular sodium concentration induced by prior activation of the Na,K,Cl cotransporter (Shuttleworth, 1983).

As the effect of hormones on Na,K-ATPase activity can be mediated by the phosphorylation of Na,K-ATPase by protein kinase A or protein kinase C or by the hormone having effects on other ion transporter/channels which increase intracellular sodium concentration and therefore increase Na,K-ATPase acitivity it would be of interest to investigate how intracellular sodium regulates Na,K-ATPase. Although it is well established that an increase in intracellular sodium concentrations can increase Na,K-ATPase activity in the rectal gland there is no information available on the mechansim by which this occurs (Shuttleworth, 1983; Lear *et al*, 1992; Marver *et al*, 1992 Greger and Schalatter, 1984). Studies by MacKenzie (1996) relate the effect of ingested sodium chloride by the dogfish to subsequent increases in Na,K-ATPase activity and expression of the Na,K-ATPase genes in the rectal gland. MacKenzie (1996) showed that an increase in ingested sodium chloride results in a 44-fold transient increase in Na,K-ATPase activity peaking 9 hours after the feeding event, however intracellular sodium concentration in the rectal gland were not mearsured. As there were no contimitten increases in $\alpha_1$ or $\beta$ mRNAs MacKenzie's results suggest

that an increase in sodium chloride can increase Na,K-ATPase activities via a mechanism other than transcription. As little information is available on the regulation of Na,K-ATPase in the shark rectal gland this study will look at the effects of changes in extracellular sodium concentration and selected hormones on Na,K-ATPase activity.

# 3.II MATERIALS AND METHODS

## 3.II Measurement of Na,K-ATPase Activity in *Scyliorhinus canicula* Rectal Gland Primary Epithelial Cell Cultures.

### 3.II a Na,K-ATPase Assay

The cells were prepared as described in Section 2. II a iii and were resuspended in growth media and seeded on collagen coated 24 well plates. When the cells formed a monolayer, approximately 5-7 days after plating, they were used to determine the effect of changes in extracellular sodium chloride concentrations on Na,K-ATPase activity in cell homogenates. The cells were incubated for various lengths of time in media (see section 3.III) containing various concentrations of NaCl and/or drugs. The cells were then washed twice with wash buffer (phosphate and phenol red-free shark Ringer see section 2.II a ii) and then 3 wells containing cells were scraped into 300 μl of homogenisation buffer (50 mM N-2-hydroxyethylpiperazine-$N^1$-2-ethanesulphonic acid (Hepes), 1 mM EDTA, 0.18 mg / ml phenyl methyl sulphonyl fluoride and 0.001 % sodium deoxycholate, pH 7.4) and homogenised using 10 full passages through a 1 ml syringe and a 19 G needle. The cell homogenates were then stored for up to 5 days at -20°C until they were assayed for Na,K-ATPase activity and for protein concentration.

Na,K-ATPase activity is defined as the ouabain-sensitive component of ATP hydrolysed by the cell homogenate in the presence of Na, K and Mg ions. Both the ratio of Na to K and the total concentration of Na plus K are important parameters in achieving optimum Na,K-ATPase activity (Esmann, 1988). The ratio of Na to K gives optimum activity at 6.5 : 1 and the total concentraton for both ions should be approximately 150 mM. The assay buffer used in these experiments consisted of a Na : K ratio of 6 : 1 and a total ion concentration of 140 mM. The presence of free Mg and an assay pH between 6.9 and 7.4 are required for optimal activity. Enzyme activity was measured in a final assay cocktail comprising of 120 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 30 mM histidine, 3 mM ATP and 1 mM Tris/HCl pH 7.4 in the presence and absence of 2 mM ouabain. The assay, total volume 500 μl, contained 150 μg of rectal gland homogenate protein and was incubated for 1 hour at 20°C and terminated by the addition of 500 μl of ice cold 10% trichloroacetic acid (TCA). After a 15 minute incubation at 4 °C the extract was centrifuged at 2400 rpm (1000 x $g_{max}$) in a MSE

Coolspin, for 5 minutes at 4°C. Three 250 µl aliquots were sampled and diluted with 750 µl of water. The phosphate content of the diluted extracts were determined by sequential addition of 200 µl molybdate reagent ( 1% ammonium molybdate, 6M sulphuric acid) and 50 µl stannous chloride reagent (0.4 % stannous chloride, 0.8 M HCl). The colour was allowed to develop for 20 minutes at room temperature before the absorbance was read at 690 nm. The amount of phosphate released was calculated from a standard curve. The standard curve consisted of a 6:4 dilution series using a top standard of 1mM $KH_2PO_4$ in 5% TCA. The activity of the enzyme was calculated from the ouabain-sensitive phosphate released and expressed as nmoles Pi / mg protein/hour.

### 3.II b Protein Assay

Bradford's reagent (0.01 % Coommassie blue-G250, 4.5 % ethanol and 5.5 % orthophosphoric acid) (1ml) was added to 50 µl of cell homogenate or 50 µl of homogenisation buffer and left for 5 minutes at room temperature to allow the colour to develop before the absorbance was read at 590 nm. The amount of protein present was calculated from a standard curve which was made from a 6:4 dilution series using a top concentration of 2 mg/ml bovine serum albumin.

### 3.II c Optimisation of the Na,K-ATPase Assay

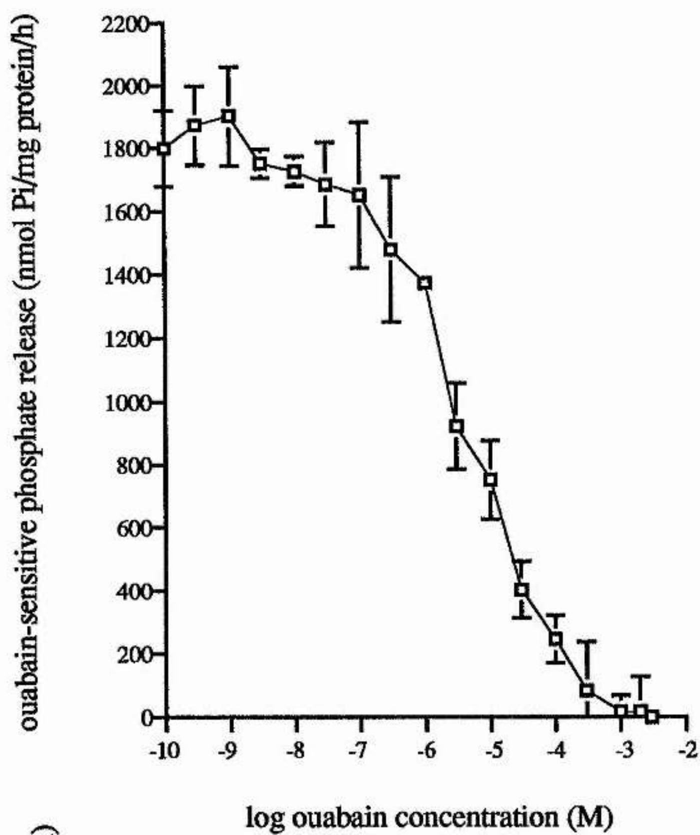
The optimal ouabain concentration to completely inhibit Na,K-ATPase activity in rectal gland epithelial cell homogenates was $1 \times 10^{-3}$ M with half maximal inhibition at $3 \times 10^{-6}$ M (Fig 3.3). A time course of ouabain-sensitive phosphate release from cell homogenates was linear for up to 90 minutes at a temperature of 20°C (Fig 3.4). In all subsequent experiments, Na,K-ATPase assay incubations were conducted for 60 minutes at temperature of 20°C.

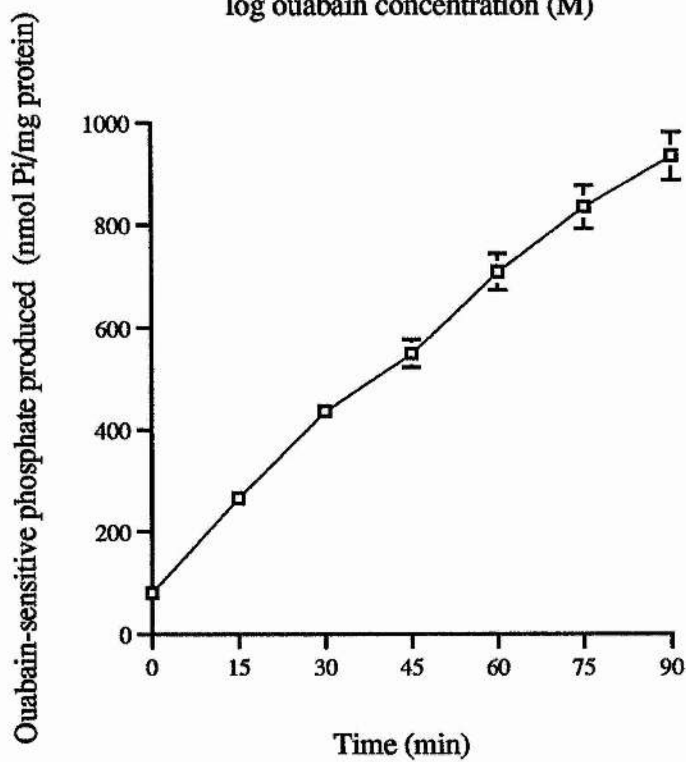
Sodium deoxycholate was added to the homogenisation buffer to permeablise any vesicles formed during homogenisation of the cells. This ensured unrestricted access of the substrates to both sides of the membrane. The effects of various sodium deoxycholate concentrations in the homogenisation buffer are shown in Fig 3.5. The lowest concentration of deoxycholate tested (0.001 %) was tenfold less than reported in most assay methods (Forbush, 1982) but proved to be the optimal concentration tested for the assay increasing activities by approximately 15 % (Fig 3.5). This suggests that very few vesicles had formed during homogenisation. Increasing the concentration of

Figure 3.3 and Figure 3.4

Figure 3.3 Ouabain dose response curve for inhibition of rectal gland Na,K-ATPase activitiy. The individual values represent the mean ± standard error of mean for 4 separate experiments.

Figure 3.4 Time course of ouabain-sensitive phosphate release, in the presence of 0.001% (w/v) sodium deoxycholate. Cells were homogenised and Na,K-ATPase activities determined from the difference in phosphate released, in the presence and absence of 2 mM ouabain as described in the methods. The values are represent the mean ± standard error of mean for 4 separate experiments.


ouabain-sensitive phosphate release (nmol Pi/mg protein/h)
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
-10
-9
-8
-7
-6
-5
-4
-3
-2
log ouabain concentration (M)


Ouabain-sensitive phosphate produced (nmol Pi/mg protein)
1000
800
600
400
200
0
0
15
30
45
60
75
90
Time (min)

the detergent above 0.001 % (w/v) resulted in a gradual reduction in maximal Na,K-ATPase activity (Fig 3.5). Therefore in all future experiments sodium deoxycholate at 0.001 % (w/v) was routinely included in the homogenisation buffer.

The effect of the age of the cell culture on Na,K-ATPase activity was investigated. Cells were plated as described in section 2. II a iii and at different times ranging from 3 to 19 days the cells were homogenised and Na,K-ATPase activities determined as described previously (Fig 3.6). Cell growth and division was evident up to the development of confluent monolayers (5-10 days depending on experiment) and thereafter a viable monolayer was maintained with little cell division for up to 19 days. Despite these changes in cell growth there was very little change in the activity of the Na,K-ATPase between cultures of all ages (fig 3.6). Cells were therefore routinely used after monolayer formation between 5 to 7 days in culture.

Once the assay was optimised a series of experiments (refer to section 3.3) were conducted to investigate the effects of increasing extracellular NaCl on the subsequent activity of Na,K-ATPase in cell homogenates.

### 3.II d Statistical Analysis

Analysis of the results obtained was performed using the statistical software package, Statview (Biosoft, Inc).

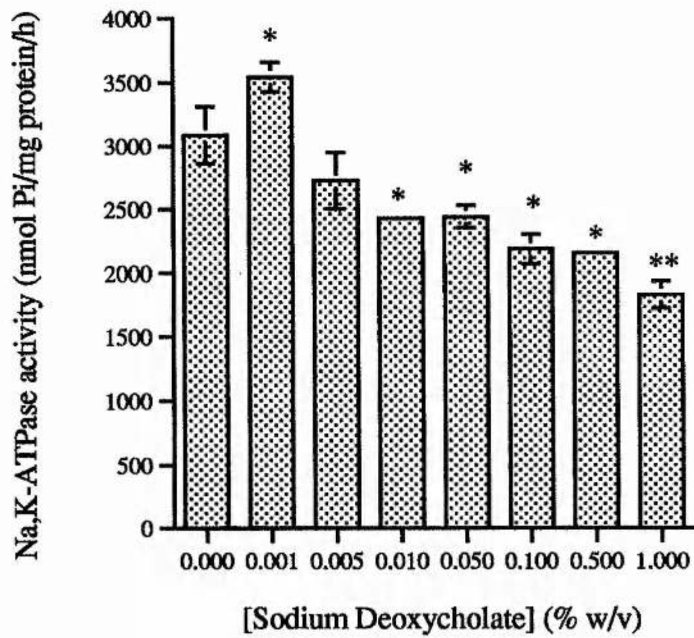
The means from each cell treatment were used to form data sets which were used for factorial two-way analysis of variance (ANOVA) to determine if there was a significant difference between the Na,K-ATPase activities in the treated cells compared to the untreated control cells. The results were displayed on an ANOVA table and the F-value(s) were compared to each other to see if the differences observed between the groups were significant.

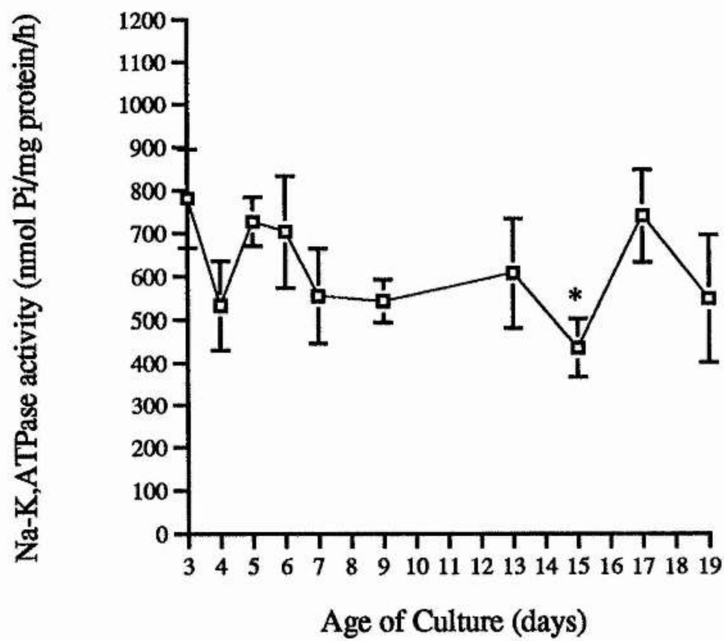
If the significant differences were assigned by ANOVA test, a post-hoc test (Bonferroni and Dunn) was applied to the results. This test may be used when the n values in the groups are unequal, as in some of the data sets analysed. The test compares, pairwise, all of the means in each experimental treatment to each other giving a p-value for each paired comparison, this is interpreted as the probability of a significant difference between the means. The p-value obtained was relevant to the degree of significant

Figure 3.5 and Figure 3.6.

Figure 3.5 The effect of inclusion of various concentrations of sodium deoxycholate in the homogenisation buffer on subsequent Na,K-ATPase activity mearsurements. The diagram shows means ± standard error of mean for 3 separate experiments. Significant differences were determined by a one way analysis of variance followed by a Bonferroni and Dunn post hoc test. The p-values obtained were relevant to the degree of significance, $p^* \leq 0.05$ significant; $p^{**} \leq 0.01$ highly significant; $p^{***} \leq 0.001$ very highly significant.

Figure 3.6 The effect of age of cell cultures on Na,K-ATPase activity. Cells were plated on day 0 and grown on 24 well plates as indicated in section 2.II a iii before being extracted and homogenised in buffer containing 0.001 % (w/v) sodium deoxycholate for determination of Na,K-ATPase activity. Individual values represent means ± standard error of mean for 8 separate experiments. Significant differences were determined by a one way analysis of variance followed by a Bonferroni and Dunn post hoc test. The p-value obtained was relevant to the degree of significant difference, $p^* \leq 0.05$ significant; $p^{**} \leq 0.01$ highly significant; $p^{***} \leq 0.001$ very highly significant.


Na,K-ATPase activity (nmol Pi/mg protein/h)
4000
3500
3000
2500
2000
1500
1000
500
0
*
*
*
*
*
*
**
0.000 0.001 0.005 0.010 0.050 0.100 0.500 1.000
[Sodium Deoxycholate] (% w/v)


Na-K,ATPase activity (nmol Pi/mg protein/h)
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
*
3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Age of Culture (days)

**difference, $p^* \leq 0.05$ significant; $p^{**} \leq 0.01$ highly significant; $p^{***} \leq 0.001$ very highly significant.**

## 3.III RESULTS

### 3.IIIa Effect of extracellular sodium chloride concentration on Na,K-ATPase activities

The Na,K-ATPase activity in homogenates of rectal gland epithelial cells transiently increased approximately 3.5 fold some 12 hours after the NaCl concentration of the growth medium was increased by 50% (Fig 3.7). The first sign of an increase in activity was seen 6 hours after the medium change with maximal activity being reached after 9-12 hours. This increase in Na,K-ATPase activity was not sustained and by 24 hours had dropped back to basal levels where activity remained for the following 2 days of the experiment even although NaCl concentrations were still elevated. The increase in Na,K-ATPase activity was dependent on an increase in both Na and Cl ions in the medium as molar equivalent additions of chloride or sodium as either choline chloride or $Na_2SO_4$ to the medium respectively, did not result in any significant increase in Na,K-ATPase activity (Fig 3.7).

Dose response curves for NaCl induced stimulation of Na,K-ATPase activity are shown in figures 3.8 and 3.9. The NaCl concentration could not be reduced below 145 mM, as this was the concentration in the commercially prepared medium. These experiments were carried out either with a constant osmolality where various mannitol concentrations were added to maintain the tonicity of the medium at approximately 1420 mOsmol/ kg (Fig 3.8) or with a varying osmolality (Fig 3.9). There was no significant difference in the results obtained using either treatment (determined by a two way ANOVA). The effect of increased extracellular NaCl concentration on homogenate Na,K-ATPase activity was independent of the osmolality of the medium as identical dose-response curves were found with increasing ionic strength or when the osmolality was held constant at 1420 mOsmol/ kg by addition of mannitol. These experiments confirm that the increase in Na,K-ATPase activity observed in figure 3.7 was due to an increase in the concentration of sodium and chloride ions in the medium and was not a result of the increased osmolality of the medium. Figures 3.8 and 3.9 show that an increase in medium NaCl concentration to just 10 % above the normal concentration (240 mM) elicits a significant (approximately 40%) increase in Na,K-ATPase activity. The enzyme activity continues to increase linearly until a plateau is reached at medium NaCl concentrations of 360 mM and above. The rate at which Na,K-ATPase activity returns to normal after 12 hours in high NaCl is not related to the amount of NaCl

Figure 3.7

Figure 3.7 Time course of Na,K-ATPase activity in homogenates of cell cultures following changes in growth medium. Cell cultures were incubated in normal medium (❑), or in medium with the addition of 120 mM NaCl (O), 60 mM $Na_2SO_4$ (◇) or 120 mM choline chloride (Δ). Figure A shows a time course from 1 to 72 hours and figure B shows a time course from 10 - 120 minutes. The individual values are means ± standard error of mean for 4 separate experiments. The control value was 954 nmols/mg protein/hour. Significant differences were determined by a two way ANOVA followed by Bonferroni and Dunn post hoc test. The p-value obtained was relevant to the degree of significant difference, $p^* \leq 0.05$ significant; $p^{**} \leq 0.01$ highly significant; $p^{***} \leq 0.001$ very highly significant.

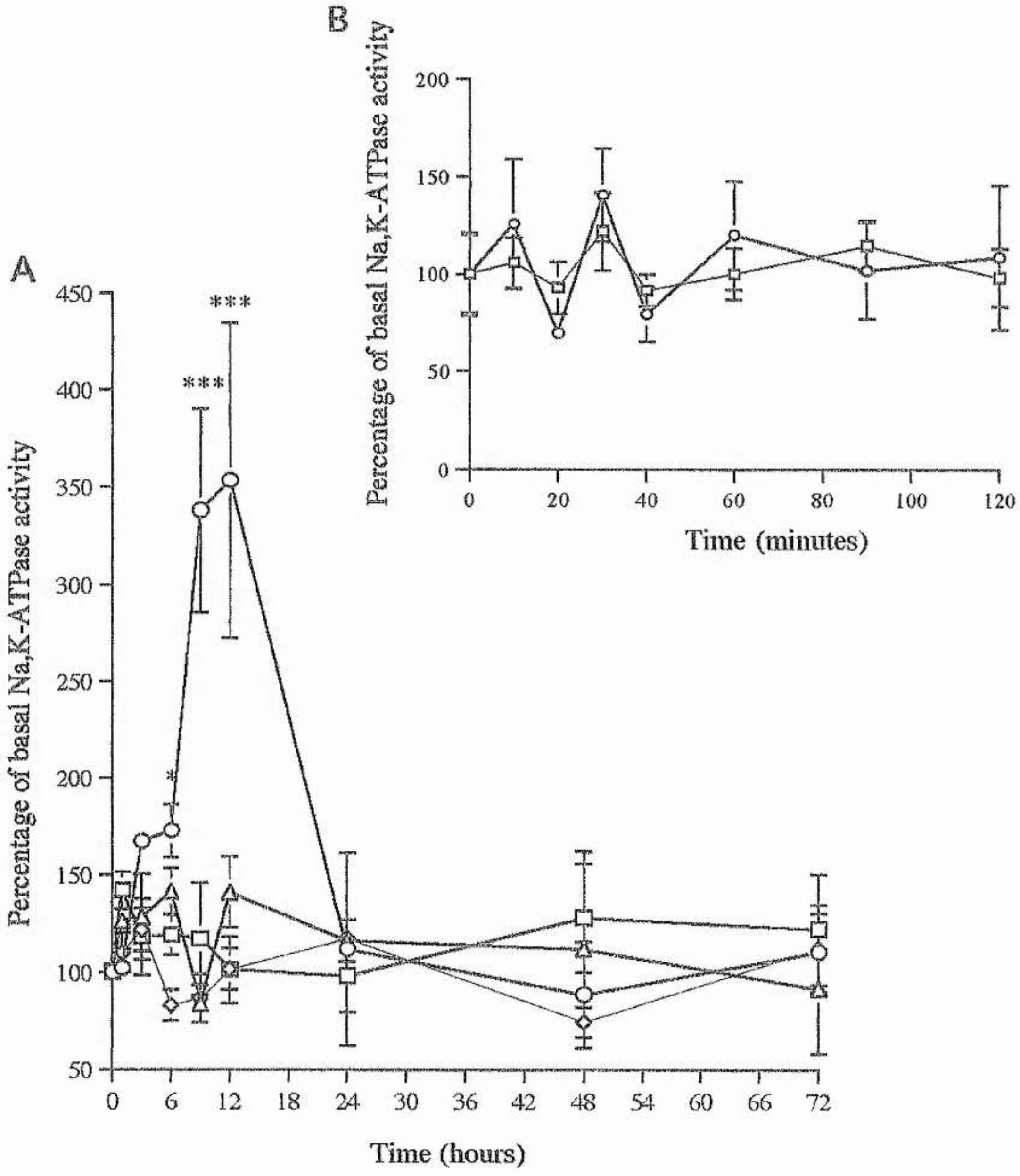
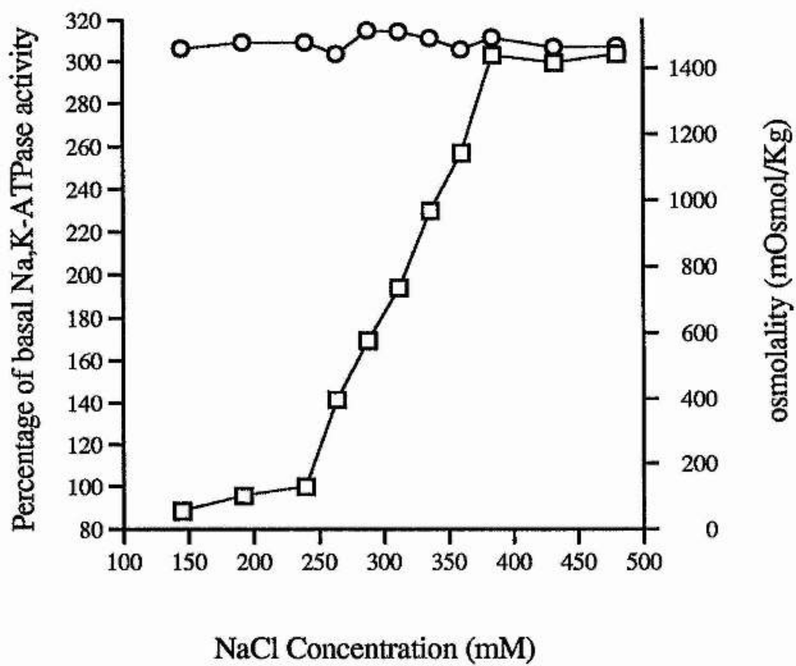

B
Percentage of basal Na,K-ATPase activity
200
150
100
50
0
0
20
40
60
80
100
120
Time (minutes)
A
***
***
*
Percentage of basal Na,K-ATPase activity
450
400
350
300
250
200
150
100
50
0
6
12
18
24
30
36
42
48
54
60
66
72
Time (hours)

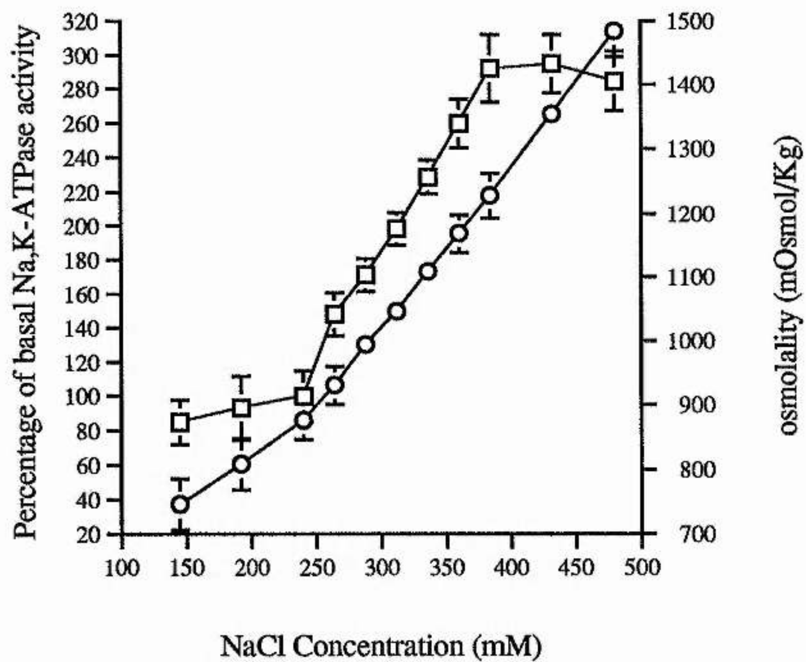
Figure 3.8 and 3.9

Figure 3.8 Dose response curve for NaCl-induced increase in cell homogenate Na,K-ATPase activity. Cells were incubated for 12 hours with medium containing the NaCl concentrations indicated. The osmolality of the medium was maintained at 1480 mOsmol/Kg by the addition of mannitol. Cells were homogenised and Na,K-ATPase activities determined. Na,K-ATPase activity (□) and osmolality (O). Values shown are percentage values compared to activities found with normal NaCl concentrations and are given as individual means ± standard error of mean for 4 separate determinations. The control value was 1083 nmols/mg protein/hour.

Figure 3.9 Dose resoponse curve for NaCl-induced increase in cell homogenate Na,K-ATPase activity. Cells were incubated for 12 hours with medium containing the NaCl concentrations indicated. The osmolality of the medium varied from 360 mOsmol/Kg to 1480 mOsmol/Kg. Cells were homogenised and Na,K-ATPase activities determined. Na,K-ATPase activity (□) and osmolality (O). Values shown are percentage values compared to activities found with normal NaCl concentrations and are given as individual means ± standard error of mean for 4 separate determinations. The control value was 1234 nmols/mg protein/hour.




NaCl Concentration (mM)




NaCl Concentration (mM)

present in the medium at this time point, as replacement of the high NaCl medium with normal NaCl containing medium after a 12 hour incubation had no effect on the rate of return of cellular Na,K-ATPase activities to control values (Fig 3.10).

### 3.IIIb Effect of treatment of cells with the sodium ionophore monensin, on Na,K-ATPase activity

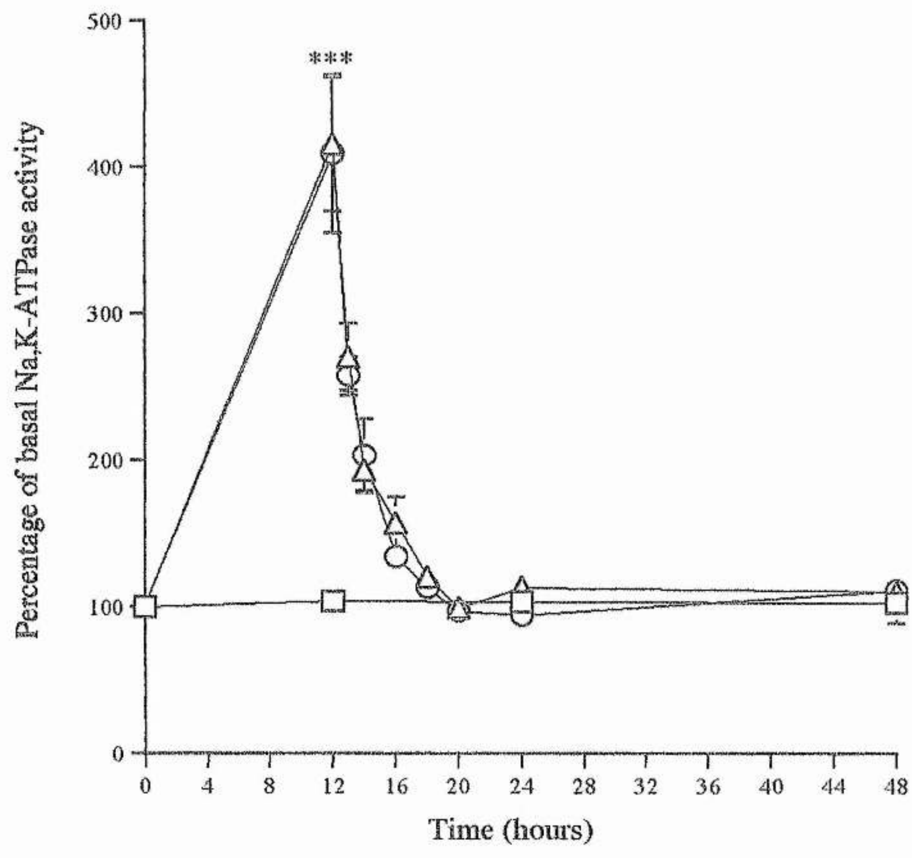
Monensin is a sodium ionophore which promotes $Na^+/H^+$ exchange across biological membranes (Rang and Dale, 1991). Cells were incubated in growth medium containing either 360 mM NaCl or normal, 240 mM NaCl medium plus 1 μM monensin for up to 72 hours and the Na,K-ATPase activities in cell homogenates determined as before. Under both conditions enzyme activities were seen to significantly increase after 6 hours of incubation (Fig 3.11). The elevated activities associated with the monensin response differed from treatment with elevated NaCl, with the enhance Na,K-ATPase activities being maintained for the duration of the experiment (Fig 3.11). A dose-response curve to monensin was then coducted (Fig 3.12). Monensin significantly increased Na,K-ATPase activities in cell homogenates by nearly 3-fold at a concentration of 1 μM, the $EC_{50}$ value for monensin was 1.5 μM, with 6 μM giving the maximum response (Fig 3.12). The Maximum response to monensin is higher than the maximal Na,K-ATPase activities observed in response to elevated NaCl concentrations. Incubations containing monensin concentrations above 6 μM resulted in loss of cells from the plate and a decrease in cell viability, at this point measured Na,K-ATPase activities fell, probably as the result of to the cell damage and death.

### 3.IIIc Transcription and translation of Na,K-ATPase.

To investigate the possibility that protein synthesis was involved in the transient increase in Na,K-ATPase activity, experiments were repeated in the presence of various concentrations of the protein synthesis inhibitor, cycloheximide (Fig 3.13). Cells were incubated for 12 hours in normal medium or normal medium supplemented with NaCl (final concentration 360 mM) in the presence or absence of various concentrations of cycloheximide. Cycloheximide, at all concentrations used, not only inhibited the NaCl-induced increase in Na,K-ATPase activity but also decreased basal levels of Na,K-ATPase activity in cell homogenates (Fig 3.13). In figure 3.13 the extent of inhibition between the different cycloheximide concentrations was not significantly different, therefore concentrations of 10 μg/ml cycloheximide are sufficient to completely inhibit

Figure 3.10

Figure 3.10 Effect of returning cells to normal NaCl medium on recovery of Na,K-ATPase activities. Cells were incubated for 12 hours in normal medium or medium containing 360 mM NaCl. The medium was then changed; cells originally in either normal medium were further incubated in normal medium for the duration of the experiment, cells in 360 mM NaCl medium were incubated in normal or 360 mM NaCl medium for a further 12 hours. At the times indicated cells were homogenised and Na,K-ATPase activities were determined. Normal medium (❑), 360 mM NaCl medium (O) and 360 mM NaCl medium to normal medium (Δ). Values shown are percentage values compared to activities found with normal NaCl concentrations and are given as individual means ± standard error of mean for 4 separate determinants. The control value was 916 nmols $P_i$/mg protein/hour. Significant differences were determined by a two way ANOVA followed by Bonferroni and Dunn post hoc test. The p-value obtained was relevant to the degree of significant difference, $p^* \leq 0.05$ significant; $p^{**} \leq 0.01$ highly significant; $p^{***} \leq 0.001$ very highly significant.

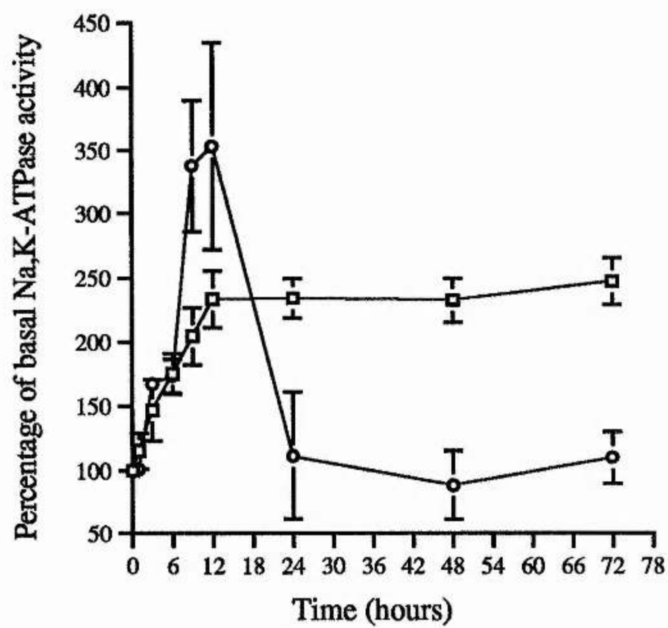


500
400
300
200
100
0
***
Percentage of basal Na,K-ATPase activity
0
4
8
12
16
20
24
28
32
36
40
44
48
Time (hours)

Figure 3.11 and Figure 3.12

Figure 3.11 Time course for the effect of monensin on cell homogenate Na,K-ATPase activity. Cells were incubated for the times indicated in the presence of 360 mM NaCl or 240 mM Nacl plus 1$\mu$M monensin Cells were then homogenised and Na,K-ATPase activities determined. Values shown are percentage values compared to activities found with normal NaCl concentrations and are given as individual means ± standard error of mean for 4 separate determinants. The control value was 853 nmols Pi/mgprotein/hour.

Figure 3.12 Dose response curve for the effects of monensin on cell homogenate Na,K-ATPase activity. Cells were incubated for 12 hours in the presence of the monensin concentrations indicated and cells were then homogenised and Na,K-ATPase activities determined. The sudden drop in activity at the 8 $\mu$M concentration coincided with loss of many cells from the culture plate. Values shown are percentage values compared to activities found with normal NaCl concentrations and are given as individual means ± standard error of mean for 4 separate determinants. The control value was 981 nmols Pi/mg protein/hour.


Percentage of basal Na,K-ATPase activity
450
400
350
300
250
200
150
100
50
0 6 12 18 24 30 36 42 48 54 60 66 72 78
Time (hours)

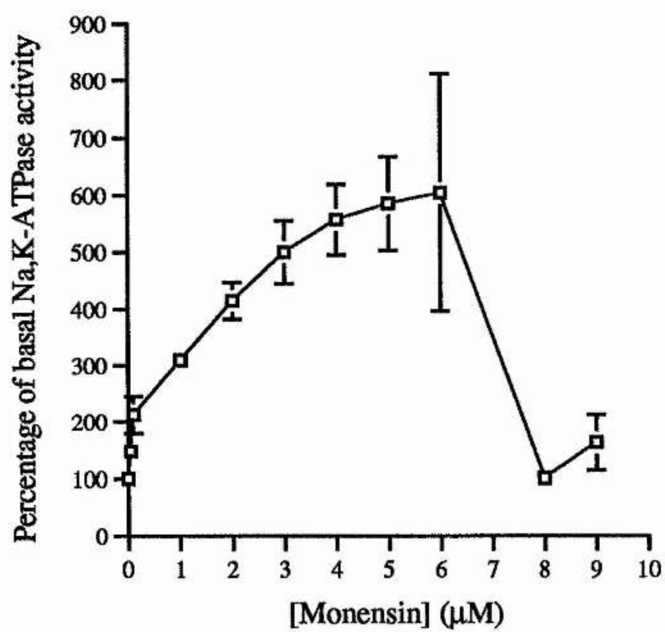
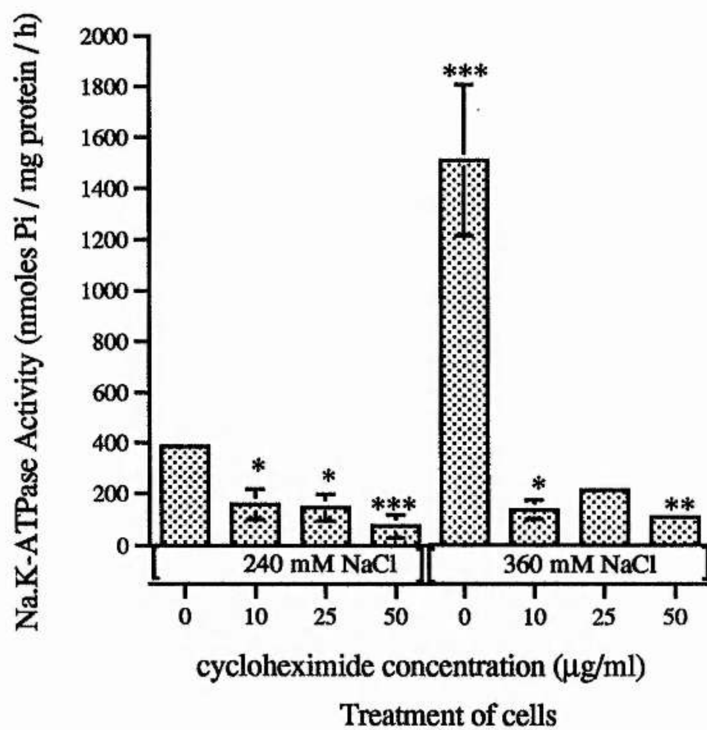
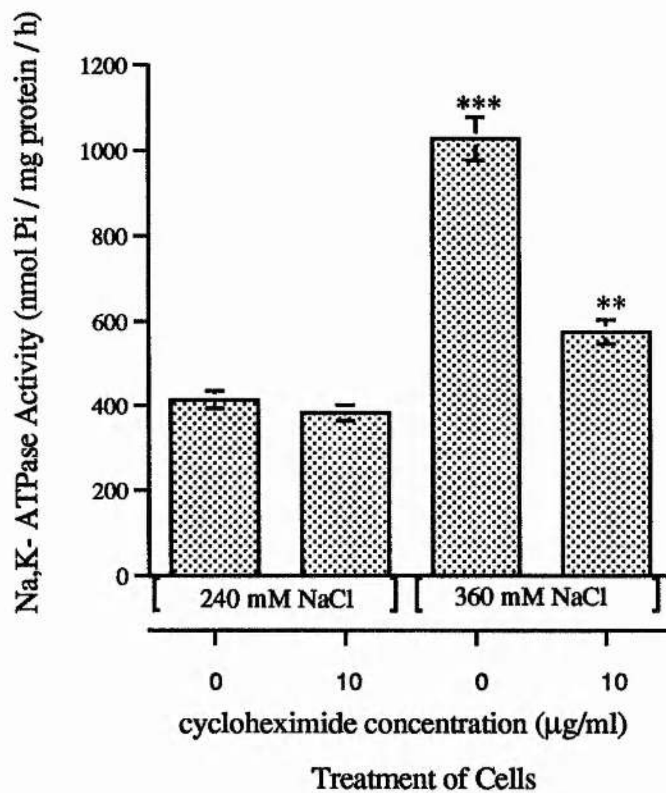

Percentage of basal Na,K-ATPase activity
900
800
700
600
500
400
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10
[Monensin] (μM)

Figure 3.13 and Figure 3.14

Figure 3.13 Effect of cycloheximide on basal and NaCl-stimulated Na,K-ATPase activities. Cells were incubated for 12 hours in the presence of cycloheximide and NaCl concentrations indicated. Cells were homogenised and Na,K-ATPase activities determined. Normal NaCl concentrations and are given as individual means ± standard error of mean for 4 separate determinants. The control value was 398 nmols Pi/mg protein/hour. Significant differences were determined by a two way ANOVA followed by Bonferroni and Dunn post hoc test. The p-value obtained was relevant to the degree of significant difference, p* $\leq 0.05$ significant; p** $\leq 0.01$ highly significant; p*** $\leq 0.001$ very highly significant.

Figure 3.14 Effect of a 6 hour incubation in cycloheximide on Na,K-ATPase activity. Cells were incubated in the presence of cycloheximide concentrations and NaCl concentrations indicated for 6 hours, medium was then removed and replaced with medium containing only the NaCl concentrations indicated for a further 6 hours. Cells were homogenised and Na,K-ATPase activities determined. Normal NaCl concentrations and are given as individual means ± standard error of mean for 4 separate determinants. The control value was 403 nmolsPi/mg protein/hour. Significant differences were determined by a two way ANOVA followed by Bonferroni and Dunn post hoc test. The p-value obtained was relevant to the degree of significant difference, p* $\leq 0.05$ significant; p** $\leq 0.01$ highly significant; p*** $\leq 0.001$ very highly significant.


Na.K-ATPase Activity (nmoles Pi / mg protein / h)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
***
*
*
***
*
**
240 mM NaCl
360 mM NaCl
0
10
25
50
0
10
25
50
cycloheximide concentration (µg/ml)
Treatment of cells


Na,K- ATPase Activity (nmol Pi / mg protein / h)
1200
1000
800
600
400
200
0
***
**
240 mM NaCl
360 mM NaCl
0
10
0
10
cycloheximide concentration (µg/ml)
Treatment of Cells

increases in Na,K-ATPase activity induced by elevations in NaCl following a 12 hour incubation. Basal Na,K-ATPase activities were also inhibited by over 50% following 12 hour incubations of cells in cycloheximide.

The possibility that enhanced protein synthesis was also involved in the abrupt fall in Na,K-ATPase activity after 12 hours in high [NaCl] medium was also investigated. The epithelial cell cultures were incubated in normal growth medium or high NaCl growth medium for 10 hours and then the media was replaced with media of the same ionic composition, but in the presence or absence of 25 μg/ml cycloheximide. The cells were incubated for a further 14 hours. The abrupt fall in Na,K-ATPase activity after 12 hours in high [NaCl] medium was not significantly affected by the addition of cycloheximide (Fig 3.15).

Sodium-mediated increases in Na,K-ATPase activity require increased protein synthesis which is delayed some 6 hours the after medium change. To investigate whether this delayed increase in protein synthesis was dependent on increased mRNA levels following a stimulation of transcription, the effect of the transcription inhibitor actinomycin D was investigated (Fig 3.16). Actinomycin D inhibits DNA-directed synthesis of RNA by RNA polymerase, but it is far less selective in inhibiting the replication of DNA by DNA polymerase (the exact mechanism by of inhibition is not yet known). As such, actinomycin D was included in the incubation medium alone or with elevated NaCl to see if this affected cell homogenate Na,K-ATPase activities (Fig 3.16). Actinomycin D was shown to have no significant effect on the observed response to elevated NaCl at a concentration of 1μg/ml after a 24 hour incubation period. Higher concentrations of actinomycin D (≥5μg/ml) were found to cause cell death and loss of cells from the plate over a 24 hour incubation period (results not shown).

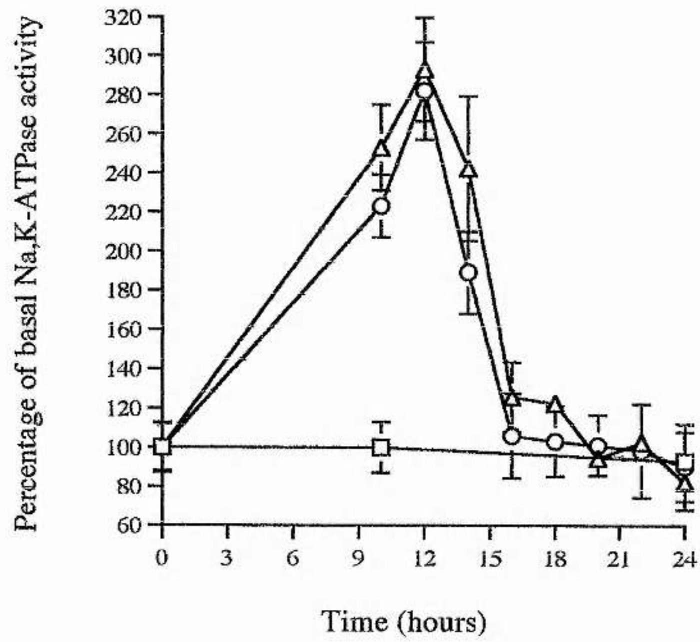
### 3.IIId Effect of inhibition of the Na,K,Cl cotransporter on Na,K-ATPase activities.

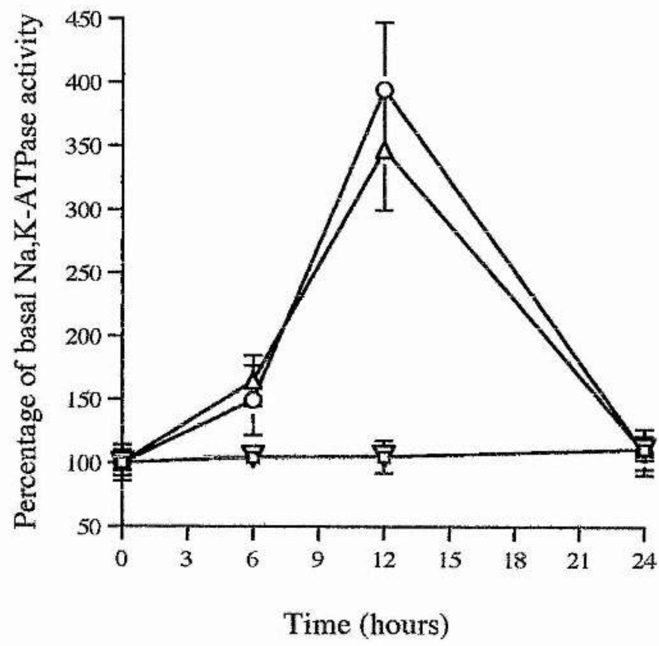
The effect of inhibition of the Na, K, Cl cotransporter on endogenous Na,K-ATPase activities was determined. Cells were incubated with the loop diuretic bumetanide, a reversible inhibitor of the Na, K, Cl cotransporter. Various concentrations of bumetanide were added to cell homogenate samples immediately prior to the assay. Bumetanide at concentrations of up to 0.1 mM did not have any significant effect on the measured Na,K-ATPase activity when added directly to the cell homogenate (Fig

Figure 3.15 and Figure 3.16

Figure 3.15 Effect of cycloheximide on the recovery of Na,K-ATPase activity following high NaCl treatment. Cells were incubated for the indicated times in the presence of 240 mM NaCl (❑) or 360 mM NaCl (Δ, O). Immediately after sampling cells at the 10 hour time point cycloheximide (25$\mu$g/ml) (Δ) was added to a subset of cells incubated in 360 mM NaCl and incubations continued the remaining 14 hours. Cells were homogenised and Na,K-ATPase activities determined. Values shown are percentage values compared to activities found with normal NaCl concentrations and are given as individual means ± standard error of mean for 4 separate determinants. The control value was 671 nmolsPi/mg protein/hour. Significant differences were determined by a two way ANOVA followed by Bonferroni and Dunnpost hoc test.

Figure 3.16 The effect of actinomycin D on basal and NaCl-mediated increases in cell homogenate Na,K-ATPase activity. Cells were incubated for the times indicated in normal culture medium (∇, ❑) or medium supplemented with NaCl (final concentration 360 mM) (Δ, O) in the presence (∇, O) or absence (Δ, ❑) of 1 $\mu$g/ml actinomycin D. Following these incubations cells were homogenised and Na,K-ATPase activities determined. Values shown are percentage values compared to activities found with normal NaCl concentrations and are given as individual means ± standard error of mean for 4 separate determinants. The control value was 1027 nmolsPi/mg protein/hour. Significant differences were determined by a two way ANOVA followed by Bonferroni and Dunn post hoc test.


Percentage of basal Na,K-ATPase activity
320
300
280
260
240
220
200
180
160
140
120
100
80
60
0
3
6
9
12
15
18
21
24
Time (hours)


Percentage of basal Na,K-ATPase activity
450
400
350
300
250
200
150
100
50
0
3
6
9
12
15
18
21
24
Time (hours)

3.17). To completely inhibit cell Na, K, Cl cotransporter activity monolayers were incubated in the presence of 0.1 mM bumetanide. As cell cultures were washed twice before homogenisation and then diluted before addition to the Na,K-ATPase assay, the maximal amount of residual bumetanide present in the Na,K-ATPase assay was estimated to be in the region of 10 nM, 10 fold lower than the lowest concentration tested in the assay (Fig 3.17). It was therefore concluded that bumetanide over the range on concentrations tested has no direct action on Na,K-ATPase activity.

The inhibitory effect of bumetanide on NaCl-induced Na,K-ATPase activity in cell homogenates is shown in figure 3.18. Cells were incubated in growth medium for 12 hours in the presence of the NaCl concentrations indicated and in the presence or absence of 0.1 mM bumetanide. Cells were then washed and homogenised as before in buffer containing 0.001 % (w/v) sodium deoxycholate and the Na,K-ATPase activities determined. Bumetanide treatment not only prevented the increase in activity seen with elevated medium NaCl concentration but also reduced the basal activity of the Na,K-ATPase by approximately 50 %, irrespective of the medium NaCl concentration. Therefore the observed NaCl-induced increase in homogenate Na,K-ATPase activity is dependent on a fully functional Na,K,Cl-cotransporter.

A time course of the effect of bumetanide on cell homogenate Na,K-ATPase activities is shown in figure 3.19. Incubation of cells with 0.1 mM bumetanide induced a rapid decrease in basal Na,K-ATPase activity with a 50% drop in activity occurring within 1 hour of treatment. As a result of the rapid drop in activity a second time course was conducted to monitor the effect of bumetanide on basal Na,K-ATPase within the first hour of treatment. The inhibition of the Na,K,Cl-cotransporter by bumetanide resulted in Na,K-ATPase activity being reduced to nearly 30% of the original value after 30 minutes of bumetanide treatment (Fig 3.20). However within 3 hours of removing the bumetanide from the incubation medium, Na,K-ATPase activity had returned to the original value (Fig 3.21). These results clearly show that incubation of cells with bumetanide reversibly inhibits Na,K-ATPase activity measured in homogenates of dogfish rectal gland epithelial cells.

Treatment of cells with bumetanide for only 30 minutes results in a complete recovery of Na,K-ATPase activity within 3 hours. However for longer treatment protocols this is not the case. The effect of the period of bumetanide administration on Na,K-ATPase recovery is shown in figure 3.22. The recovery of cell homogenate Na,K-ATPase activity following inhibition of the Na, K, Cl cotransporter with bumetanide is

Figure 3.17

Figure 3.17 The effect of bumetanide added directly to the Na,K-ATPase assay. Cells were homogenised and bumetanide was added directly to cell homogenates to give the concentrations indicated. Individual values are means ± standard error of mean (0 bumetanide n=3, 0.0001 mM bumetanide n=3, 0.001 mM bumetanide n=4, 0.01 mM bumetanide n=2 and 0.1 mM bumetanide n=3). Significant differences were determined by a two way ANOVA.

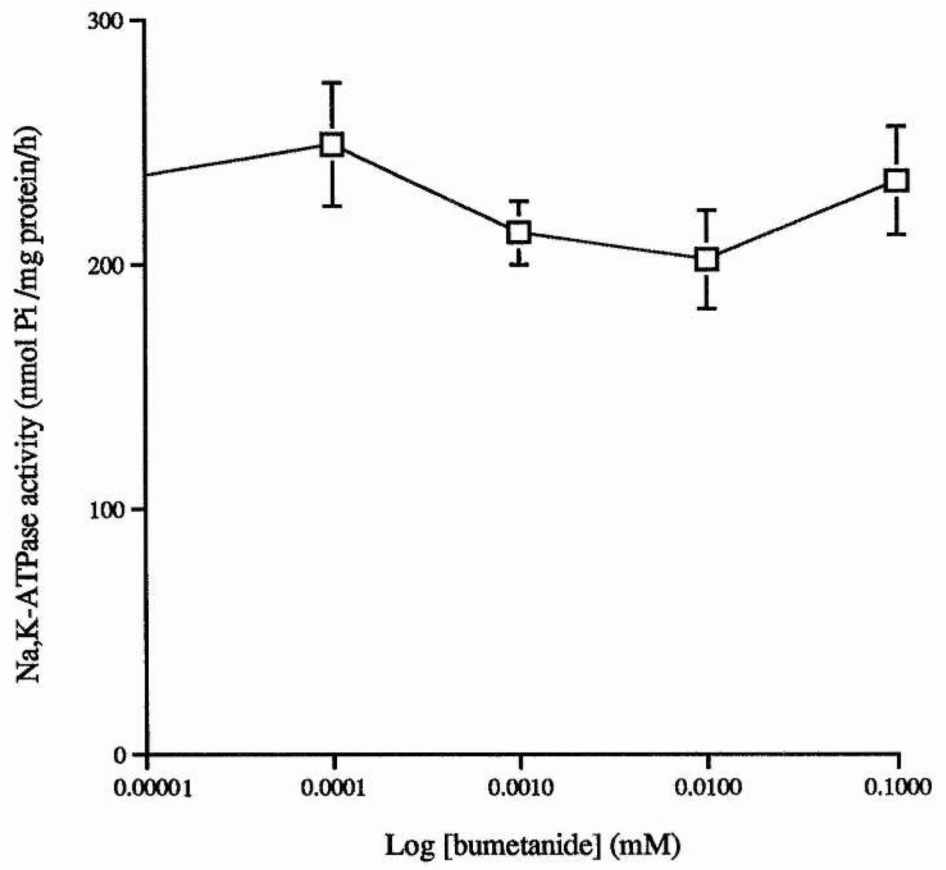
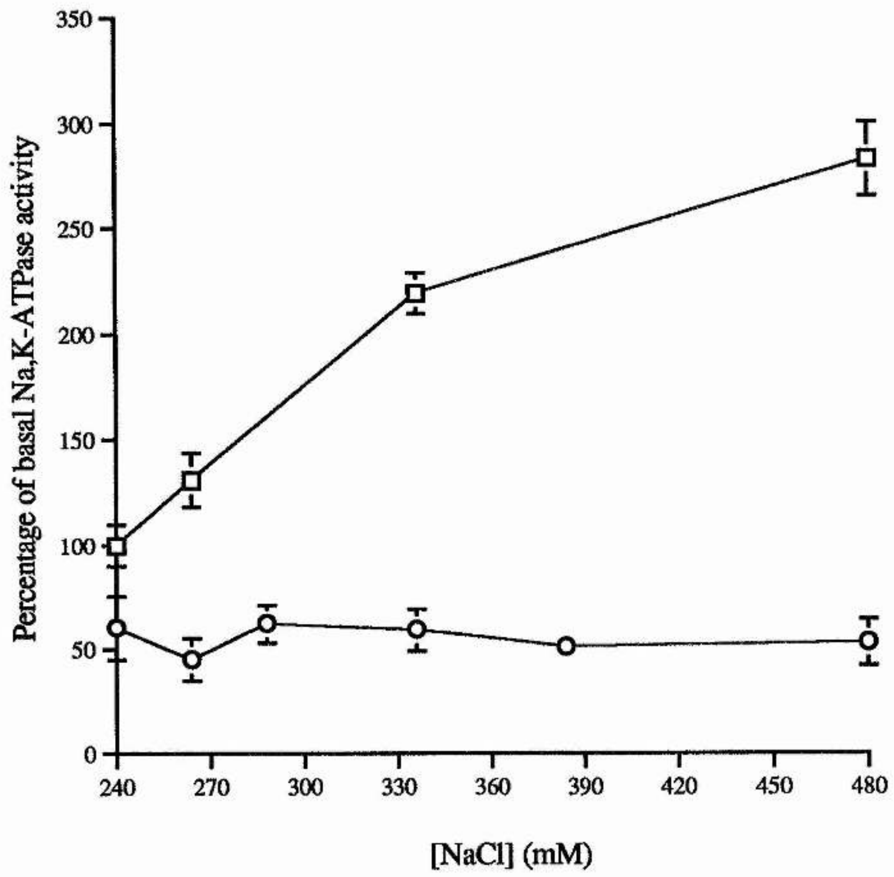

300
200
100
0
Na,K-ATPase activity (nmol Pi /mg protein/h)
0.00001
0.0001
0.0010
0.0100
0.1000
Log [bumetanide] (mM)

Figure 3.18

Figure 3.18 The effect of bumetanide on the NaCl-induced upregulation of Na,K-ATPase activities in cell homogenates. Cells were treated with medium containing the NaCl concentrations indicated in the absence (❑) or presence (o) of 0.1 mM bumetanide for 30 minutes. Cells were homogenised and Na,K-ATPase activities determined. Values shown are percentage values compared to activities found with normal NaCl concentrations and are given as individual means ± standard error of mean for 4 separate determinants. The control value was 1468 nmols Pi/mg protein/hour. Significant differences were determined by a two way ANOVA followed by Bonferroni and Dunnpost hoc test. The p-value obtained was relevant to the degree of significant difference, $p^* \leq 0.05$ significant; $p^{**} \leq 0.01$ highly significant; $p^{***} \leq 0.001$ very highly significant.

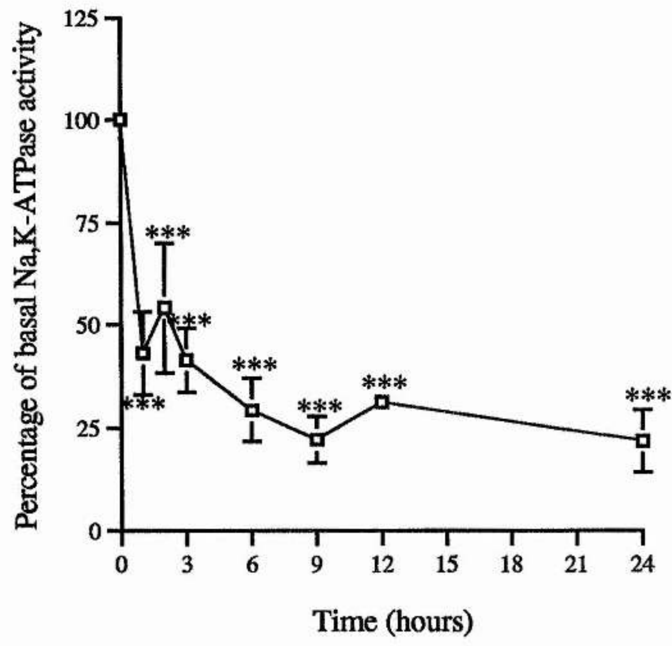


350
300
250
200
150
100
50
0
240
270
300
330
360
390
420
450
480
Percentage of basal Na,K-ATPase activity
[NaCl] (mM)

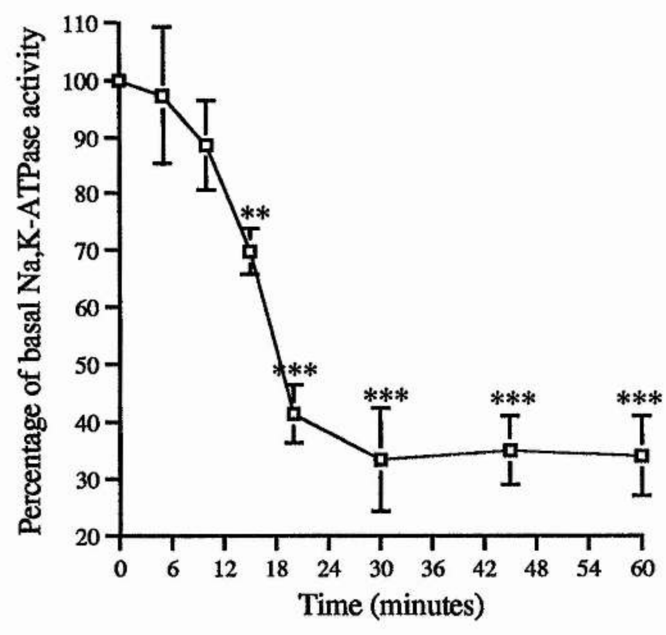
Figure 3.19 and Figure 3.20

Figure 3.19 Time course showing the effect of incubating cells in the presence of 0.1 mM bumetanide on subsequent Na,K-ATPase activities in cell homogenates. Values shown are percentage values compared to activities found with normal NaCl concentrations and are given as individual means ± standard error of mean for 4 separate determinants. The control value was 657 nmolsPi/mg protein/hour. Significant differences were determined by a two way ANOVA followed by a Bonferroni and Dunn post hoc test. The p-value obtained was relevant to the degree of significant difference, $p^* \leq 0.05$ significant; $p^{**} \leq 0.01$ highly significant; $p^{***} \leq 0.001$ very highly significant.

Figure 3.20 Time course showing the effect of incubating cells in the presence of 0.1 mM bumetanide on subsequent Na,K-ATPase activities in cell homogenates. Values shown are percentage values compared to activities found with normal NaCl concentrations and are given as individual means ± standard error of mean for 4 separate determinants. The control value was 1134 nmolsPi/mg protein/hour. Significant differences were determined by a two way ANOVA followed by a Bonferroni and Dunn post hoc test. The p-value obtained was relevant to the degree of significant difference, $p^* \leq 0.05$ significant; $p^{**} \leq 0.01$ highly significant; $p^{***} \leq 0.001$ very highly significant.



Percentage of basal Na,K-ATPase activity
125
100
75
50
25
0
***
***
***
***
***
***
***
0
3
6
9
12
15
18
21
24
Time (hours)

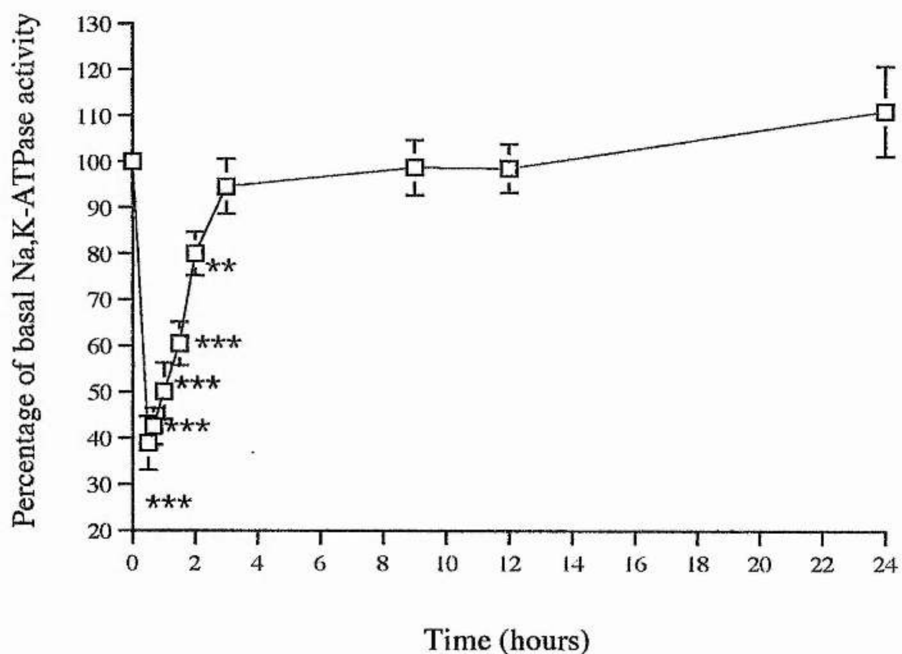



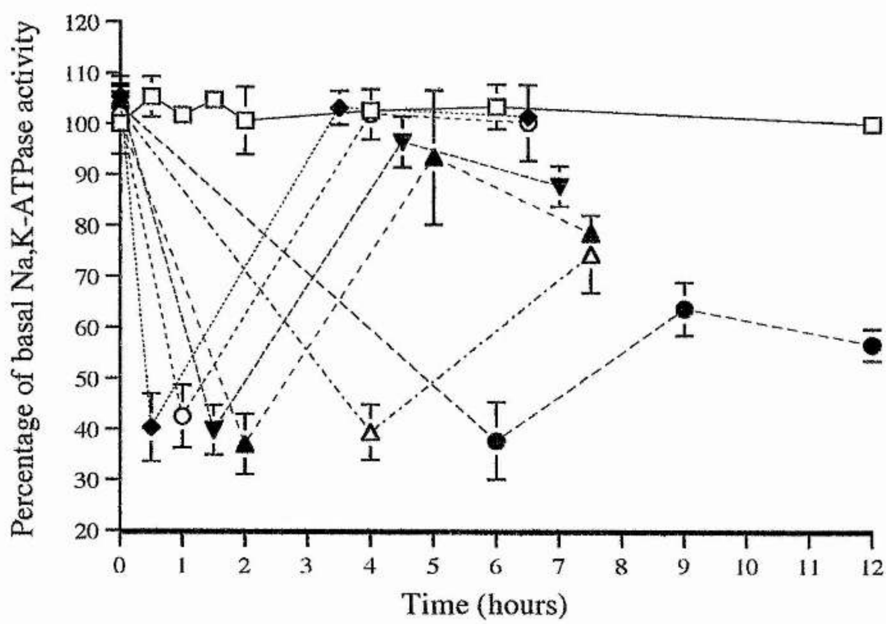
Percentage of basal Na,K-ATPase activity
110
100
90
80
70
60
50
40
30
20
**
***
***
***
***
0
6
12
18
24
30
36
42
48
54
60
Time (minutes)

Figure 3.21 and 3.22

Figure 3.21 Inhibition and recovery of Na,K-ATPase activities in cell homogenates following addition and then removal of 0.1 mM bumetanide from the growth medium. Cells were incubated in the presence of bumetanide for 30 minutes before washing the cells and replacing with normal growth medium for the remaining duration of the experiment. Values shown are percentage values compared to activities found with normal NaCl concentrations and are given as individual means ± standard error of mean for 4 separate determinants. The control value was 789 nmolsPi/mg protein/hour. Significant differences were determined by a two way ANOVA followed by Bonferroni and Dunn post hoc test. The p-value obtained was relevant to the degree of significant difference, $p^* \leq 0.05$ significant; $p^{**} \leq 0.01$ highly significant; $p^{***} \leq 0.001$ very highly significant.

Figure 3.22 Time course of Na,K-ATPase inhibition and recovery following bumetanide treatment for varying lengths of time. Cells were untreated (□) or incubated in 0.1 mM bumetanide for 30 mins (◆), 1 hour (O), 1.5 hours (▼), 2 hours (▲), 4 hours (Δ) and 6 hours (●). The bumetanide medium was then removed, the cells washed and the growth medium was replaced with normal growth medium for the remaining duration of the experiment. Values shown are percentage values compared to activities found with normal NaCl concentrations and are given as individual means ± standard error of mean for 4 separate determinants. The control value was 1431 nmolsPi/mg protein/hour.


Percentage of basal Na,K-ATPase activity
130
120
110
100
90
80
70
60
50
40
30
20
**
***
***
***
***
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (hours)


Percentage of basal Na,K-ATPase activity
120
110
100
90
80
70
60
50
40
30
20
0
1
2
3
4
5
6
7
8
9
10
11
12
Time (hours)

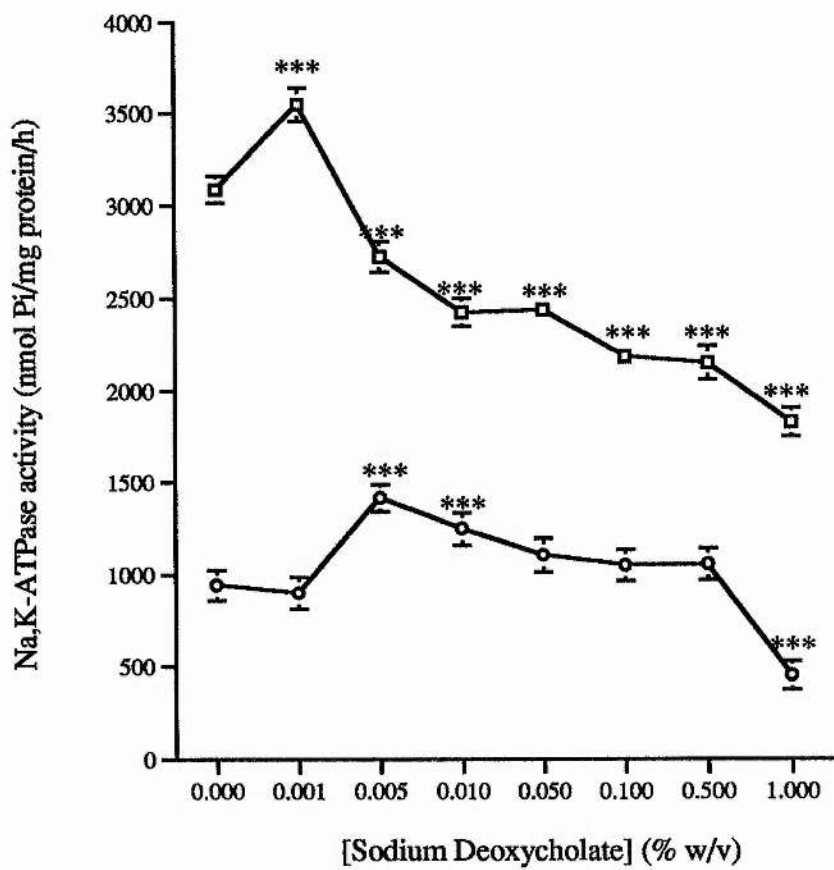
dependent on the period of inhibition; the longer the period of inhibition the less Na,K-ATPase activity was recovered (Fig 3.22). In cases of prolonged bumetanide treatment (4 and 6 hours) the activity never returned to the original value.

Sodium deoxycholate was routeinely included in the homogenisation buffer to disrupt any naturally occurring vesicles which formed during homogenisation. This treatment should also permeablilise any endogenous subcellular vesicles within the cells. Therefore if active Na,K-ATPase units were removed from the membrane following bumetanide treatment and transferred to some vesicular compartment, the detergent treatment should allow the assay to measure the total Na,K-ATPase activity present in the whole cell, not only the plasma membrane associated Na,K-ATPase. To determine if indeed the standard sodium deoxycholate concentration of 0.001 % was sufficient to unmask all intracellular Na,K-ATPase activity associated with some vesicular compartment, a dose response curve for sodium deoxycholate was conducted to assess Na,K-ATPase activities in homogenates of cells treated with bumetanide (Fig 3.23). The sensitivity of the Na,K-ATPase assay to sodium deoxycholate was found to be different between control and bumetanide treated cells. In control cells incubated in normal growth medium, concentrations of sodium deoxycholate in the homogenisation buffer above 0.001% resulted in the gradual decrease in activity. This was not the case in the bumetanide treated cells with concentrations of 0.005% and 0.01% sodium deoxycholate increasing activity. However this activity was still significantly lower than the basal value measured with 0.05 % (w/v) deoxycholate in the untreated cells.

To further examine the possibility that Na,K-ATPase enzyme units were internalised following bumetanide treatment the effects of the anti-microtubule agent colchicine was investigate. The cell monolayers were pre-incubated in normal growth medium or growth medium containing 0.1 μg/ml colchicine for 1 hour. After this time 0.1 mM bumetanide was added to both cell groups and the incubations continued. Cells were then homogenised and assayed for Na,K-ATPase activity at the times indicated in figure 3.24. This experiment showed that pre-treatment with colchicine completely suppressed the bumetanide-induced inhibition of Na,K-ATPase activity (Fig 3.24). The effect of colchicine on the recovery of Na,K-ATPase activity following bumetanide removal was also investigated (Fig 3.25). Cells were incubated for 60 minutes in growth medium containing 0.1 mM bumetanide. At the 30 minutes time point colchicine (0.2 μg/ml) was added to one group of cells. After 60 minutes the bumetanide containing media was removed from the both groups and the cells were washed twice, then incubated in normal media for a further 3.5 hours. Treatment with

Figure 3.23

Figure 3.23 Effect of varying sodium deoxycholate concentrations in the homogenisation buffer on Na,K-ATPase activites measure in homogenates of untreated cells (❑) and cells incubated in bumetanide for 30 minutes (O). Following incubation for 30 minutes in the presence or absence of bumetanide cells were washed twice and then homogenised in the homogenisation buffer, Na,K-ATPase activities were then determined. Individual values are means ± standard error of mean for 4 separate experiments. Significant differences were determined. The p-value obtained was relevant to the degree ofsignificant difference, $p^* \leq 0.05$ significant; $p^{**} \leq 0.01$ highly significant; $p^{***} \leq 0.001$ very highly significant.

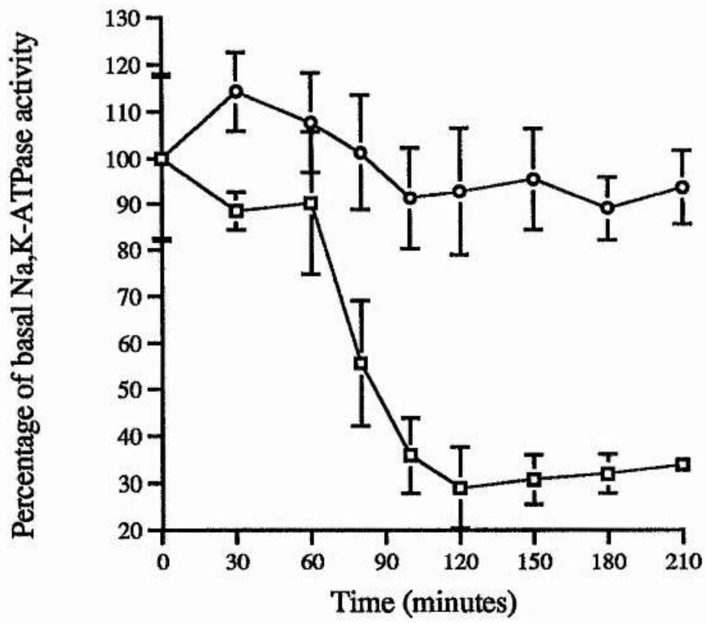


Na,K-ATPase activity (nmol Pi/mg protein/h)
4000
3500
3000
2500
2000
1500
1000
500
0
***
0.000
0.001
0.005
0.010
0.050
0.100
0.500
1.000
[Sodium Deoxycholate] (% w/v)

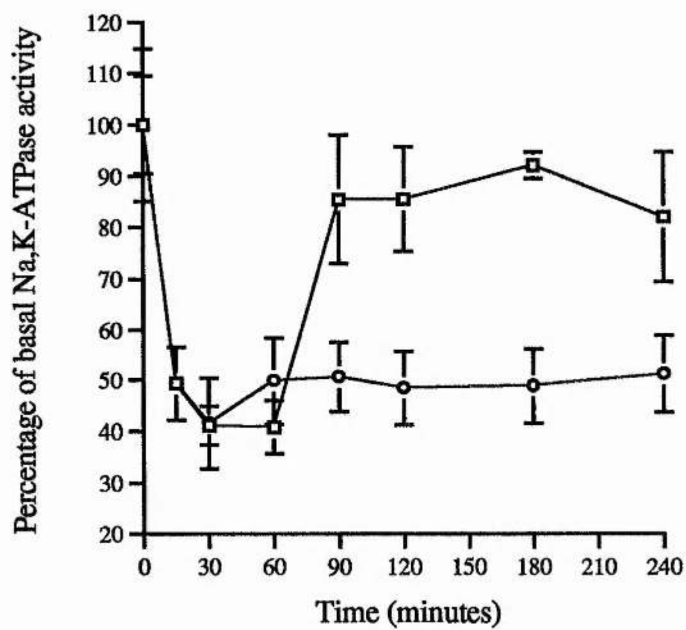
Figure 3.24 and 3.25

Figure 3.24 The effect of colchicine on bumetanide induced inhibition of Na,K-ATPase activity in cell homogenates. Cell cultures were treated with 0.1 mM bumetanide from the 60 minute time point (❑), or were pretreated with 0.1 $\mu$g/ml colchicine for 1 hour and then with 0.1 mM bumetanide and 0.1 $\mu$g/ml colchicine together (O) from the 60 minute time point. Cells were homogenised and Na,K-ATPase activities determined. Values shown are percentage values compared to activities found with normal NaCl concentrations and are given as individual means ± standard error of mean for 4 separate determinants. The control value was 1005 nmolsPi/mg protein/hour.

Figure 3.25 The effect of colchicine on recovery of bumetanide induced inhibition of Na,K-ATPase activity in cell homogenates. Cell cultures were pretreated with 0.1 mM bumetanide for 30 minutes, then growth medium was replaced with normal growth medium (❑) or medium containing 0.1 $\mu$g/ml colchicine (O) for the times indicated. Cells were homogenised and Na,K-ATPase activities determined. Values shown are percentage values compared to activities found with normal NaCl concentrations and are given as individual means ± standard error of mean for 4 separate determinants. The control value was 872 nmolsPi/mg protein/hour.



Percentage of basal Na,K-ATPase activity
130
120
110
100
90
80
70
60
50
40
30
20
0
30
60
90
120
150
180
210
Time (minutes)

Percentage of basal Na,K-ATPase activity
120
110
100
90
80
70
60
50
40
30
20
0
30
60
90
120
150
180
210
240
Time (minutes)

colchicine completely suppressed the recovery of Na,K-ATPase activity after bumetanide removal (Fig 3.25)

### 3.IIIe Effect of calcium of Na,K-ATPase activity

As calcium is known to be an important intracellular second messenger which is involved in a number of processes from activation of protein kinases to vesicle movement the possible involvement of this divalent cation on the NaCl up-regulation and the bumetanide down regulation of Na,K-ATPase activities was investigated. Cells were incubated in calcium-free medium ( commercialy bought medium contained 10 μM calcium, therefore it was necessary to treat this medium with 5 mM EGTA) in the presence of elevated NaCl concentrations for up to 72 hours. The cells were then homogenised and the Na,K-ATPase activities determined as before. Removing calcium from the medium inhibited the NaCl-mediated increase in Na,K-ATPase activities normally observed (Fig 3.26). The cells incubated in the calcium-free medium did not only fail to respond to the increased NaCl concentration, but also exhibited a fall in Na,K-ATPase activity compared to basal levels if incubated in calcium-free medium for prolonged periods (>24 hours) (Fig 3.26).

The effect of raising intracellular calcium concentrations on Na,K-ATPase activities was also investigated. A dose-response curve was constructed to investigate the effects of the calcium ionophore A23187 on cellular Na,K-ATPase activities. However A23187 at all concentrations used did not significantly change basal Na,K-ATPase activities in the cell homogenates after a 12 hour incubation period (Fig 3.27). Although it was not possible to measure intracellular calcium and confirm its elevation by A23187 these results suggest that the transient increase in Na,K-ATPase activity associated with an increase in growth medium NaCl concentration is dependent on the presence of extracellular, and presumably intracellular, calcium but is not itself induced by increases in intracellular calcium.

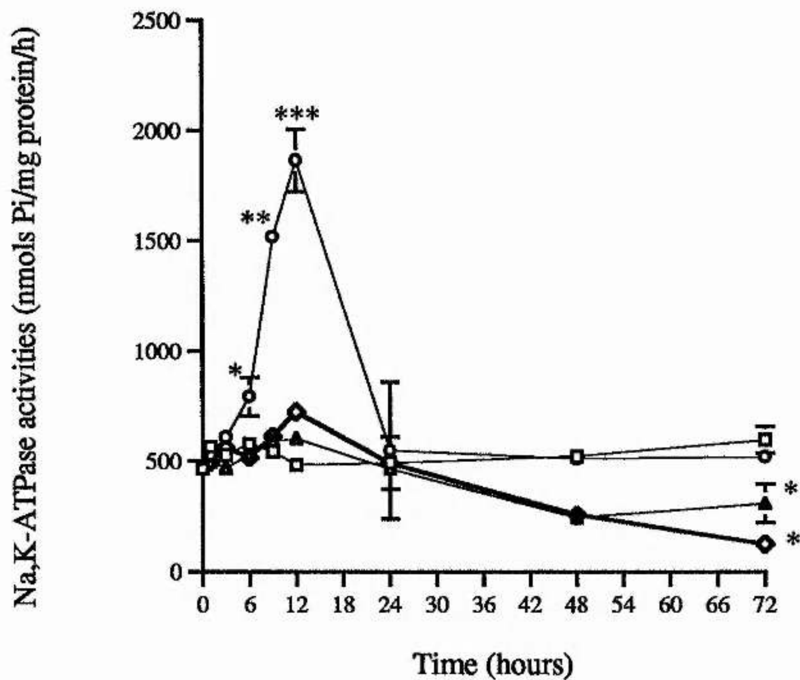
### 3.IIIf Effects of extracellular NaCl on Na,K-ATPase activity in cells grown in suspension

Cells grown in suspension as described in section 2.II a iii were incubated for various time periods in the presence of normal growth medium containing 240 mM NaCl or in medium containing 360 mM NaCl for up to 24 hours. These cells however did not exhibit the same response to increases in medium NaCl concentrations as the cells

Figure 3.26 and Figure 3.27

Figure 3.26 Effect of extracellular calcium on NaCl-mediated increases in Na,K-ATPase activity. Cells were incubated in normal growth medium (□), 360 mM NaCl growth medium (O), calcium-free normal growth medium (Δ) or calcium-free growth medium containing 360 mM NaCl (◊) for the times indicated. Cells were homogenised and Na,K-ATPase activities determined. Individual values are means ± standard error of mean for 4 separate experiments. Significant differences were determined by two way ANOVA followed by a Bonferroni and Dunn post hoc test. The p-value obtained was relevant to the degree of significant difference, $p^* \leq 0.05$ significant; $p^{**} \leq 0.01$ highly significant; $p^{***} \leq 0.001$ very highly significant.

Figure 3.27 The effect of the calcium ionophore A23187 on Na,K-ATPase activity in cell homogenates. Cells were incubated in the presence of A23186 at the concentrations indicated for 12 hours, after which cells were homogenised and Na,K-ATPase activities determined. Individual values are means ± standard error of mean for 4 separate experiments. Significant differences were determined by two way ANOVA. The control value was 654 nmolsPi/mg protein/hour.


Na,K-ATPase activities (nmols Pi/mg protein/h)
2500
2000
1500
1000
500
0
***
**
*
*
*
0 6 12 18 24 30 36 42 48 54 60 66 72
Time (hours)

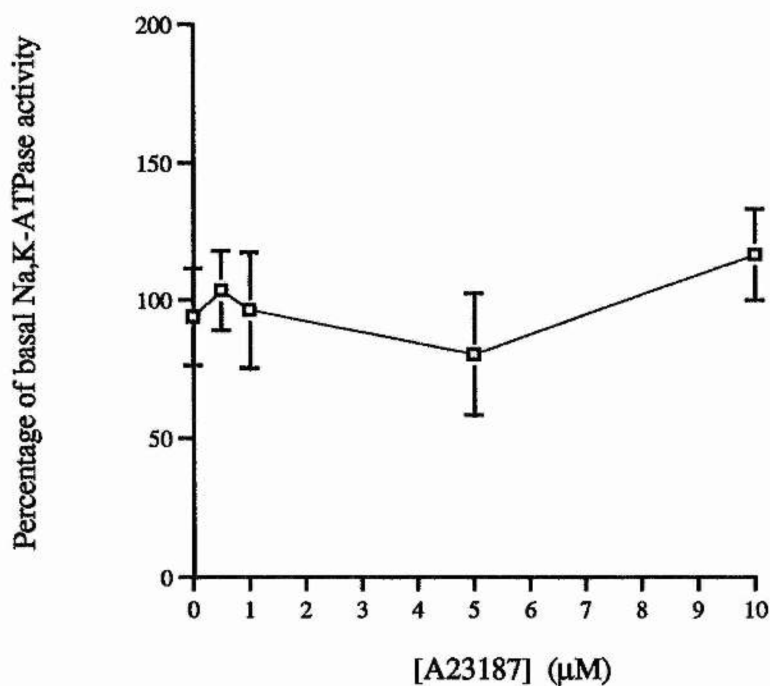
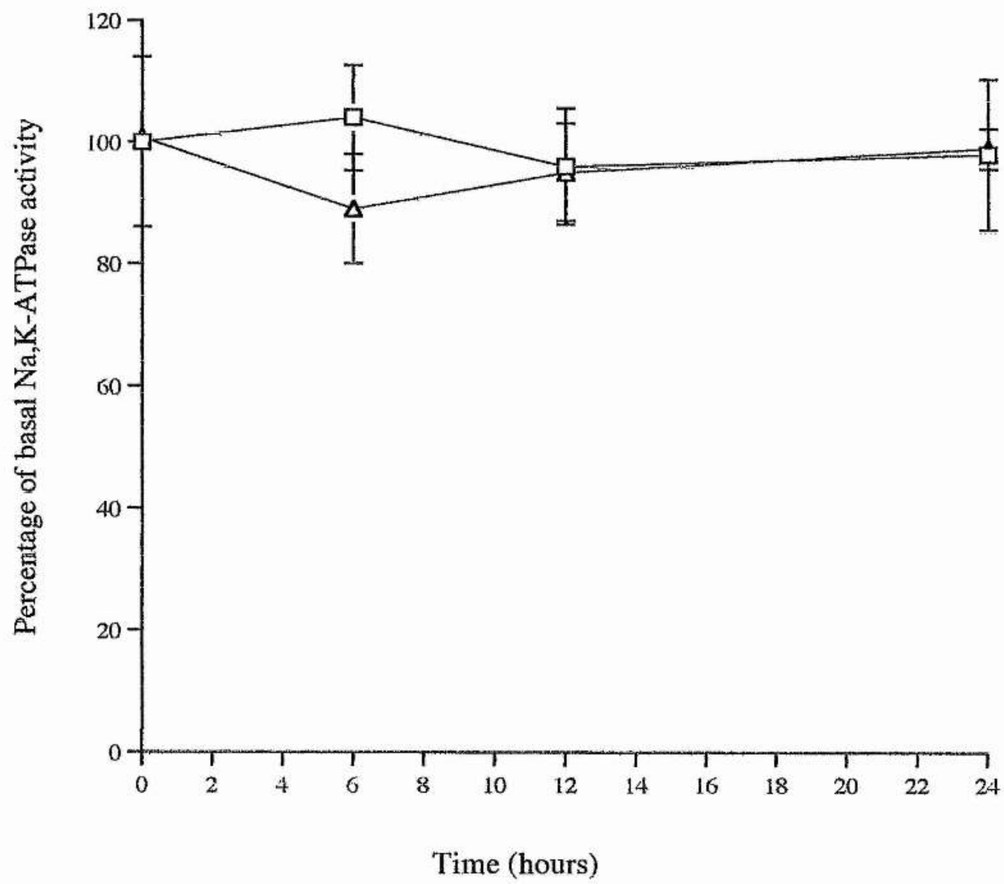

Percentage of basal Na,K-ATPase activity
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10
[A23187] (μM)

Figure 3.28

Figure 3.28 Effect of increased extracellular NaCl concentrations on Na,K-ATPase activity in rectal gland cells grown in suspension. Cells were grown in suspension for 13 days and then incubated in medium either containing 240 mM NaCl (□) or containing 360 mM NaCl medium (Δ) for the times indicated. The cells were pelleted by centrifugation at 4°C, 200 $x_g$ for 90 seconds on an MSE coolspin washed in shark Ringer and then homogenised and Na,K-ATPase activities determined. Values shown are percentage values compared to activities found at zero timewith normal NaCl concentrations and are given as individual means ± standard error of mean for 3 separate determinants. The control value was 308 nmolsPi/mg protein/hour.



120
100
80
60
40
20
0
Percentage of basal Na,K-ATPase activity
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (hours)

grown on collagen plates and there was no significant change in Na,K-ATPase activities of cell homogenates in response to elevated NaCl in cells grown in suspension (Fig 3.28). However the basal Na,K-ATPase activities measured in the cells grown in suspension were similar to the basal Na,K-ATPase activities measured in attached cells.

## 3.IV DISCUSSION

The regulation of Na,K-ATPase activity has been widely investigated in a number of tissues or cell types originating from various species (Bertorello, *et al*, 1993; Beguin, *et al*, 1996; Simmons, *et al*, 1986; Ikeda *et al.* 1991; Oguchi *et al.* 1993, Farman *et al.* 1994; McDonough *et al.* 1988). Factors which result in the raising of intracellular sodium concentration are known to cause an up-regulation of functional Na,K-ATPase enzyme units on the surface of the cell (Lamb, 1988; Tirupattur *et al.* 1993). The up-regulation has been reported to be the combined result of increases in the transcription of α and β- subunits (Oguchi *et al.* 1993; Farman *et al.* 1994; Blot-Chabaud *et al.* 1990), recruitment of newly synthesised proteins to the plasma membrane (Ismail-Beigi *et al.* 1993) and a reduction in the rate of enzyme removal and turnover (Geering *et al.* 1990).

We have investigated the effects of raising extracellular sodium concentration on Na,K-ATPase activity in cultured dogfish rectal gland epithelial cells. These cells have been used to demonstrate that an increase in extracellular NaCl concentration can transiently increase Na,K-ATPase activity by more than 3 fold, the activity peaking some 12 hours after the initial increase in medium NaCl and then returning to basal within 24 hours of incubation. This response was dependent on an increase in both Na and Cl ions in the medium as equivalent concentrations of chloride as choline chloride or sodium as $Na_2SO_4$ to the medium did not result in any significant increase in Na,K-ATPase activity. It was confirmed that the increase in Na,K-ATPase activity was due to increases in both sodium and chloride ions in the medium and was not an osmotic effect as NaCl exhibited a dose-dependent increase in Na,K-ATPase activity when the osmolality of the medium was held constant by addition of mannitol. The NaCl dose response curve showed that under normal conditions when the growth medium contained 240 mM NaCl, changes in Na,K-ATPase became very sensitive to relatively small increases in extracellular sodium chloride levels. For example extracellular NaCl concentrations were increased from 240 mM to 260 mM, Na,K-ATPase activities in cell homogenates rose by 40 % after a 12 hour incubation. This could have physiological relevance as normal extracellular NaCl concentrations in elasmobranchs are around 240 mM and normal NaCl plasma concentration can vary by ± 10 % when the fish feed.

The experiments conducted in the presence of the drug cycloheximide provided valuable evidence that the lag period of 6 hours before Na,K-ATPase activity started to increase in response to an increase in extracellular NaCl concentration was the result of increased protein synthesis, of presumably either or both $\alpha$ and $\beta$ subunits of the Na,K-ATPase. If the cycloheximide was present for the full duration of the incubation in 360 mM NaCl, the NaCl-stimulated Na,K-ATPase activity was completely inhibited. Cycloheximide did not only inhibit NaCl-stimulated Na,K-ATPase activity but also decreased the normal endogenous activity to below basal. It was concluded from these results that protein synthesis was involved in the response of Na,K-ATPase to increased extracellular [NaCl]. These experiments also showed that as cycloheximide decreased Na,K-ATPase activities below basal levels, cycloheximide reduced normal synthesis of Na,K-ATPase required for enzyme turnover .

Although the cycloheximide experiments show that protein synthesis was required for the time delayed and dose-dependent NaCl- stimulated increase in Na,K-ATPase activity, results using the transcription inhibitor, actinomycin D, suggest that increases in activity were not dependent on increased mRNA levels brought about by transcription. This is in agreement with previous work from our laboratory which showed that the transient increases in rectal gland Na,K-ATPase activities after feeding dogfish with high sodium chloride diets were not accompanied by increases in mRNAs for $\alpha$ or $\beta$ subunits of the enzyme (MacKenzie, 1996). The mechanism(s) responsible for this increase in Na,K-ATPase activity were not investigated, however it was shown that the high dietary sodium chloride loads did cause an increase in plasma sodium chloride concentration by approximately 10 % (260 mM to 280 mM). The exact mechanism(s) by which Na,K-ATPase activity is increased in response to extracellular NaCl is not yet known and requires further investigation. Considering the results of both studies it is possible that the increase in Na,K-ATPase activity seen in response to an increase in extracellular sodium chloride concentration is due to an concomitant increase in intracellular sodium and/or chloride concentrations. However, this increase in activity is not associated with an increase in $\alpha$ or $\beta$ subunit mRNA levels. It has been previously reported by other workers that factors which result in the raising of intracellular sodium concentrations are known in mammals to cause an up-regulation of functional Na,K-ATPase enzyme units on the surface of the cell (McDonough *et al.* 1988). The up-regulation has been reported to be the results of increases in the recruitment of newly synthesised proteins to the plasma membrane and a reduction in the rate of enzyme removal and turnover (Geering *et al.* 1990; Ideda *et al.* 1991). Although in the present study intracellular sodium concentrations were not measured, it

is assumed that elevations in intracellular sodium concentrations are implicated in increases in rectal gland epithelia cell Na,K-ATPase activity as sodium is required for the response and monensin also causes an increase in Na,K-ATPase activity. In addition the cycloheximide and actinomycin D studies suggest that the increase in activity although not associated with increases in mRNA levels is dependent upon protein synthesis.

Increases in Na,K-ATPase activity in dogfish rectal gland cells have previously been shown to be linked to sodium uptake via the Na,K,Cl cotransporter (Shuttleworth 1983). Shuttleworth, (1983) reported that cAMP-stimulated increases in Na,K-ATPase activity were blocked by furosemide or by the absence of extracellular chloride, and concluded that cAMP stimulation of the Na,K-ATPase occured via chloride-coupled sodium entry into the cell, which increases intracellular sodium levels and in turn leads to an activation of the Na,K-ATPase as ascertained by enyme assay of Na,K-ATPase in rectal gland homogenates and via ouabain binding and ouabain-sensitive oxygen consumption in rectal gland slices. Observations from our own study (the need for both extracellular sodium and chloride) suggested that activation of the Na,K-ATPase in response to increased extracellular sodium chloride concentrations is linked to the Na,K,Cl-cotransporter. These observations led to a series of experiments that employed the drug bumetanide, a specific inhibitor of the Na,K,Cl-cotransporter,which provided strong evidence that the Na,K,Cl-cotransporter is central to the stimulatory effect of high extracellular NaCl concentrations on Na,K-ATPase. In addition bumetanide reversibly reduced basal Na,K-ATPase activities in cell homogenates by 70%. These observations combined with the results of the monensin experiments lead to the conclusion that the increase in Na,K-ATPase activity is linked to an increase in intracellular sodium concentration which enters the cell via the bumetanide sensitive Na,K,Cl-cotransporter. This increase in intracellular sodium concentration can by an unknown mechanism, induce increased protein synthesis which in turn is an essential pre-requisite for the insertion of functional Na,K-ATPase units into the membrane. These observations are in agreement with those made by Shuttleworth and Thompson (1980), who reported that in rectal gland slices from the European dogfish, cAMP stimulation of the Na,K-ATPase occured indirectly via an increase in intracellular sodium concentration which entered the cell via the Na,K,Cl cotransporter. Greger and Schlatter, (1984) also showed that, in dissected rectal gland tubules, intracellular sodium concentration increased prior to an increase in Na,K-ATPase activity following cAMP treatment. The Na,K,Cl-cotransporter has also been shown to be involved in Na,K-ATPase regulation in 3T3-L1 cells when Na,K-ATPase is stimulated by insulin

(Haussinger *et al.* 1994). Insulin stimulated ouabain-sensitive $Rb^+(K^+)$ uptake can be prevented by bumetanide in this cell type (Haussinger *et al.* 1994).

Pre-treatment of cells with the anti-microtubular agent, colchicine, suppressed the inhibitory effect of bumetanide on cell homogenate Na,K-ATPase activities. Colchicine was also able to inhibit the recovery of Na,K-ATPase activity following bumetanide removal. It is possible that colchicine may inhibit Na,K-ATPase recovery by a method previously reported in MDCK cells. Na,K-ATPase in MDCK cells is co-localised with two cytoskeletal actin binding proteins, ankyrin and fodrin, which appear to stimulate Na,K-ATPase in a dose-dependent manner, and colchicine has been shown to inhibit this interaction (Morrow *et al.* 1989). However in the present study it seems unlikely that colchicine acts by inhibiting cytoskeletal stimulation of Na,K-ATPase as it is also inhibited the fall in activity seen in response to bumetanide treatment. Therefore these results suggest that active Na,K-ATPase units may be removed from the membrane when the intracellular sodium concentration falls and stored in some intracellular compartment in an inactive form. If the intracellular sodium concentration is subsequently increased these inactive Na,K-ATPase units may then be reactivated and re-inserted back into the plasma membrane. The recovery of cell homogenate Na,K-ATPase activity following transient inhibition of the Na,K,Cl cotransporter was dependent on the period of bumetanide inhibition. The longer the period of inhibition the less Na,K-ATPase activity was recovered. In experiments where the treatment lasted longer than 4-6 hour the activity never returned to the original value. These results indicate that the inactivated and internalised Na,K-ATPase enzyme units, if not immediately re-inserted back into the plasma membrane are degraded with a half life of 4-5 hours. In the longer term (12 hours) this intracellular pool is totally degraded and when cells are grown in normal sodium chloride concentrations (240 mM) there is no intracellular pre-formed stores of inactivated enzyme units which can be rapidly activated and returned to the plasma membrane on raising extracellular NaCl (see Fig 3.7B).

Sodium deoxycholate was included in the homogenisation buffer to disrupt any vesicles which formed during homogenisation. To check whether enough sodium deoxycholate was present under these conditions a dose response curve for sodium deoxycholate was conducted to assess Na,K-ATPase activities in cell homogenates pre-treated with bumetanide. If active Na,K-ATPase units were removed from the membrane on bumetanide treatment and transferred to a vesicular compartment the detergent treatment should allow the assay to measure all of the Na,K-ATPase activity present in the whole

cell not only the plasma membrane bound component of Na,K-ATPase. More sodium deoxycholate was necessary to measure optimal Na,K-ATPase activities in bumetanide treated cells than in untreated cells (0.005 % (w/v) compared to 0.001 % (w/v)). However activity in the presence of bumetanide was still significantly lower than the basal value measured in untreated cells. Therefore if Na,K-ATPase is taken up in an active form by the cells into vesicular stores sodium deoxycholate can not release this. Since this is highly unlikely this suggests that any intracellular stored enzyme must be in an inactive form .

Internalisation and storage of inactivated Na,K-ATPase enzyme units has previously been reported. Blot-Chad and coworkers (1990) reported that a pool of latent Na,K-ATPase enzymes are present in rabbit kidney cells. These pumps are rapidly mobilised to the basolateral membrane under the influence of intracellular sodium. Whether the latent pool of Na,K-ATPase was composed of inactive enzyme units in the membrane or of intracellular pumps was not confirmed. Translocaton of Na,K-ATPase units to the plasma membrane have been reported in response to insulin stimulation in the frog skeletal muscle (Omatsu-Kanbe and Kitasato, 1990) and in mammalian skeletal muscle (Hundal *et al.* 1992). Intracellular stores of Na,K-ATPase units were shown to exist in experiments measuring Na,K-ATPase activities and ouabain binding in different cell fractions isolated from frog (*Rana cayesbeiana* ) skeletal muscle (Omatsu-Kanbe and Kitasato, 1990). The translocation of the Na,K-ATPase units to the plasma membrane in response to insulin was hypothesised to be due to increased intracellular sodium concentrations as insulin is known to induce elevation of intracelluar sodium in 3T3-L1 adipocyte cells (Haussinger *et al.* 1994). The exact mechanism by which the increase in sodium causes translocation of Na,K-ATPase units to the plasma membrane remains to be established although, it was suggested that the gain in salt could increase cell volume, and changes in cell volume in response to insulin, have been implicated in subsequent metabolic control (Haussinger *et al.* 1994). However rectal gland epithelial cells under normal circumstances do not appear to have a pre-formed intracellular pool of inactive Na,K-ATPase units explaining why in this particular tissue there is a delayed response to sodium chloride loading. This delayed response is likely to be due to the need for synthesis of new Na,K-ATPase enzyme units.

The rapid fall in cellular Na,K-ATPase activity that occurs after the 12 hour peak activity associated with incubations of cells in high NaCl-containing medium is not linked to the synthesis of some inhibitory protein as the addition of cycloheximide at this time point does not affect the fall in activity observed. The activity of the Na,K,Cl-

cotransporter is implicated in the return of Na,K-ATPase activity to basal levels as monensin induced upregulation of the enzyme (which essentially short circuits the sodium influx via the cotransporter) is not refractory and activities remain elevated. It is therefore possible that sodium entry into the cells via the Na,K,Cl-cotransporter may be reduced over this time period resulting in a fall in intracellular sodium concentration back to control values despite the elevated extracellular concentrations. The drop in Na,K-ATPase activity might therefore be linked by an unknown mechanism, to the reduced entry of sodium into the cell via the suppressed activity of the Na,K,Cl-cotransporter. Recent evidence suggests that the Na,K,Cl-cotransporter can be regulated by feedback control mechanism(s) that sense and prevent increases or decreases in cytoplasmic chloride concentrations (Haas *et al.* 1992). How a change in intracellular chloride concentration is perceived and translated into a corrective Na,K,Cl-cotransport flux is unknown, however it is thought to involve phosphorylation of the transporter itself. This information lead me to hypothesis that the decrease in Na,K-ATPase activities in dogfish rectal gland cells observed following a 12 hour incubation is due to the progressive inhibition of the Na,K,Cl cotransporter as a result of the accumulation of intracellular chloride. This in turn may suppress sodium entry into the cell and hence intracellular sodium concentrations return to basal levels resulting in a fall in elevated Na,K-ATPase activities back to control values.

As calcium is known to be an important intracellular second messenger which is involved in a number of cellular processes from the activation of protein kinases to vesicle movement the possible involvement of this divalent cation on up-and down-regulation of Na,K-ATPase activities was investigated. Although it was shown that calcium was necessary to elicit the NaCl-stimulated increase in Na,K-ATPase activity, addition of the calcium ionophore A23187 did not significantly effect Na,K-ATPase activity in cell homogenates. Based on these observations it is unlikely that the NaCl-mediated effect is the sole result of increases in intracellular calcium concentrations. Shuttleworth, (1983) reported that stimulation of the Na,K-ATPase by cAMP is blocked by verapamil a calcium channel blocker. However Na,K-ATPase activity was shown not to be stimulated by calcium alone as Shuttleworth reported that in the absence of any change in cAMP concentrations A23187 (calcium ionopnore) failed to induce an increase in ouabain binding or ouabin-sensitive oxygen consumption in rectal gland slices. The implication being that calcium does not have a direct effect on the regulation of Na,K-ATPase acivity. Shuttleworth's observations therefore agree with this study, i.e. calcium is necessary for regulation of the Na,K-ATPase, however many other factors are also involved in the regulation of this enzyme's activity.

Cells grown in suspension did not, however, exhibit the same response to increases in extracellular NaCl as collagen-attached cells. A possible explanation for this is that the cells need to establish some kind of polarity before they are able to respond. When grown in suspension the cells form groups or aggregates with a tubular type structure (refer to chapter 2 section III for description of the morphology), however the cells may not form the correct polarity and differentiation of intracellular and extracellular membrane compartments necessary for them to respond to increases in extracellular NaCl. An explanation for the lack of response to sodium chloride by the cells grown in suspension could be that the tubular morphology they adapt results in the exposure of the basolateral membrane rather than the apical membrane to the growth medium. Cells grown as monolayers on collagen coated plates have their apical surfaces exposed to the growth media, however it might be that in cells grown in suspension the apical surface of the cells is not experiencing an increase in sodium choride and the cells therefore do not respond. However without further experimental evidence it is very difficult to give an explanation for the lack of response to NaCl for the cells grown in suspension.

These results lead me to conclude that the concentration of extracellular sodium chloride in the growth medium of rectal gland epithelial cells is important for regulation of Na,K-ATPase activity. Increasing extracellular sodium chloride concentration leads to increased synthesis and recruitment of active Na,K-ATPase units to the plasma membrane and probably result in a delayed inactivation of the Na,K,Cl -cotransporter. Inhibition of the Na,K,Cl-cotransporter using the drug bumetanide resulted in the rapid inactivation and uptake of Na,K-ATPase into some intracellular store. It is probable that these effects are due to changes in intracellular sodium concentrations due to the entry of extracellular sodium via the Na,K,Cl-cotransporter. If this is true then the intracellular sodium concentration is an important regulator of de novo synthesis and / or cycling of Na,K-ATPase units between the plasma membrane and some intracellular vesicular compartment.

# Effects of increased extracellular sodium chloride concentration on the expression of Na,K-ATPase α-subunit mRNA in rectal gland epithelial cell cultures

## 4.I INTRODUCTION

Ribonucleic acid (RNA) synthesis (DNA transcription) is a highly selective process. In most mammalian cells, for example, only about 1% of the entire chromosomal nucleotide sequence is transcribed into functional RNA sequences (Alberts *et al.* 1989). The selectivity occurs at two levels:- 1. only part of the DNA sequence is transcribed to produce nuclear RNAs and 2. only a minor proportion of the nucleotide sequences in nuclear RNAs survives the RNA processing steps that precede the export of RNA molecules to the cytoplasm (Alberts *et al.* 1989).

RNA polymerase, the enzyme that catalyses DNA transcription, is a complex molecule containing many polypeptide chains. In eukaryotic cells there are three RNA polymerases, designated polymerases I, II, and III; they are evolutionary related to one another and to bacterial RNA polymerase (Alberts *et al.* 1989). After initiating transcription, each enzyme is thought to release one or more initiation factors and to bind elongation factors that are required for RNA elongation, termination and modification (Alberts *et al.* 1989).

Most cellular messenger RNA (mRNA) is produced by a complex process beginning with the synthesis of heterogeneous nuclear RNA (hnRNA) (Alberts *et al.* 1989). The primary hnRNA transcript is synthesised by RNA polymerase II. Following RNA synthesis, intron sequences (segments of largely unknown function within a gene, present in the initial transcript, but not found in functional mRNA) are removed from the hnRNA resulting in a decrease in the size of the mRNA. In mammalian cells about 80-85% of total cellular RNA is ribosomal RNA (rRNA) (chiefly 28S, 18S and 5S incomplete ribosomal subunits) and most of the remaining 15-20% consists of a variety of low molecular weight species (transfer RNA (tRNA) and small nuclear RNAs). These latter RNAs are of defined size and sequence. In contrast, mRNA, which makes up approximately 3% of the total cellular RNA, is heterogeneous in both size and sequence (Alberts *et al.* 1989). Different types of RNA are present in the cell for different purposes, the RNA transcripts that direct the synthesis of protein molecules

are the mRNA. The codons in a mRNA molecule do not directly recognise amino acids, therefore the translation of mRNA into protein depends on tRNA which recognise both an amino acid and a group of three nucleotides. The codon recognition process by which genetic information is transferred from mRNA via tRNA to protein depends the presence of rRNA, the ribosomes are where tRNA molecules position themselves so as to read the mRNA molecule.

A change in mRNA levels can be controlled by gene expression. Gene expression at the protein level is dependent on the rates of transcription of specific mRNAs, their stability and therefore half life within the cell, regulates the rates of translation and final stability of the proteins. The stability of a mRNA can be changed in response to extracellular signals. Thus, for example, steroid hormones can affect target cells not only by increasing the transcription of specific genes, but also by increasing the stability of several of their mRNAs (Alberts *et al.* 1989). Measuring mRNA levels for a specific protein in a cell enables you to determine whether an increase in gene expression in response to an external stimuli is associated with an increase in mRNA content either as a result of increased transcription, an increase in RNA stability. The method normally employed to measure specific mRNA levels in cells is called Northern blotting. This method will give only a qualitative or at best a semi-quantitative, measure of a specific mRNA transcript present, whereas the related technique of slot or dot blotting is more quantitative, as the RNA is blotted on to one spot of the filter and is not run on a gel first separating RNA's of different sizes and spreading it over a large area.

Northern blotting involves denaturing the RNA (which has been isolated from the organ, tissue or cells of interest) by heating in a solution containing the denaturants formamide and formaldehyde. The individual RNA species in the sample are then separated on the basis of size using gel electrophoreses. This system of gel electrophoresis allows good size separation and resolution of single-stranded RNAs. Following electrophoresis the RNAs can then be transferred to a nylon filter by a blotting procedure, which results in the trapping of the denatured RNA on the filter by general electrostatic interaction. After the blotting period the filter can either be baked in an oven overnight or treated with an ultra violet light to covalently cross link and permanently attach the RNA to the filter. During the subsequent hybridisation procedure radio-labelled nucleic acid probe is used to hybridise to the specific mRNA(s) in the original sample. The final step of autoradiography then provides an accurate record of the image of the radioactive probe hybridised to the filter. Dot blot

hybridisations are performed by spotting a small sample of the RNA preparation onto a nylon or cellulose acetate filter using a filtration manifold which is designed to accept a large number of samples. The manifold fits onto a suction platform which supports the filter onto which the samples are deposited. The filter is then removed from the dot blotter, dried, and the RNA covalently-cross linked to the filter support before hybridising with a specific radio-labelled nucleic acid probe. The filter is then exposed to X-ray film and the autoradiographic images on the X-ray film are analysed using a flying spot scanning densitometer.

## 4.I ai Regulation of Na,K-ATPase expression

Hormones can cause increases in Na,K-ATPase activity, by increasing the total number of available sodium pump units. This is caused by regulation at both transcriptional and post-transcriptional levels. The latter level may involve not only changes in mRNA stability but likely changes in Na,K-ATPase protein half-life (Bertorello, 1993). A hormone that typically increases the total amount of pump units is thyroid hormone (Azuma *et al.* 1983), but this effect may also be elicited by sustained exposure to aldosterone ( Blot Chabaud *et al.* 1990), and in certain tissues insulin (Beguin *et al.* 1994). The effects of all three hormones are manifest on both $\alpha$ and $\beta$-subunits and depending on the tissue, on all isoforms of each subunit. The exact mechanism(s) underlying these actions is not known. However changes in the rate of transcription and or stability of mRNA of the $\beta$-subunit can lead to higher levels of $\alpha\beta$ complexes, due to stabilisation of $\alpha$-proteins that otherwise would be degraded if uncomplexed by the $\beta$-subunit ( McDonough *et al.* 1988; Geering *et al.* 1990).

Aldosterone, a steroid hormone synthesised and released by the adrenal cortex, is known to stimulate Na,K-ATPase activity in MDCK cells (Shahedi *et al.* 1993), rat cardiocytes (Ikeda *et al.* 1991), vascular smooth muscle (Oguchi *et al.* 1993) and hippocampus (Farman *et al.* 1994). Aldosterone increases the activity of the sodium pump by inducing the synthesis of new $\alpha$ and $\beta$-subunits. This has been observed in diverse tissues including toad bladder (Geering *et al.* 1990), rabbit kidney (Blot-Chabaud *et al.* 1990) and colon (Wiener*et al.* 1993), and in rat kidney (Palmer *et al.* 1993), heart (Ideda *et al.* 1991) and skeletal muscle (Oguchi *et al.* 1993). This response presumably involves interaction of the aldosterone receptor complex with specific hormone-responsive elements in the promoter regions of the $\alpha$ and/ or $\beta$-subunit genes. Treatment of rabbits with aldosterone for several days via a minipump implant markedly induced the expression of the $\alpha_1$-subunit in kidney cortical collecting

tubules, without evidence of the appearance of $\alpha_2$ in this tissue (Welling *et al.* 1993). Similarly, addition of aldosterone to vascular smooth muscle primary cultures led to the accumulation of $\alpha_1$ and $\beta_1$-mRNAs (Oguchi *et al.* 1993). In contrast, the $\alpha_3$-isoform, but not the $\alpha_1$, was induced in rat hippocampus 48 hours after injection of aldosterone (Farman *et al.* 1994). Together, these findings suggest that the effect of aldosterone on the Na,K-ATPase gene expression is highly dependent on the tissue involved.

Evidence suggests that thyroid hormone does not alter the catalytic properties of the enzyme but stimulates overall Na,K-ATPase activity by increasing the number of cellular pump molecules expressed on the cell surface (Ismail-Beigi *et al.* 1993). The input from thyroid hormone occurs both at the level of $\alpha$ and $\beta$ gene transcription and also at the level of mRNA stability (Ismail-Beigi *et al.* 1993). Indeed, treatment with thyroid hormone causes increased levels of Na,K-ATPase mRNA subunits in several tissues, eg increases $\alpha_1$ and $\alpha_2$ mRNA in rat muscle (Azuma *et al.* 1993), increases $\alpha_1$ and $\beta_1$ mRNAs in rat kidney (McDonough *et al.* 1988) and increases $\alpha_2$, $\alpha_3$ and $\beta_1$ mRNA in cardiac myocytes (Orlowski *et al.* 1990). These examples highlight the specificity of thyroid hormone action on the pump isoforms in different tissues.

Although insulin is known to acutely stimulate Na,K-ATPase activity by increasing intracellular concentrations of sodium, the long-term actions of insulin have been studied to a lesser extent. The effect of sustained exposure to insulin (1 and 6 hours) has been examined in vascular smooth muscle cell cultures derived from rat thoracic aorta (Tirupattur *et al.* 1993). In these cells the level of $\alpha_2$-mRNA was increase by 30% without any change in $\alpha_1$ mRNA. The consequence of this modest increase on the levels of protein $\alpha_2$ subunits was however not assessed. Insulin has been reported to increase $\alpha_2$-subunit and $\beta_1$-subunit expression in frog skeletal muscle (Russo and Sweadner, 1993).

It is also well established that conditions which cause a chronic elevation in intracellular sodium concentrations can also lead to an upregulation of sodium pump density in a variety of cells (Lamb, 1988). In cells where the intracellular sodium concentration is raised by growth in low-potassium media, the increased pump density is partly due to a decrease in the rate of internalisation and degradation of existing pump units (Lewis *et al.* 1981) and partly to a transient increase in synthesis associated with an increase in the abundance of mRNA for the $\alpha$-subunit of the sodium pump (Cramb *et al.* 1987). In cells where sodium is artificially raised by addition of the Na/H antiporter monensin, an increase in mRNA with no reduction of the internalisation rate is seen (Lewis *et al.*

1981). This suggests that the monensin upregulation is wholly due to the rise in pump synthesis (Lewis *et al.* 1981) or may be a specific effect of monensin in this cell type.

In conclusion increases in sodium pump activity due to stimulation by hormones or by increases in intracellular sodium concentrations can, in certain circumstances, be related to increases in mRNA production for both $\alpha$ and $\beta$ subunits. This increase in mRNA subsequently leads to an increase in protein synthesis and hence to an increase in functional sodium pump units on the cell surface.

## 4.I aii Dogfish rectal gland Na,K-ATPase expression

Complete cDNAs for both the $\alpha$ and $\beta$-subunits of *Torpedo californica* , a related elasmobranch to dogfish, have been cloned (Kawakami *et al.* 1985 and Noguchi *et al.* 1986). The larger $\alpha$-subunit which has a catalytic role is composed of 1,022 amino-acid residues (Kawakami *et al.* 1985). The sequence of amino-acid residues from 2-27 is homologous to the amino-terminal sequence for the sheep and avian Na,K-ATPase $\alpha$-subunits (Kawakami *et al.* 1985) and analysis of the full putative amino-acid sequence predicts at least ten transmembrane segments forming presumably $\alpha$-helical structures (Peluffo *et al.* 1996). The Na,K-ATPase $\beta$-subunit is a glycoprotein, the complete amino acid sequence has been deduced by cloning and sequencing the cDNA (Noguchi *et al.* 1986). It was concluded that the $\beta$-subunit of the *Torpedo californica* consists of 305 amino acid residues (Noguchi *et al.* 1986). The predicted secondary structure of the $\beta$-subunit is identical to the cloned mammalian $\beta$-subunits in having only one potential membrane spanning region near the amino terminus (Noguchi *et al.* 1986). More recently a partial cDNA of the *Scyliorhinus canicula* $\alpha$-subunit from the rectal gland (between nucleotide positions 1471-2137 of the Torpedo sequence) was cloned in our laboratory (Dr C Cutler).

The regulation of dogfish rectal gland Na,K-ATPase activity and expression has been reviewed in Chapter 3. To date there have been no studies examining both the regulation of Na,K-ATPase activity and the expression of Na,K-ATPase genes in the dogfish rectal gland. However recent studies by MacKenzie (1996) have shown that increases in rectal gland Na,K-ATPase activity and expression are related to dietary intakes of sodium chloride by the dogfish. MacKenzie (1996) reported that following a single feeding event with 6 % (w/w) sodium chloride-supplemented pellets, dogfish rectal gland Na,K-ATPase activities transiently increased 44-fold over a 6-12 hour period. This increase in Na,K-ATPase activity was not paralleled by a concomitant rise

in the levels of mRNA of either the $\alpha_1$ or $\beta_1$ Na,K-ATPase subunits within this time period, suggesting that the up-regulation of the enzyme must occur at some point downstream of transcriptional regulation. Strangely, small (approximately 50%) increases in $\alpha_1$ and $\beta_1$ mRNA expression were found some 48 hours after the initial feeding event when rectal gland Na,K-ATPase activity had returned to normal values.

MacKenzie (1996) also reported that following repeated feeding studies, when fish were fed with a pellet diet containing 6% (w/w) sodium chloride every alternate day over a period 4 weeks, maximal Na,K-ATPase activities in rectal gland homogenates were over 3-fold higher than activities from starved fish. Again no significant increase in rectal gland $\alpha_1$ or $\beta_1$ Na,K-ATPase mRNA levels were observed. However after repeated feeding with a squid diet containing approximately 3 % (w/w) sodium chloride, a 40 % increase in $\beta_1$ subunit Na,K-ATPase mRNA expression was observed however this was not accompanied by any increase in rectal gland Na,K-ATPase activity (MacKenzie, 1996). The reason for the discrepancies between mRNA expression and Na,K-ATPase activities was not known.

In summary in studies using *Scyliorhinus canicula* MacKenzie (1996) showed that an increase in ingested sodium chloride results in a transient 44-fold increase in Na,K-ATPase activity in rectal gland homogenates peaking some 9 hours after the feeding event. This increase in Na,K-ATPase activity was not paralleled by a concomitant rise in the levels of mRNA of the $\alpha_1$ or $\beta_1$ Na,K-ATPase subunits. These results suggest that an increase in dietary sodium chloride can increase Na,K-ATPase activities in the rectal gland by some mechanism independent of transcriptional regulation increases mRNA. As reported in Chapter 3 of this study, a transient up-regulation of Na,K-ATPase activity (over 4-fold) was found in rectal gland epithelial cell homogenates at 9-12 hours after cells were exposed to a 50% increase in sodium chloride in the growth medium. Possible explanations for this increase in activity include:- 1) activation of existing pre-formed sodium pumps or 2) an increase in catalytic turnover of the enzyme or 3) there is an increase in transcription and/or translation of one or both subunits resulting in increased synthesis of Na,K-ATPase. An increase in mRNA levels of Na,K-ATPase subunits can be tested using suitable homologous cDNA probes. Earlier experiments with actinomycin D (Chapter 3) already suggest that the increase in Na,K-ATPase activity associated with the increase in extracellular sodium concentration is not due to an increase in transcrption. The aim of this chapter was to confirm these results. In this chapter experiments were designed to determine if mRNA levels for the $\alpha_1$-

subunit were altered following incubation of cells with elevated sodium chloride concentrations.

## 4.II MATERIALS AND METHODS

To obtain viable preparations of eukaryotic mRNA, it is necessary to minimise the activity of RNAses liberated during cell lysis by using inhibitors of RNAses or methods that simultaneously inactivate RNAses. Consequently, it is also important to avoid the introduction of RNAses from other potential sources in the laboratory, therefore some special precautions are necessary when preparing RNA. These are listed below:-

1. As general laboratory glassware is often contaminated with RNAses from various sources, separate glassware was kept for RNA work. The glassware was treated by baking at 180°C for 4 hours to destroy any RNAses present. All plastic containers used were sterile, disposable and RNAse free.

2. Disposable gloves were worn during the preparation of all materials and solutions used for the isolation and analysis of RNA and during manipulations involving RNA as skin RNAses are a potential source of contamination.

3. All the solutions used in the RNA work were prepared using DEP-treated water (1 ml of diethylpyrocarbonate (DEP) (Sigma) was added to 1 litre of filter sterilised water. This was stirred vigorously overnight and then autoclaved twice to eliminate the DEP).

### 4.IIa Extraction and analysis of RNA from *Scyliorhinus canicula* rectal gland primary epithelial cell monolayer cultures.

Two methods were used for extraction of RNA from rectal gland cell cultures. These were a lithium chloride precipitate method reported by Cathala *et al.* (1983) and a commercially produced RNA extraction protocol associated with the RNeasy total RNA kit (Qiagen). The initial stage of removal of cells from the collagen coated plates was varied. In all RNA extraction procedures, the cells were prepared and plated onto 9 cm diameter collagen coated plates as described in section 2.II. When the cells formed a monolayer, approximately 5-7 days after plating, they were incubated for 9 hours in normal shark Ringer containing 240 mM sodium chloride or high salt shark Ringer containing 360 mM sodium chloride (8 plates were used per condition). Following this

incubation, the plates were washed twice with normal shark Ringer at room temperature and the cells were then either, 1) scraped from the culture dish using a plastic scraper directly into 0.3 ml lysis buffer (5 M guanidinium thiocyanate, 50 mM Tris/HCl pH 7.5 10 mM EDTA and 1.12 M ß-mercaptoethanol) and then homogenised by 7 serial passages through a 19 G needle using a 1 ml syringe, or 2) 0.3 ml lysis buffer was added directly to the cell monolayers before scraping the cells from the plate and then homogenised by 7 serial passages through a 19 G needle using a 1 ml syringe or 3) each plate was incubated for 2 hours at room temperature in 3 ml of calcium and magnesium free shark Ringer containing 1% trypsin (w/v) to produce a cell suspension. An equal volume of normal shark Ringer was then added to the cells and the cells were pelleted by centrifugation at 200 x $g_{max}$ for 90 seconds at 4 °C in an MSE Coolspin. The supernatant was then aspirated and the cells washed twice in normal shark Ringer (1 ml) with centrifugation as above. After the final wash, the shark Ringer was aspirated and the surface of the tube wiped with tissue to remove as much of the solution as possible. Lysis buffer (170 µl) was added to the cell pellet and then homogenised by 7 serial passages through a 19 G needle using a 1 ml syringe.

### 4.II ai RNA extraction using lithium chloride precipitation method

Samples prepared by the methods described above were then transferred to siliconised 1.5 ml microfuge tubes and 1.35 volumes of 6 M LiCl added. The samples were mixed by inversion 4-5 times and left overnight at 4 °C to precipitate the RNA. Tubes were centrifuged at 15,000 rpm, (23,550 x $g_{max}$) $4^0$C for 60 minutes using a JA-18.1 rotor in a Beckman centrifuge, model J2-MC. The supernatant was discarded, the tube drained and the pellets resuspended in 2 volumes of 3 M LiCl using a 1 ml syringe fitted with a 19 G needle. The samples were then recentrifuged as above to pellet the RNA. The supernatant was then aspirated and the pellet resuspended in 1 volume TNESDS (1 mM EDTA, 150 mM NaCl, 10 mM Tris/HCl and 0.2 % (w/v) SDS pH 7.5) containing 250 µg/ml proteinase K. This was then incubated at 37°C for 1 hour to digest any protein in the sample.

Following the proteinase K digestion the samples were extracted by addition of an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and samples were centrifuged for 10 minutes at 13,000 rpm (14,926 x $g_{max}$) at room temperature in a bench top microfuge (Heraeus Sepatech, Biofuge A). The upper aqueous layers were transferred into siliconised 1.5 ml microfuge tubes and extracted once again with phenol/chloroform/isoamyl alcohol (25:24:1) as described above. The extraction

procedure was repeated until the interface between the two solutions showed no signs of precipitate and then the aqueous phase was extracted with an equal volume of chloroform/isoamyl alcohol (24:1). The upper phase was again removed following centrifugation and RNA was precipitated from the samples by sequential addition of 0.1 volume of 3 M sodium acetate pH 5.2 and 2.5 volumes of absolute ethanol at -20 °C, mixed well and incubated at -20 °C overnight. The RNA samples were then pelleted by centrifugation at 15,000 rpm, (23,550 x $g_{max}$) 4°C for 60 minutes using a JA-18.1 rotor in a Beckman centrifuge, model J2-MC. The pellets were washed with 1 volume 70 % (v/v) ethanol at -20 °C and recentrifuged for 30 minutes as above. The ethanol was discarded and the pellets allowed to dry at room temperature for 30 minutes. The extracted RNA was resuspended in 50 µl DEP treated water and the RNA quantified as described in section 4.II b.

### 4.II aii RNA extraction using RNeasy extraction kit (Qiagen)

The RNeasy total RNA kit (Qiagen) provides a fast and simple method for the preparation of up to 100 µg of total RNA from tissues, cultured cells, and bacteria. This kit uses a specialised high salt buffer system to bind the RNA to a silica gel-based membrane, and following the removal of contaminants by efficient use of wash buffers, total RNA is then selectively eluted in 30 µl or more of water.

Following incubation and washing of cells with normal shark Ringer, 350 µl of Qiagen lysis buffer was added to each 9 cm plate (containing 10 µl of ß - mercaptoethanol per 1 ml lysis buffer), the cells detached by repeated pipetting and the samples were then transferred into siliconised microfuge tubes before homogenisation by 7 serial passages through a 19 G needle using a 1 ml syringe. Complete homogenisation was very important to optimise yields, and to avoid clogging of the RNeasy spin column. The lysate was centrifuged for 3 minutes at 13,000 rpm (14,926 x $g_{max}$) at room temperature in a bench top microfuge (Heraeus Sepatech, Biofuge A). To the supernatant, 1 volume of 70% (v/v) ethanol was added and mixed by repeated pipetting and the sample was applied to the RNeasy spin column (Qiagen). The maximal loading volume to the column is 750 µl. Columns were centrifuged at 8,000 x $g_{max}$ for 15 seconds in a bench top microfuge (Heraeus Sepatech, Biofuge A) and the flow-through discarded. The column was then washed with 700 µl of RW1 (reaction wash buffer 1 provided in the kit) and the column was re-centrifuged as before at 8,000 x $g_{max}$ and the eluate discarded. The column was then washed with 500 µl of RPE wash buffer and centrifuged as described above, the supernatant was discarded. The wash with

RPE wash buffer was then repeated but the centrifugation period was extended to 2 minutes at full speed (14,926 x $g_{max}$). The RNA was then eluted from the column by the application of 30 µl of DEP-treated water. The water was pipetted directly onto the RNeasy spin column membrane and centrifuged for 60 seconds at 8,000 x $g_{max}$ in the bench top microfuge. This elution stage was then repeated, the elutes were combined and the RNA quantified as described in section 4.IIb.

## 4.IIb Quantification of RNA

Samples (4µl) of resuspended RNA were diluted to 400µl with DEP treated water and quantified by absorption at 260 nm using a UV-spectrophotometer (Philips). At 260 nm, 1 O.D. unit is equivalent to 43.17 µg/ml of RNA (Ausubel *et al.* 1987) and the 260 nm/280 nm O.D. unit value ratio gives an estimation of the purity of the sample i.e. a pure RNA sample would give a 260/280 nm ratio of 1.8, with lower values indicative of contamination (Ausubel *et al.* 1987).

## 4.IIc Denaturing agarose gel electrophoresis

Gel electrophoresis is a method whereby charged molecules in solution migrate in response to an electrical field. Their rate of migration or mobility through the electrical field depends on the strength of the field, on the net charge, size, and the shape of the molecules, and, also, on the ionic strength, viscosity, and temperature of gel that the molecules are moving through.

Two basic electrical equations are important in electrophoresis. The first is Ohm's law, $I=V/R$, which states that electrical current (I) is directly proportional to the voltage (V) and inversely proportional to the resistance (R). The second is $P=VI$, which states that power (P), a measure of the amount of heat produced in watts, is the product of voltage and current. In electrophoresis, one electrical parameter, either current or voltage is always held constant.

Agarose is a highly purified polysaccharide derived from agar which dissolves readily when added to boiling aqueous solutions. It remains in a liquid state until the temperature is lowered to approximately $40^{0}$C, at which point it gels. The gel is stable and does not melt again until the temperature is raised back to around $100^{0}$C. The pore size of the gel may be predetermined by adjusting the concentration of agarose: the higher the concentration, the smaller the pore size.

To prepare a 30 ml gel, 0.3g of agarose powder (Gibco BRL) was dissolved in 21.6 ml Milli Q water by boiling for 5 minutes. The agarose was dissolved and allowed to cool to 50-55°C before addition of 3 ml 10 x MOPS (0.2 M 3-[N-morpholino]propanesulfonic acid, 0.5 M sodium acetate and 0.01 M EDTA, pH 8.0) and 5.4 ml formaldehyde. The gel solution was mixed gently and poured over a glass plate into a gel casting tray with sample comb and allow to solidify at room temperature before placing in a refrigerator for 15 minutes. The sample comb was removed and the glass plate and gel were then carefully extracted from the casting tray and placed in the flat bed electrophoresis tank. The gel was submerged with electrophoresis buffer (1x MOPS) and the samples loaded directly into the sample wells. RNA samples ( 5µg in 1µl) or 1.5 µl standard RNA 100 bp ladder (Gibco BRL) were diluted with 20 µl of formamide, 7 µl formaldehyde, 4 µl of 10 x MOPS and DEP treated water to 40 µl, heated in a dry block at 65°C for 10 minutes to denature the RNA, and centrifuged for 10 seconds at top speed (14926 x $g_{max}$) at room temperature in a bench top microfuge (Heraeus Sepatech, Biofuge A). Loading dye (50 % glycerol, 1 mM EDTA (pH 8), 0.25 % (w/v) bromophenol blue and 0.25 % (w/v) xylene cyanol) (4 µl) was then added to each sample, mixed and then applied to the gel wells.

Electrophoresis was conducted at 100 volts for 3-5 minutes to run samples into the gel and then continues at 70 volts for a further 1.5 hours. Following electrophoresis the gel was washed in Milli Q water for 30 minutes and then the outside lanes containing RNA size markers were excised. The excised gel containing the RNA size ladder was then stained in 0.1 M ammonium acetate containing 0.5 µg/ml ethidium bromide on a shaking platform for 30-45 minutes. The gel was destained at room temperature in 2 x 100 ml 0.1 M ammonium acetate for 60-90 minutes and viewed on the transilluminator. The gel was photographed for future reference. The $\log_{10}$ of the size of the RNA fragments against the Rf (distance moved by RNA fragment/distance moved by bromophenol blue dye) was plotted. The resulting curve was used to calculate the sizes of any mRNA species detected with cDNA probes after blotting and hybridisation.

### 4.IIdi Northern blotting

RNA samples were separated on the basis of size by denaturing formaldehyde agarose gel as described in 4.IIb. The separated RNAs were transferred from the gel to a nylon filter (Zeta Probe GT-Biorad) by electroblotting. Following electrophoresis the gel was first transferred to a clean dish where any lanes containing RNA size ladders or unused

areas of the gel were trimmed with a razor blade. The top right-hand corner of the gel was cut off to serve as a marker for orientation of the gel during the subsequent operations. The gel was submerged in transfer buffer (1xTAE) (0.04 M Tris:acetate and 0.002 M EDTA) and left for 20 minutes at room temperature to equilibrate. The gel was placed onto the nylon (Zeta Probe GT (Biorad)) filter and sandwiched between two layers of Whatman 3 MM paper and 2 Scotch Brite foam pads as show in fig 4.1. This entire process was carried out under 1xTAE buffer.

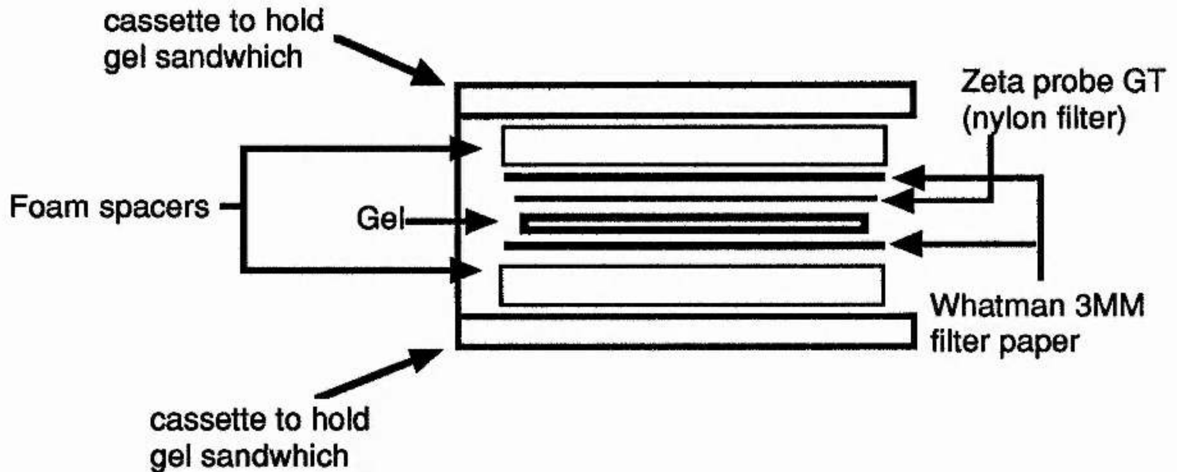



Figure 4.1 Gel sandwich used in Northern blotting

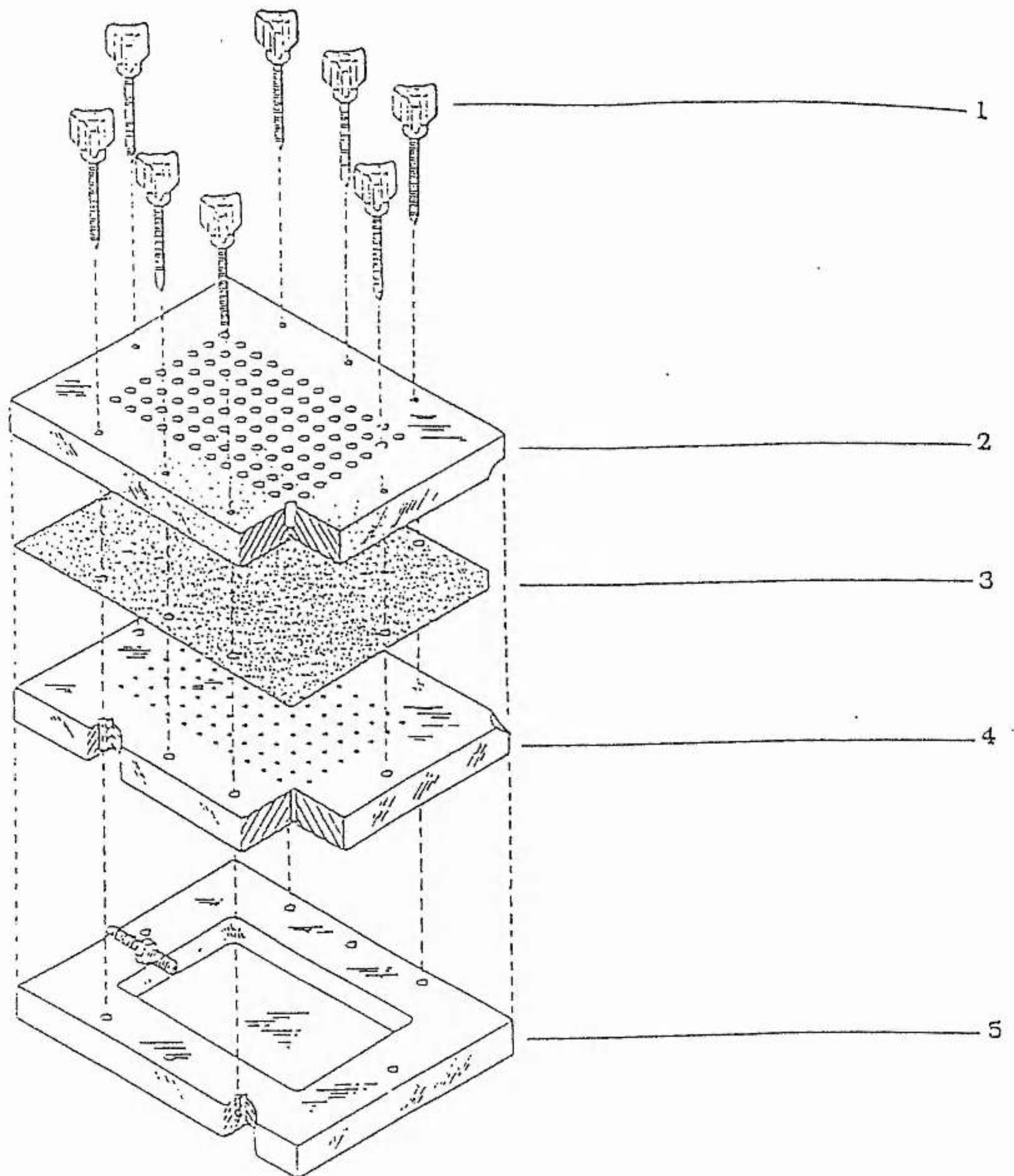
The gel sandwich was transferred to the blotting apparatus tank and electrophoresis run overnight at 20 volts, 0.45 amps. Following removal of the nylon filter from the apparatus the transferred nylon filter was air dried and the RNA was cross-linked to the filter using U.V. radiation from a Strata-linker (Stratagene), set for optimal cross-linking (1200 Joules/$cm^2$).

## 4.IIdii Dot blotting

Quantification of RNA in each sample was performed by a Dot blot method based on that reported by Davis *et al.* (1986). The RNA samples were applied to the Zeta-Probe GT nylon membrane (under a vacuum of 5 inches of Hg) using a dot blotting vacuum manifold (Gibco/BRL) (Fig 4.2). The blotting vacuum manifold consists of 3 stacked blocks of perspex held together with 8 locating bolts. A grid of 96 wells, 4 mm in diameter (8 x12) were made in the top two blocks The Zeta Probe GT membrane was mounted between the top 2 blocks after prior equilibration in 10 x SSC (66% w/v NaCl, 33% w/v tri sodium citrate, pH 7.0). Dot blots require a high salt concentration

Figure 4.2

Figure 4.2 Structural view of the BRL dot blot manifold: 1. Locating bolts, 2, Sample well template (block 1), 3. Zeta-probe GT membrane or Whatman 3MM paper, 4. Filter support template (block 2), 5. Vacuum chamber (block 3).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to facilitate the effective transfer of RNA from solution to membrane as this alters the physical state of the RNA making it "sticky". Once in close proximity to the membrane there will also be some charge interaction with the membrane which has a positive charge which increases binding of the RNA to the membrane. To facilitate uniform suction and prevent leakage into adjacent wells a piece of Whatman 3 MM paper, also equilibrated in 10 x SSC, was placed between the first and second perspex blocks under the membrane. RNA samples (500 ng) in a denaturing loading buffer (consisting of 20 μl of formamide, 7 μl formaldehyde, 4 μl of 10 x MOPS and 19 μl DEP treated water) were heated to 65°C for 15 minutes and rapidly cooled on ice. Ten volumes (400 μl) of 10xSSC were then added to the RNA samples and then loaded into the wells on the manifold and the vacuum (5 inches of Hg) applied. The wells were rinsed with two washes of 10 x SSC (400 μl) to wash the filter and remove any RNA which adhered to the side of the wells. The membrane was removed from the manifold and dried in an oven and RNA cross-linked as in described in section 4.IIdii.

## 4.II e Nucleic acid Hybridisation

Following transfer of the RNA to the Zeta Probe GT by either Northern blotting or dot blotting, specific RNA species can be identified in terms of size and relative amount by hybridising the RNA to a specific radio-labelled DNA probe. In this study the probe used was a radiolabelled 673 bp cDNA of the $\alpha_1$-subunit of Na,K-ATPase cloned from Scyliorhinus *canicula* rectal gland using reverse transcriptase polymerase chain reaction (RT-PCR). The cDNA fragment is located between nucleotide positions 1471-2137, amino acid positions 490-714, as aligned with the *Torpedo californicus* sequence reported by Kawakami *et al.* (1985).

### 4.II ei Prehybridisation

Prehybridisation is a process where the filter produced by Northern or dot blotting is incubated in a solution which reduces the non-specific binding of a radio-labelled probe by blocking potential non-specific adsorption sites and other components of the solution denatured the RNA. Non-specific binding sites for nucleic acids are bound with sheared, denatured DNA and/or RNA from a species unrelated to the biological origin of the experimental RNA: in this case calf thymus DNA and yeast tRNA were used as non-specific blockers. After crosslinking the RNA to the filter, the nylon filter is placed in a hybridisation bottle (Techne) containing 20 ml of prehybridisation buffer, comprising, 50% (w/v) formamide, 5 x Denhardts solution (0.25 % (w/v) Ficoll 400,

0.25 % (w/v) polyvinylpyrrolidone and 0.25 % (w/v) BSA), 1% (w/v) SDS, 1 M NaCl, 0.05 M phosphate buffer pH 8.0, 5 mM EDTA and 1 mg each of heat denatured sonicated calf thymus DNA and yeast tRNA. The hybridisation bottle containing the filter and prehybridization buffer was then placed into a hybridisation oven (Techne) and incubated overnight with constant rotation at 42°C.

#### 4.II eii Radio-labelling the cDNA probe and hybridisation

The most commonly used radio-isotope used to label nucleic acids is $^{32}P$, this can become an integral part of the nucleic acid structure. It emits energetic ß particles and has a fairly short half-life (14 days). The cDNA probe was radiolabelled with [$\alpha^{32}P$] dCTP by random-sequence oligonucleotide-primed synthesis. This is a type of primer extension in which a mixture of small oligonucleotides, acting as primers, anneal to a heat-denatured double-stranded cDNA template. The annealing primers ultimately become part of the probe itself as the Klenow fragment extends the primers in the 3 prime direction, and in doing so incorporates radiolabelled [$^{32}P$] $\alpha$ dCTP. To label the cDNA using this random priming method a MEGAPRIME kit was purchased from Amersham Int. A 30 µl reaction mix was prepared containing 25 ng cDNA (denatured by boiling for 15 minutes), 50 µCi [$\alpha$-$^{32}P$]dCTP, dNTPs comprising 5 mM ATP, 5 mM GTP and 5 mM UTP, random primer solution (containing 50 mM Tris/HCl, pH 7.5, 10mM $MgCl_2$) and 1 unit of the Klenow enzyme. Once prepared this reaction cocktail was incubated at 37°C for 30 minutes. During this time the [$\alpha$-$^{32}P$] dCTP was incorporated into the probe (Ausubel *et al.* 1987).

Following the radiolabelling reaction the mixture was briefly centrifuged before applying to a Sephadex G50 column pre swollen in TE (10mM Tris/HCl, 1 mM EDTA, pH 8.0). The column was prepared using a 2 ml siliconised glass pipette stopped with siliconised glass wool and siliconised glass beads and packed with Sephadex G50. The column was equilibrated with TE buffer. The radiolabelling mixture was added to the column and the progress of the radiolabelling was monitored through the column using a Geiger counter (Mini Instruments Ltd). As the radiolabel reached the bottom of the column the eluate was collected into 1.5 ml microfuge tubes at 4 drops / tube. The radioactive profile of the eluate fractions was determined by taking a 1 µl sample from each tube into 3 ml of water. Radioactivity was determined by Cerenkov counting using a Packard Canberra scintillation counter (1600 TR). The labelled cDNA routinely eluted in the first 4 fractions and a second radioactive peak of unincorporated $\alpha$ $^{32}P$

dCTP eluted in later fractions. The percentage incorporation of the label and the specific activity of the cDNA was calculated to be in the region of 40-50%.

Once the probe was radiolabelled and purified by column chromatography, the tubes containing the peak radiolabelled fractions were combined and boiled for 5 minutes to ensure complete denaturation. The probe was snap cooled on ice for 5 minutes centrifuged briefly and then immediately added to the hybridisation bottle which already contained the nylon filter and prehybridisation solution, and mixed well. The filter was then hybridised overnight (12-26 hours) at 42 °C with constant rotation.

#### 4.II e iii Post hybridisation, washing of filter.

Following the hybridisation incubation the solution was poured from the hybridisation bottle and the filter and bottle rinsed once with 10-20 ml wash solution 1 (44.5 mM NaCl and 0.5 % SDS) at 42 °C. With all processes maintained at 42°C the filter was sequentially washed by incubation in 10-20 ml wash solution 1 for 30 minutes, 10-20 ml wash solution 2 (44.5 mM NaCl and 0.1 % SDS) for 30 minutes, 10-20 ml wash solution 3 (23.9 mM NaCl and 0.1 % SDS) for 30 minutes and 10-20 ml wash solution 4 (12.8 mM NaCl and 0.1 % SDS) for 10 minutes. After the washes the filter was removed from bottle and sealed in a plastic bag.

### 4.II f Autoradiography

The autoradiograph provides an accurate record of the image of the radioactive probe hybridising to the filter. Alternatively the distribution of radiolabelled probe on the film can be viewed by electronic autoradiography using the Packard Canberra Instant Imager. Electronic autoradiography is 10 to 100 times more sensitive than autoradiography (exposing the filter to X-ray film) and gives a linear output of radioactivity over 5 orders of magnitude. Autoradiography however gives better resolution than the Instant Imager (10 µm compared to > 400 µm), but is less sensitive and only gives a graded, non-linear response to radioactivity over 2 orders of magnitude. In this study both methods were used.

The autoradiographic images on the X-ray film were analysed using a flying spot scanning densitometer (Shimadzu model CS-9000, Shimadzu Corp., Kyoto, Japan). The scanner moves the developed X-ray film over a light beam and measures the absorption of light at a pre-determined wavelength; in this case a wavelength of 550 nm

was used. In the case of the dot blots, the absorption was calculated and the area under the peak of absorbance was calculated. The amount of radioactivity contained on the image was quantified against a set of radioactive standard dot blots which were incubated at the same time as the experimental membrane so the area obtained using the densitometer was expressed as counts per minute (cpm) per dot.

## 4.III RESULTS and DISCUSSION

Using isolated cell suspensions prepared by trypsinization, approximately 20 μg of RNA was extracted per sample. Following denaturing gel electrophoresis and staining with ethidium bromide, it was apparent that this RNA was degraded and did not result in a good quality Northern blot (Fig 4.3). However the degraded RNA was used for dot blots (Fig 4.4) and was quantified by scanning densitometry.

Table 4.1

| Cell preparation (9 hour treatment) | Quantity of RNA detected by scanning densitometry |
|---|---|
| normal treated cells (240 mM NaCl) | 4.02 cpm/μg ± 1.334 |
| high salt treated cells (360 mM NaCl) | 3.45 cpm/μg ± 0.18 |

The reason for the RNA degradation was thought to be due to the prolonged trypsinization period resulting in cell damage and hence exposing the RNA to any RNAses present. It was therefore necessary to look for another method of RNA extraction. Scraping the cells from the culture dish using a plastic scraper was an alternative method employed for the rapid detaching of cells from their culture substrata. However in this case, cell damage resulting from the scraping process presumably resulted in the release of RNAses causing a substantial degradation of cellular RNA before the cells could be homogenised in the guanidinium lysis buffer. Very low yields of RNA (too low for Northern analysis) were obtained using this method. The next method used in an attempt to extracted RNA from the dogfish rectal gland epithelial cells involved applying lysis buffer directly to the culture dish. This method immediately denatured any RNAses released on disruption of the cell. However this method also provided a very low yield of RNA, thought to be due to the large volume of lysis buffer required to cover the total surface area of the cell plates, hence diluting the RNA too much for optimal precipitation with lithium chloride.

As the above mentioned methods were not successful in extracting viable RNA, a spin column preparation method was attempted using a Qiagen kit. When using this kit, lysis buffer was applied directly to the culture dish therefore overcoming any problems with RNA degradation caused by exposure to RNAases released on cell disruption.

Figure 4.3

Figure 4.3 Photograph of Northern blot of dogfish rectal gland epithelial cells mRNA extracted using trypsinzation method. Lane 1 represents control cells (5$\mu$g total RNA) and lane 2 represents cells incubed in high salt for 9 hours (5$\mu$g total RNA).

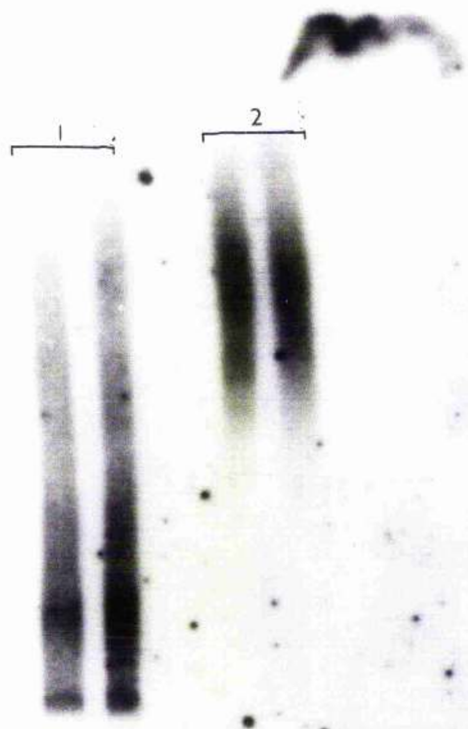
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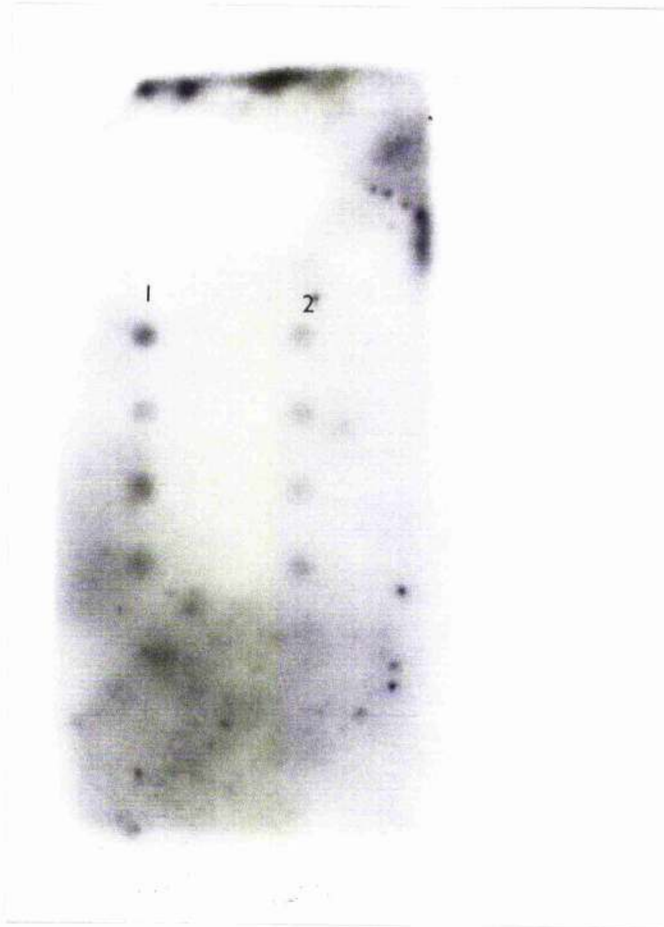
Figure 4.4

Figure 4.4 Photograph of Dot blot of dogfish rectal gland epithelial cells mRNA extracted using trypsinzation method. Lane 1 represents control cells and lane 2 represents cells incubed in high salt for 9 hours. Each sample contains 500 ng total RNA.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The possible problem of precipitation of low concentrations of RNA in the lysis buffer was solved as the kit claimed that the column would bind any total RNA present even if it was at very low concentrations. Although use of this kit did provide a small amount of viable RNA the yield was not high (0.9 $\mu$g RNA per 9cm plate). However when RNA was analysed by either Northern or dot blot analysis there was no evidence of Na,K-ATPase $\alpha$-subunit present. It was concluded from this observation that the extracted RNA from the cultured cells was incorrectly quantified with the spectrophotometer reading at 260 nm (due to some contaminants associated with this isolation method) and there was insufficient quantities of RNA isolated for Northern analysis. The main limitations when extracting RNA from dogfish rectal gland epithelial cell cultures was thought to be due to the small amount of cell cytoplasm present in this cell type compared to the area the cells cover. Photographs and electron micrographs demonstrating this characteristic of primary cultured dogfish rectal gland epithelial cells may be found in chapter 3 section III. The low cytoplasm to cell area ratio means that relatively large volumes of lysis buffer were used resulting in very dilute concentrations of extracted RNA. Very dilute concentrations of RNA are very difficult to precipitate using standard methods ie lithium chloride or salt /ethanol.

Because the methods gave low yields of RNA (too low to run Northern blots) the original method of trypsinization which produced degraded RNA (mainly in the normal salt incubations) was the only method which produced any result. Although the RNA was degraded there was no significant change in relative $\alpha_1$-subunit Na,K-ATPase signal between normal and high salt treated cells. This suggests that at the 9 hour incubation point a 50% increase in medium sodium chloride concentration does not affect the Na,K-ATPase $\alpha_1$-subunit mRNA levels in rectal gland epithelial cells. This conclusion was reached as although the RNA was degraded, this degradation occurred mostly in the normal treated cell sample and not the high salt treated cell sample (Fig4.3), suggesting if anything that the levels of mRNA observed in the normal treated cell sample may be lower than the true value. This therefore supports the view that a 50% increase in medium sodium chloride concentration does not affect the Na,K-ATPase $\alpha$-subunit mRNA levels. These results agree with the earlier observations showing that actinomycin D has no effect on the high salt induced increase in Na,K-ATPase activity as described in section 3.III. Although these preliminary experiments support this view, more work is required to conclusively prove that a increase of sodium chloride in the medium of the cells does not cause any increase in $\alpha$- or $\beta$-subunit mRNA abundance. Confirmation of these results would support the view that upregulation of the Na,K-ATPase in the dogfish rectal gland in response to increased

sodium chloride concentration occurs at some point down stream of transcriptional regulation The observation made in this study concerning the Na,K-ATPase $\alpha$-subunit mRNA levels in response to sodium chloride concentration are in agreement with the earlier in vivo studies carried out by MacKenzie, (1996) in our laboratory. MacKenzie reported that dogfish rectal gland Na,K-ATPase activities increased 44-fold 9 hours after a single feeding episode with sodium chloride pellets (6% w/w), and this increase in Na,K-ATPase activity was not associated with any increase in $\alpha$-subunit mRNA abundance. The results found both in this study and in MacKenzie's are in contrast to many long-term studies looking at the effect of intracellular sodium concentration on Na,K-ATPase expression. A number of studies have found that increasing intracellular sodium concentration results in an increase in abundance of one or all of the Na,K-ATPase subunits ( Allen *et al.* 1986, Emanuel *et al.* 1987, Gick *et al.* 1988, Schmalzing *et al.* 1989, Baehlen *et al.* 1991, and Welling *et al.* 1991), however none of these studies were conducted in elasmobranchs. Many hormones have also been reported to increase Na,K-ATPase activity by increasing gene transcription of the pump e.g. thyroid hormone in rat smooth muscle (Azuma *et al.* 1993), aldosterone in rabbit colon (Wiener *et al.* 1993) and insulin in rat thoracic aorta (Tirupatture *et al.* 1993). Insulin is of particular interest as the short term actions of insulin are reported to involve increases in intracellular sodium concentrations, however it is unknown whither the long term actions of insulin which result in increased levels of $\alpha$-subunit mRNA also involve intracellular sodium levels. It therefore appears that the effect of extracellular sodium (presumed to result in an increase in intracellular sodium concentration) on Na,K-ATPase activity in dogfish rectal gland cells in contrast to the above mentioned studies is regulated by a mechanism other than increased abundance of mRNA levels for the Na,K-ATPase $\alpha_1$-subunit in response to extracellular sodium concentration. However as this study only looked at mRNA levels of the $\alpha_1$-subunit, mRNA levels of the $\beta$-subunits must also be investigated. There may be an abundance of $\alpha$-subunits and a lack of $\beta$-subunits, meaning that only more $\beta$-subunit mRNA are required for an increase in Na,K-ATPase expression. However experiments looking at $\beta$-subunit mRNA levels in response to sodium chloride were not investigated in this study as the $\beta$-subunit mRNA is present at lower concentrations than the $\alpha$-subunit mRNA and as this is not detechtable it would be infesible to look for $\beta$-subunit mRNA. An alternative explanation for the increase in Na,K-ATPase activity seen in this study is that the increase in extracellular sodium chloride concentration is associated with the translation of a protein or proteins which activates pre-formed sodium pumps or the increase in extracellular sodium leads to an increase in intracellular sodium

concentration which might activate translation of new sodium pump proteins. These points are further discussed in chapter 3.

# Hormonal regulation of cyclic nucleotide levels in the perfused rectal gland and cultured rectal gland epithelial cells.

## 5.I INTRODUCTION

There is a large number of different types of external signals to which a cell can be exposed. These signals include hormones, neurotransmitters, autocoids, foreign proteins and cells (e.g. antigens or viruses), extracellular structures (e.g. surfaces of neighbouring cells) and environmental elements (e.g. light and toxins). The cell may respond to these different extracellular signals in many ways, however the two main mechanisms by which external signals are received by cells are through receptors located on the external surface of the plasma membrane that respond to hormones and neurotransmitters and sequence-specific DNA-binding receptor proteins located within the cell that respond to steroid and thyroid hormones. Once a cell has received an extracellular signal it responds by triggering pathways of intracellular communication involved in the control of the cells functions.

Adenosine 3',5'-cyclic monophosphate (cAMP) and guanosine 3',5'-cyclic monophosphate (cGMP) have evolved as intracellular messengers in animal cells. The intracellular concentration of each of these cyclic nucleotides is controlled by the relative rates of their syntheses, catalysed by adenylate or guanylate cyclases, and their degradation, catalysed by cyclic nucleotide phosphodiesterases ( Fig 5.1). An agonist can alter the intracellular concentration of cAMP or cGMP by increasing the rate of synthesis or changing the rate of degradation of the cyclic nucleotide.

The discovery of cAMP as an intracellular messenger was first reported by Sutherland and his colleagues during the period between 1955 and 1960 (as cited in Rang and Dale, 1990). They showed that ATP and $Mg^{2+}$ were required for the conversion of isolated liver glycogen phosphorylase B to phosphorylase A (Rall *et al.* 1956). This enabled Rall *et al.* (1957) to develop a cell-free system in which the addition of adrenaline or glucagon to a liver homogenate in the presence of ATP and $Mg^{2+}$ caused an increase in phosphorylase activity. After searching for a compound which might be formed as a result of the action of the hormones, a heat-stable factor was isolated from liver slices treated with hormone. This factor was shown to activate glycogen phosphorylase in liver homogenates incubated in the absence of hormones. Also

during this time period Lipkin was investigating the chemistry of compounds formed when ATP was treated with $Ba(OH)_2$ (Lipkin *et al.* 1959). One of the products of the ATP-$Ba(OH)_2$ reaction was found to be similar in biological properties to the heat-stable activator of phosphorylase. The two groups collaborated in the elucidation of the structure of the activator which was subsequently identified as cAMP (Sutherland and Rall, 1958; Lipkin *et al.* 1959). It was subsequently shown that almost all the intracellular effects of cAMP are mediated through the activation of a cAMP-dependent protein kinase known as protein kinase A (PKA) (Barritt, 1992).

Studies of the distribution of cAMP in extracellular fluids in animals led to the discovery, in mammalian urine, of another cyclic nucleotide, cGMP (Ashman *et al.* 1963). The subsequent detection of cGMP in many cells, and of cGMP-dependent protein kinase, suggested that this cyclic nucleotide may have a role as an intracellular messenger similar to that of cAMP. The first description of a clearly defined function of cGMP as an intracellular messenger was in photoreceptor cells. Here the role of cGMP was to transfer light-induced signals (Miki *et al.* 1973). It was not until 1984 that definitive evidence for an intracellular messenger function for cGMP in a system other than the photoreceptor cell was obtained. This observation was that atrial natriuretic peptide (ANP) stimulates guanylate cyclase resulting in the formation of cGMP in smooth-muscle cells (Winquist *et al.* 1984).

Synthesis of cAMP or cGMP is regulated by controlling the activity of adenylate cyclase or guanylate cyclase respectively (fig 5.1). Adenylate cyclase is an integral membrane protein with its catalytic centre located on the cytoplasmic side of the plasma membrane. The activity of adenylate cyclase is principally controlled by extracellular agonists binding to specific receptors which act through either $G_s$, the stimulatory GTP-binding regulatory protein, or $G_i$, the inhibitory GTP-binding regulatory protein to stimulate or inhibit respectively adenylate cylase activity of the cell. The G proteins are heterdimers which are composed of three polypeptides:- an $\alpha$ chain which binds and hydrolyses GTP, and a tight complex of a $\beta$ chain and a $\gamma$ chain which anchors $G_s$ or $G_i$ to the cytoplasmic face of the plasma membrane. The $\alpha\beta\gamma$ complex dissociates into free $\alpha$ and $\beta\gamma$ subunits when activated. The $\alpha$ subunit of the $G_s$ protein stimulates adenylate cyclase activity by allowing the active site of adenylate cyclase to bind ATP. The inhibition of adenylate cyclase through the actions of $G_i$ is mediated by released $\beta\gamma$ subunits and by the interaction of $\alpha$ with adenylate cyclase. In contrast to adenylate cyclase which is located only on the plasma membrane, there are both membrane-bound and soluble forms of guanylate cyclase. Both of these are dual function proteins, acting

as both the receptor for extracellular ligands and also as the enzyme producing the second messenger. Therefore the G proteins are involved in these signal transduction pathways. The plasma-membrane guanylate cyclases are activated by the binding of an agonist to the extracellular domain of the guanylate cyclases which induces a dimerisation and conformational change in the polypeptide chain which, in turn, stimulates the activity of the cytosolic catalytic centre of the guanylate cyclase. The cytoplasmic form of guanylate cyclase which is activated by nitric oxide is composed of two subunits with molecular weights of 82 and 70 kDa (Winquist *et al.* 1984). Activation of cytoplasmic guanylate cyclase by nitric oxide involves combination of this molecule with a haem group resulting in a conformational change in the guanylate cyclase enabling it to bind GTP and hence increase intracellular cGMP levels (Winquist *et al.* 1984),

Degradation of cAMP and cGMP is regulated by controlling the activity of the cAMP-dependent and cGMP-dependent phosphodiesterases respectively (Barritt, 1992). Many isoforms of these phosphodiesterases exist. The activities of cAMP-dependent phosphodiesterases are regulated by several different mechanisms e.g. cAMP-dependent protein kinases and calcium. The activation of cAMP-dependent phosphodiesterases by cAMP-dependent protein kinase is probably a negative-feedback mechanism by which the increase in cAMP induced by an agonist is tightly controlled. In cells in which type I or type II calmodulin-activated cAMP phosphodiesterase is present, an increase in intracellular [$Ca^{2+}$] stimulates the degradation of cAMP (Barritt, 1992). Some forms of cAMP phosphodiesterase may be responsible for the degradation of cGMP as well as cAMP, the only difference being the affinity for each cyclic nucleotide.

Cyclic GMP phosphodiesterase enzymes are composed of three subunits, $\alpha$, $\beta$, and $\gamma$ (Barritt, 1992). Enzyme activity resides in the $\alpha$ and $\beta$ subunits while the $\gamma$ subunit acts as an inhibitory regulatory subunit whereby activation of the phosphodiesterase involves dissociation of the $\gamma$ subunit from the $\alpha$ and $\beta$ subunits (Barritt, 1992).

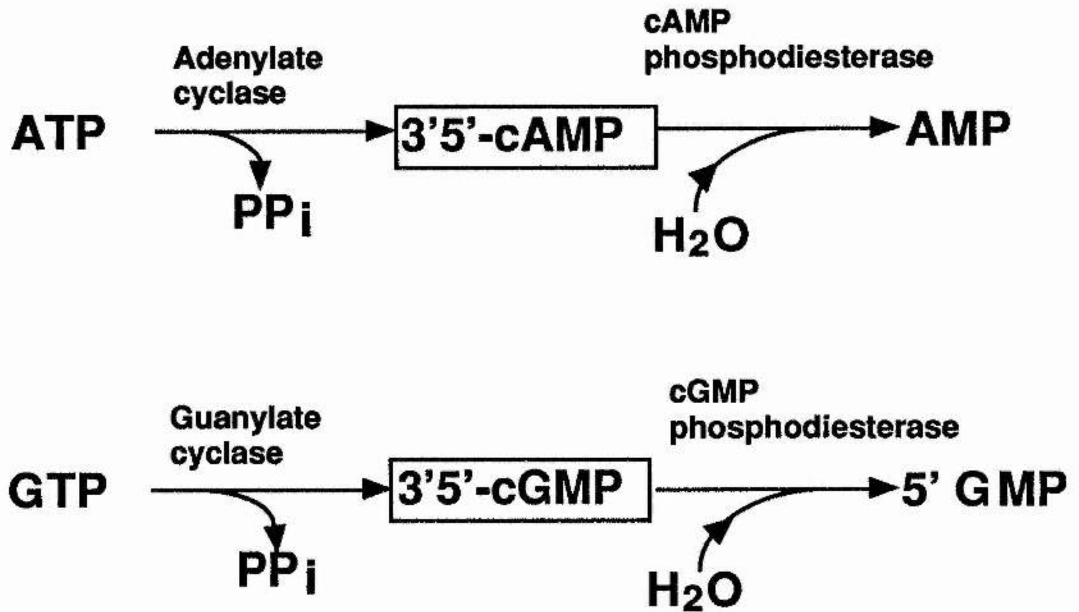



Figure 5.1 The reactions involved in the synthesis and degradation of cAMP and cGMP. The concentration of the cyclic nucleotide which is present in a given region of the cytoplasmic space at any given time is determined by the balance of the biosynthetic and degradative reactions.

Almost all the intracellular effects of cAMP are mediated through the activation of cAMP-dependent protein kinase known as protein kinase A (PKA). An exception is the action of cAMP in olfactory cells in which the cyclic nucleotide binds to an allosteric site on a cation channel (Barritt, 1992). An increase in the concentration of cGMP acts as an intracellular signal for the activation of cGMP-dependent protein kinases eg myofibril relaxation is caused by a decrease in the cytoplasmic free calcium concentration which in turn is due to the activation, by cGMP-dependent protein kinase, of the plasma-membrane (Ca-Mg) ATPase. Cyclic GMP also has direct actions in cells eg. in the photoreceptor cell, cGMP activates cGMP-gated cation channels in the plasma membrane which results in membrane depolarisation and the release of a neurotransmitter at the synapse (Barritt, 1992).

## 5.I a The actions of cAMP in the dogfish rectal gland.

Although it is well documented that cAMP stimulates transepithelial sodium chloride transport in the dogfish rectal gland (Siegel *et al.* 1976, Riordan *et al.* 1994, Erlig and Rubio, 1986, Moran and Valentich 1993, Valentich and Forrest, 1991, Stoff *et al.* 1977) the exact mechanism or mechanisms involved are still under investigation. Cyclic AMP and cell permeable analogues of cAMP, such as dibutyryl cAMP in association with compounds that inhibit the breakdown of intrinsic cAMP by inhibiting phosphodiesterases eg. theophylline and 3-iso butyl-1-methyl xanthine (IBMX), have been used as investigative tools to study the intracellular mechanisms involved in salt secretion from the rectal gland.

It is known that addition of cAMP plus theophylline to perfused rectal glands isolated from *Squalus acanthias* (Silva *et al.* 1977) and *Scyliorhinus canicula* (Shuttleworth and Thompson, 1986) results in the stimulation of sodium chloride secretion. However it has been suggested that this effect, especially in *Scyliorhinus canicula,* is due to the action of the phosphodiesterase inhibitor theophylline and not cAMP. Anderson (1995) reported that cAMP alone has no effect on sodium chloride secretion rates from perfused rectal glands isolated from the *Scyliorhinus canicula*, although the phosphodiesterase inhibitor IBMX was able to stimulate sodium chloride secretion and this secretion was not potentiated by simultaneous addition of cAMP. Similarly Shuttleworth and Thompson (1980), reported that cAMP did not significantly stimulate ouabain sensitive oxygen consumption in rectal gland slices from the *Scyliorhinus canicula*, but the addition of theophylline to the media caused a significant rise in ouabain-sensitive oxygen consumption. Again the theophylline stimulated increase was not potentiated by cAMP, indicating that cAMP is without effect on Na,K-ATPase activity and presumably sodium chloride secretion (Shuttleworth and Thompson 1980). Therefore cAMP alone seems unable to stimulate sodium chloride secretion in the *Scyliorhinus canicula* rectal gland. However forskolin which specifically activates adenylate cyclase and hence increases intracellular cAMP concentration has been reported to increase sodium chloride secretion rates by 10 fold in the *Squalus acanthias* (Lear *et al.* 1995).

The apparent discrepancy between these studies might be due to species differences, ie the effect reported in *Squalus acanthias* is due to cAMP, however in *Scyliorhinus canicula,* cAMP is without effect and the increase in sodium chloride secretion rate

appears to be due to phosphodiesterase inhibitors having some unknown effect which is independent of cAMP. As yet there have been no reports on the effect of perfusing rectal glands isolated from the *Scyliorhinus canicula* with forskolin. Such a study would hopefully confirm or disprove the concept that cAMP is without effect on secretion of sodium chloride from the rectal gland of *Scyliorhinus canicula.*

Recently, the elasmobranch hormones, shark c-type naturetic peptide (sCNP) and Scyliorhinin II (Scy II) have become available. These hormones have previously been implicated in ionic regulation of elasmobranchs (Anderson, 1995), as has cAMP, however the second messenger system(s) used by these hormones are not yet known. Therefore this study has investigated the effects of sCNP and scyliorhinin II on cAMP concentrations in rectal gland cell monolayers and in the perfusate of rectal glands isolated from *Scyliorhinus canicula.*

5.I a i Hormonal control of rectal gland secretion via increases in intracellular cAMP

Numerous hormones, neurotransmitter and autocoids eg adenosine, forskolin, phorbol esters and VIP (Lear *et al.* 1995, Stoff *et al.* 1979) have been shown to stimulate sodium chloride secretion from the rectal gland of *Squalus acanthias* by as much as 40 fold via an increase in cAMP ( Forest *et al.* 1983, Greger *etal.* 1984 and Valentich *et al.* 1991). However few studies have directly investigated the effect of hormones on cAMP levels in the rectal gland of the *Scyliorhinus canicula..* For further information on the actions of VIP, scyliorhinin II, rectin and adenosine refer to chapeter 1 section 1.IV a.

Prostaglandins

Prostaglandins are biologically active lipids present in almost all mammalian tissues and body fluids (Sagi *et al.* 1995). Recent evidence suggests that this group of ecosinoids might also have a biological role in many marine dwelling organisms e.g. prostaglandin $E_2$ ($PGE_2$) and prostaglandin $F_2$ ($PGF_2$) levels increase in Floridian cray fish during ovulation (Spaziani *et al.* 1993). It has also been shown that $PGE_2$ stimulates an increase in intracellular cAMP concentrations in ovarian tissue of the prawn *Macrobranchium roserbergii* (Sagi *et al.* 1995). The effect of $PGE_2$ on sodium chloride secretion by the *Squalus acanthias* rectal gland has also been investigated, although $PGE_2$ did not increase sodium chloride secretion in the isolated perfused rectal

gland, this prostaglandin was reported to induce up to a 5-fold increase in intracellular cAMP concentrations in primary rectal gland epithelial cell cultures (Forrest *et al.* 1994).

Inhibition of sodium chloride transport in the shark rectal gland

Several hormones have been shown to inhibit cAMP-stimulated chloride secretion. Somatostatin inhibits accumulation of cAMP in the *Squalus acanthias* rectal gland by inhibiting adenylate cyclase (Silva *et al.* 1988). In addition this hormone must act downstream of cAMP as it is reported to also inhibit dibutyryl cAMP stimulated chloride secretion (Silva *et al.* 1988). Neuro Peptide Y (NPY) inhibits sodium chloride secretion in the forskolin-stimulated dogfish rectal gland distal to cAMP generation as dibutyryl cAMP-stimulated chloride secretion is also inhibited (Forrest *et al.* 1992). Cadmium (250 μM) completely reversed the ability of somatostatin and NPY to inhibit forskolin stimulated chloride transport (Forrest *et al.* 1992), however it is not able to stimulate or directly block sodium chloride secretion by the rectal gland. Cadmium therefore appears to block the receptor mediated inhibition (but not stimulation) of chloride transport, suggesting that this metal selectively interferes with the inhibitory signal transduction system(Forrest *et al.* 1992).

5.I a ii Action of cAMP on chloride channels present in dogfish rectal gland epithelial cells.

Cyclic AMP is generally accepted as one of the second messengers involved in sodium chloride secretion by the *Squalus acanthias* rectal gland. Theoretically cAMP stimulation directed via PKA might act on any of the four sites in the rectal gland cell that is involved in transepithelial sodium chloride transport, namely the Na,K,Cl-cotransporter, the sodium pump, the CFTR like chloride channel (sCFTR) or the potassium channel (fig 1.1). However the seminal event in this chloride secretory process has been shown to be an increase in the apical membrane chloride ion conductance (Greger *et al.* 1984). The increased activity of sCFTR chloride channel was shown to be responsible for the cAMP-stimulated secretion chloride in the dogfish rectal gland as the current observed in response to cAMP chloride stimulated secretion in the dogfish rectal gland cells is the same size and has the same characteristics i.e. functions as a cAMP/PKA activated chloride channel (Riordan *et al.* 1993, Rich *et al.* 1990, Marshall 1991) and it has a linear current-voltage relationship independent of time or voltage. However there is also a 50-pS channel present in the rectal gland cells

that is cAMP dependant and may some how, by an as yet unknown mechanism, be involved in chloride secretion when cells are transporting sodium chloride (Greger *et al.* 1987).

5.I a iii Cyclic AMP and Na,K-ATPase and Na,K,Cl cotransporter activity in dogfish rectal gland epithelial cells.

Although cAMP-stimulated sodium chloride secretion has been shown to involve the increased apical chloride conductance via sCFTR this may not be the only site at which cAMP acts via PKA when sodium chloride secretion is stimulated in the shark rectal gland. There is evidence that cAMP also increases the activity of the sodium pump which is also involved in transepithelial chloride transport in the rectal gland cell, i.e. an increase in cAMP has been shown to increase ouabain-sensitive $^{86}Rb$ uptake in dispersed rectal gland cells (Silva *et al.* 1983) and to increase ouabain-sensitive oxygen consumption in the whole gland (Silva *et al.* 1979). The mode of activation of the sodium pump by cAMP, is however unclear. An increase in Na,K-ATPase activity might be driven either by a rise in the rate of Na,K-ATPase activity or by an increase in the number of enzyme units, or both. There is conflicting evidence in the literature on whether cAMP via PKA acts on the pump or whether the increased Na,K-ATPase activity found after cAMP-stimulation is secondary to an increase in intracellular sodium concentration (Lear *et al.* 1992, Shuttleworth 1983, Silva *et al.* 1979, Greger and Schlatter, 1984). By using classical chemical methods to calculate the intracellular electrolyte content of whole tissue Silva *et al.* (1979), reported a fall in intracellular sodium and chloride concentration in the isolated perfused rectal glands of *Squalus acanthias* harvested 30 minutes after stimulation with 1 mM cAMP and 0.5 mM theophylline. This was subsequently confirmed using electron probe analysis, a technique that permits the measurement of ion content in individual cells (Lear *et al.* 1992). Lear *et al.* (1992), reported that when primary epithelial cell cultures from the *Squalus acanthias* rectal gland are treated with 1 mM cAMP and 0.5 mM theophylline a 30% decrease in intracellular sodium and an 11 % decrease in intracellular chloride was observed after eight minutes. Intracellular potassium levels did not significantly change with cAMP-stimulation at the eight minute time point but did fall by 6% after 33 minutes. Lear *et al,* (1992), also reported that ouabain-sensitive oxygen consumption of the cultured rectal gland cells increase from $3.3 \pm 5$ to $41 \pm 4$ μmol $0_2$ /mg wet wt/hour within 3 minutes of exposure to 1 mM cAMP and 0.5 mM theophylline, therefore concluding that Na,K-ATPase activity had increased. It was assumed from these results that the increase in pump activity observed was not driven by high levels of

internal sodium, suggesting cAMP or PKA has a direct action on the sodium pump. In contrast to these reports Greger and Schlatter (1984), using microelectrode measurements of single isolated perfused rectal gland tubules from the *Squalus acanthias*, found an increase in cell sodium activity between 2 and 4 minutes after addition of cAMP. They concluded that the primary event after cAMP addition was an increase in apical chloride conductance, resulting in a fall in intracellular chloride activity. This was followed by an increase in sodium entry through the Na,K,Cl cotransporter and a consequent rise in intracellular sodium concentration which stimulated the sodium pump. However Lear *et al*, (1992), showed that the intracellular sodium content had fallen below the basal level present in resting cells by eight minutes and this change could not be accounted for by a concomitant increase in apical chloride conductance or membrane polarisation because of the absence of sodium channels in this tissue (Marver *et al.* 1986) and therefore points strongly to direct stimulation of the sodium pump. The difference in the reports of these two groups could be due to differences in preparation (dissected tubules versus cultured cells), combined with the fact that both groups measured sodium concentrations at different time points following cAMP treatment. The measurements of intracellular sodium content by Lear and coworkers was initiated at eight minutes after cAMP addition whereas Greger and Schlatter made their sodium measurements after 2 minutes. Further evidence for a direct effect of hormonal stimulation on Na,K-ATPase comes from ouabain binding studies. Ouabain binding to a high-affinity site is increased in *Squalus acanthias* rectal gland slices ( Silva *et al.* 1983) and dispersed cells (Marver *et al.* 1986) after treatment with cAMP and theophylline. The increase in ouabain binding is demonstrable even in the presence of furosemide and after the removal of sodium or chloride from ambient solutions (manoeuvres that block active transport ) (Marver *et al. 1986*). Such experiments suggest a direct action of cAMP or cAMP-dependent PKA on the sodium pump. However studies involving the *Scyliorhinus canicula* suggest that cAMP regulates the sodium pump indirectly. Shuttleworth, (1983) reported that cAMP-stimulated increases in sodium pump activity in *Scyliorhinus canicula* rectal gland slices as determined by ouabain binding and ouabain-sensitive oxygen consumption is blocked by furosemide or by the absence of extracellular chloride, this indicates that cAMP exerts its effect by altering the activity of furosemide-sensitive, chloride-coupled entry into the cell. This in turn leads to the direct activation of Na,K-ATPase due to an increase in intracellular sodium concentration or activation of previously inactive pumps.

These conflicting results suggest that cAMP may simultaneously activate a number of separate membrane events in rectal gland cells that operate together to produce active secretion of sodium chloride. It had been well documented that cAMP-stimulated activation of chloride channels results in an increase in apical chloride conductance and membrane depolarisation (Marshall, 1991, Fuller. 1992, Kushman *et al.* 1992) In addition activity of the sodium pump itself appears to be enhanced by cAMP stimulation either directly (Lear *et al.* 1992) or as a result of increased intracelular sodium arising from stimulation of the Na,K,Cl cotransporter (Shuttleworth, 1983) which has also been reported to be stimulated by cAMP (Shuttleworth, 1983).

5 I a iv Vascular actions of cAMP in the dogfish rectal gland

Cyclic AMP has also been reported to have physiological actions on the vasculature of the rectal gland (Shuttleworth, 1983 and Shuttleworth, 1991), this is discussed in detail in chapter 1 section 1.IV.

## 5.I b The actions of cGMP in the dogfish rectal gland.

Sodium chloride transport in the perfused rectal gland isolated from the Squalus *acanthias* can be stimulated by hormones known to increase intracellular cGMP concentrations eg atriopeptin (Karnaky *et al,* 1991). These hormones have also been shown to increase intracellular cGMP concentration in monolayers of shark rectal gland cells (Moran *et al.* 1993).

5.Ib i sCNP

The first natriuretic peptide discovered was atrial natriuretic peptide (ANP). This was first discovered in granules in human atrial myocardial cells (Windgard *et al.* 1991), and subsequently was found to act on the mammalian kidney to inhibit sodium reabsorption. Blood concentrations of ANP have been reported to increase when blood pressure rises, or when dietary intake of salt is raised (Rang and Dale, 1990). This hormone causes vasodilatation and increases renal blood flow and glomerular filtration rate. Its vasodilatory effect in mammals is known to be mediated by cGMP (Windgard *et al.* 1991). Since the discovery of ANP two further members of this natriuretic peptide family have been isolated, these are known as B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). Mammalian ANP has also been reported to stimulated sodium chloride secretion in the perfused rectal gland isolated from *Squalus*

*acanthias*, however it has no effect on isolated tubules or single cells isolated from *Squalus acanthias* suggesting that a whole gland preparation is required for heterologous natriuretic peptide-stimulation of sodium chloride secretion (Silva *et al.* 1987). However, since then, ANP has been shown to act directly on shark rectal gland cells in culture and to elevate cGMP levels and stimulate transepithelial chloride transport (Karnaky *et al.* 1991). Bumetanide inhibits the action of ANP in shark rectal gland cells demonstrating that Na, Cl, K cotransporter activity is associated with ANP-dependent chloride secretion (Karnaky *et al.* 1991).

C-type natriuretic peptide (CNP) has recently been isolated from the dogfish rectal gland and is known as shark CNP (sCNP). This endogenous peptide has been shown to stimulate sodium chloride secretion in the isolated perfused rectal gland of *Squalus acanthias* (Solomon *et al.* 1992) and *Scyliorhinus canicula* (Anderson, 1995). Binding studies have recently shown that 2 classes of natriuretic receptors exist in shark rectal gland. The first is a non guanylate cyclase linked receptor (Gunning, *et al* . 1993) and the second is a guanylate cyclase-linked receptor, which preferentially binds CNP (Gunning, *et al* . 1993). As sCNP has been shown to stimulate sodium chloride secretion and is able to stimulate guanylate cyclase activity it was predicted that the second messenger cGMP would stimulate sodium chloride secretion from rectal gland epithelia. However, perfusion of rectal glands isolated form either *Squalus acanthias* (Silva *et al.* 1987) or *Scyliorhinus canicula* (Anderson, 1995) with the cell permeable analogue 8-bromo cGMP failed to stimulate secretion. Likewise addition of 8-bromo cGMP to the medium of cell cultures isolated from the *Squalus acanthias* also failed to have any effect on sodium chloride secretion (Silva *et al.* 1993). However, the addition of $10^{-7}$ M ANP to cell cultures of *Squalus acanthias* was found to increase intracellular levels of cGMP by 10 fold (Karnaky *et al.* 1991). Therefore it appears that sCNP does not mediate sodium chloride secretion via its receptor linked to guanylate cyclase, however the action of ANP does involve increasing intracellular cGMP concentrations via the guanylate linked receptor.

The mechanisms of action of sCNP in the rectal gland are poorly understood. Although sCNP is able to increase intracellular cGMP concentrations, this alone does not seem sufficient to stimulate sodium chloride secretion in the isolated perfused gland as in cultured cells. Whether sCNP acts directly on the epithelial cells or acts via the vascular smooth muscle to stimulate secretion remains to be determined. This study will look at the effect of sCNP on cGMP concentrations in the isolated perfused rectal

gland and primary rectal gland epithelial cell monolayers isolated from *Scyliorhinus canicula* .

Many hormones eg VIP (Forrest *et al.* 1983) scyliorhinin II (Anderson *et al.* 1995) and adenosine (Kelley *et al.* 1992) affect the rate of sodium chloride secretion by the dogfish rectal gland and these hormones may use either cAMP or cGMP as second messengers. The intracellular pathways themselves are not well characterised and the nature of interaction of the cyclic nucleotides with the proteins involved in this sodium chloride secretion pathway require investigation. The aims of this chapter are therefore to investigate if sCNP or scyliorhinin II increase cAMP or cGMP intracellular levels in the rectal gland epithelial cells or do they act via the glands vascular.

## 5.II MATERIALS AND METHODS

### 5.II a Radioimmunoassay (R.I.A.)

R.I.A. was first developed in 1959 for the assay of insulin, as described by Patrono and Peskar, 1987. It is still the current method of choice for measuring very low concentrations of cyclic nucleotides in biological extracts. The three main requirements that are necessary to perform a R.I.A. are:-

1. An antibody which binds specifically and with high affinity to the antigen being assayed.
2. A radioactively labelled analogue of the antigen to be assayed.
3. A method of separating bound from free antibody in the solution.

The general principles of the assay are that a variable amount of specific compound eg cAMP or cGMP, is introduced into a system which contains a constant amount of radioactive compound eg $^{125}$I succinyl cAMP or $^{125}$I succinyl cGMP and a limiting concentration of antibody for that compound eg an antibody to cAMP or cGMP. The non radiolabelled compound will compete with the radioactive compound for the limited number of antibody binding sites (Fig. 5.2). This interaction between an antigen and antibody provides the basis of the assay of antigen concentration.

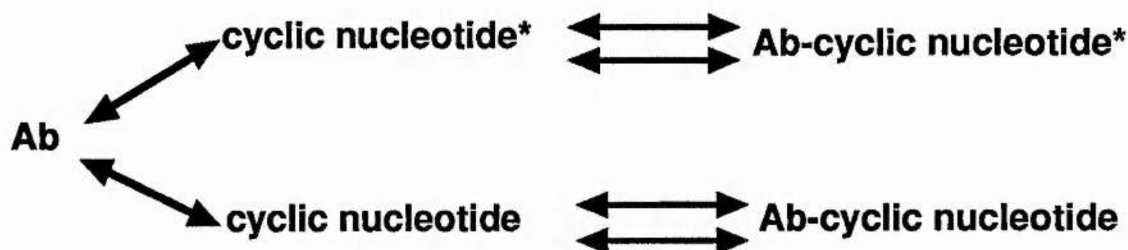



Figure 5.2 Competitive equilibrium between radiolabelled (*) and unlabelled cyclic nucleotide for the antibody (Ab).

The amount of radioactively labelled antigen bound to the antibody decreases as the concentration of the unlabelled antigen increases. A standard curve can therefore be prepared using a range of known concentrations of unlabelled cAMP or cGMP. From this curve the concentration of cAMP or cGMP in any biological extract can be determined by reference to the standard curve.

5.II a i Preparation of polyclonal antibodies to cAMP.

Preparation of Antigen (in association with Dr G Cramb).

2'[0-succinyl]-cyclic AMP (ScAMP) was conjugated to human thyroglobulin by a modification of the method of Skowsky and Fisher (1972). Crosslinking of ScAMP to thyroglobulin involved coupling of ScAMP to the free amino groups on thyroglobulin using the water soluble 1-ethyl-3(3-dimethylamine propyl) carbodiimide (EDC). The efficiency of the coupling is enhanced by the addition of the stabiliser N-hydroxysulpho-succinimide (sulpho NHS). An aqueous solution was prepared containing 2 mg ScAMP ( approximately 4 μ moles), 4 mg EDC (21 μ moles), 11 mg sulpho NHS (51 μ moles) in 1 ml of activation buffer (0.1 M morpholine ethane sulphonic acid and 0.5 M sodium chloride pH 4.4). This was incubated at room temperature for 15 minutes. Human thyroglobulin (10 mg) was dissolved in 1 ml activation buffer (as above) and added drop-wise to the ScAMP/ EDC/ Sulpho NHS mix with constant stirring. The reaction mixture was then incubated in the dark at room temperature for 3 hours. The mixture was then dialysed against four changes of 2 litres of water over a 48 hours period, at 4°C. (Dialysis tubing was prepared prior to this step by boiling in 2 changes of 40 mM EDTA and 25 mM sodium bicarbonate, pH 6 for 1 hour before finally washing with Milli Q water. Following dialysis the thyroglobulin ScAMP conjugation product precipitated in the dialysis tube. The precipitate was collected by centrifugation at 30,000 $g_{max}$ for 30 minutes at 4°C and dissolved in 5 ml phosphate buffered saline (PBS).

The ultra violet spectra of thyroglobulin (0.25 mg/ml in PBS), ScAMP (0.05 mg/ml in PBS) and the thyroglobulin-ScAMP conjugation product (approximately 0.2 mg/ml in PBS) were then compared using a dual beam spectrophotometer (Perkin Elmer) (Fig 5.3). In all cases the blank was PBS. The spectrum of the conjugate product was similar to the ScAMP and thyroglobulin spectras when superimposed on each other and therefore a successful conjugation was assumed to have taken place (Fig 5.3). Aliquots (500 μl) of the reaction product (approximately 0.2 mg/ml) were frozen in liquid nitrogen and stored at -70°C.

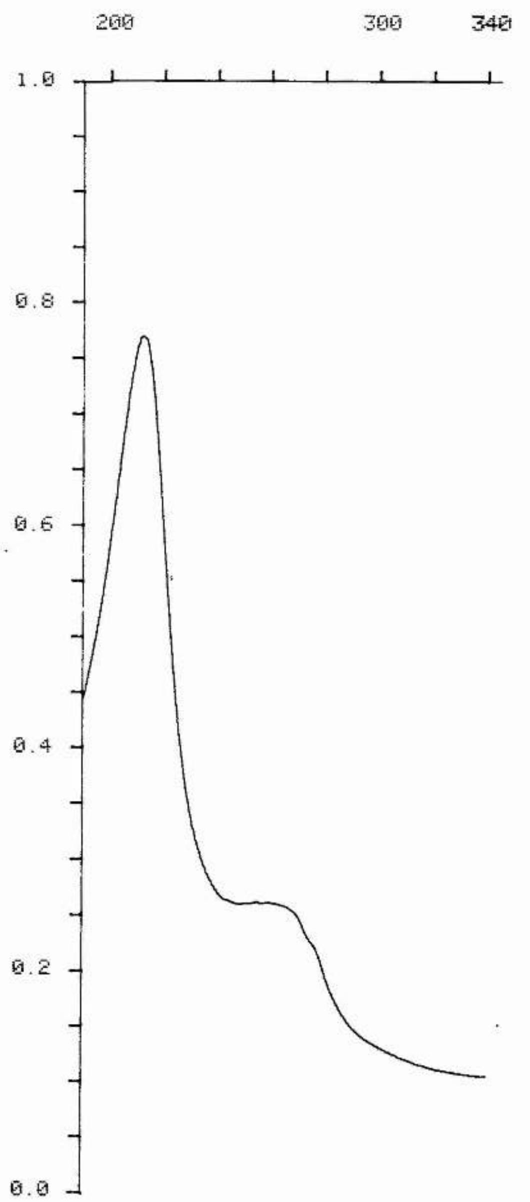
Immunisation Procedure (in association with Dr G Cramb).

The adjuvant was prepared as described by Sommerville, (1972). The first step in this method was to prepare an oil emulsion by adding 9 parts of DRACEOL-6VR

Figure 5.3

1, 2 + 3 need labelling on fig

Figure 5.3 The ultra violet spectra of thyroglobulin (0.25 mg/ml) in PBS (1), ScAMP (0.05 mg/ml) in PBS (2) and thyroglobulin ScAMP conjugation product (approximately 0.2 mg/ml thyroglobulin) (3).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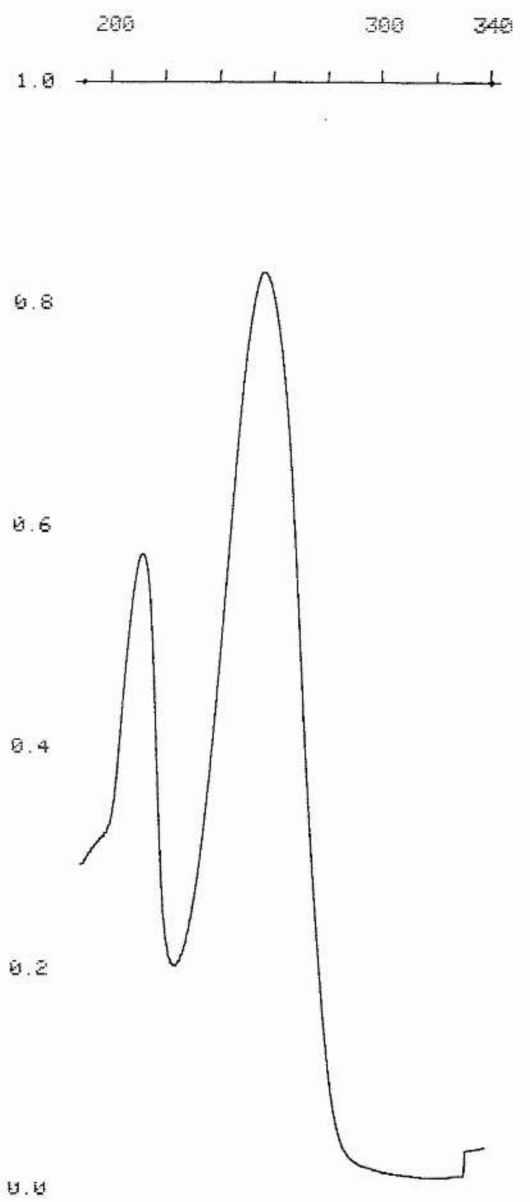
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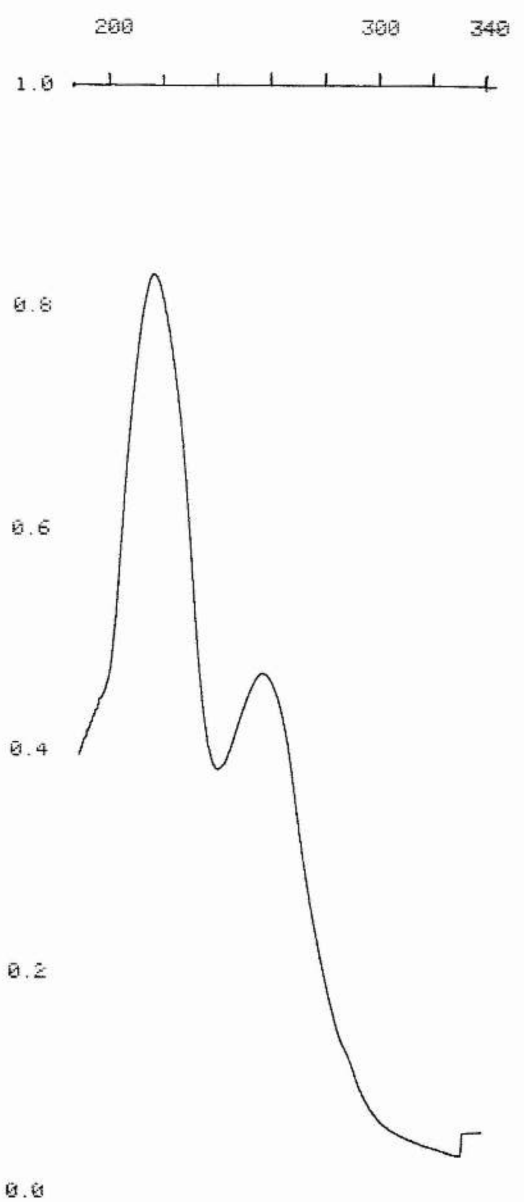
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(Pennsylvania Refining Company USA) to 1 part ARLACEL-A (Atlas Producer Co. USA). The cyclic nucleotide conjugate (1ml of 0.2 mg/ml) was then added to an equal volume of the oil emulsion (1ml) and the sample mixed thoroughly by cycling the mixture through a hypodermic syringe fitted with a 0.6 mm bore needle, this continued until a thick viscous emulsion was obtained. To this emulsion, an equal volume of 2% TWEEN 80 in 0.9% saline was added and the mixture re-cycled again through the syringe and needle until a free flowing liquid was obtained. The emulsion was injected into New Zealand white female rabbits. Each rabbit was injected subcutaneously at 4 sites with 0.5 ml of the emulsion, booster injections were administered every 14 days by the same method as previously described, ie 2 ml per rabbit. Animals were bled from an ear vein 7 to 10 days after each booster. The blood was allowed to clot for 2 hours at room temperature. Then using a sealed pipette it was separated from the side of the centrifuge tube and incubated overnight at 4°C . The blood was centrifuged at 1720 x$g_{max}$, 4°C for 20 minutes. The serum was removed from the blood sample and stored aliquots tube at -70°C. This procedure was performed with the help of Dr G. Cramb and the antibody for the cGMP R.I.A. was made by Dr. G. Cramb using an identical procedure.

Determination of Antibody titre (in association with Dr G Cramb).

The dilution of the serum to give a suitable antibody concentration in the R.I.A. was ascertained by adding 400 µl of serum to 1.6 ml of sodium acetate buffer (50 mM sodium acetate, 0.5 % BSA pH 4.75 with glacial acetic acid). A 1:1 dilution series was then made with the diluted serum and acetate buffer up to 1: 327, 680. From each step in the dilution series duplicate 250 µl samples were placed into a RT-30 tube and 200 µl of $^{125}$I-ScAMP-TME (15,000 cpm) in acetate buffer was added. This mixture was incubated overnight at 4°C and 2 ml of ice cold ethanol was added. The tubes containing the resulting precipitate were incubated at room temperature for 30 minutes before being centrifuged at 1720 x $g_{max}$, 4°C for 30 minutes. The supernatant was discarded and the tubes drained before the radioactivity in the pellet was determined on a Packard Cobra II auto gamma counter. The percentage radioactivity bound at different dilutions was plotted against dilution factor. The serum dilution which resulted in half maximum of total counts bound as extrapolated from the graph was found to be 1 in 5 000 which is equivalent to a 1:9 000 dilution in the final assay.

Antibody specificity was ascertained by determining the concentrations of nucleotides and nucleosides (ATP, ADP and AMP either acetylated or non acetylated) required to

displace the $^{125}$I-ScAMP-TME from the cAMP rabbit antiserum at a final concentration of 1 in 9 000. A graph was constructed showing % Specific Bound against nucleoside or nucleotide concentrations (Fig 5.4). Figure 5.4 shows that the antibody was 100 fold more specific for cAMP than any other nucleoside. As expected the acetylated samples had higher affinity for binding to the antibody than the non-acetylated samples.

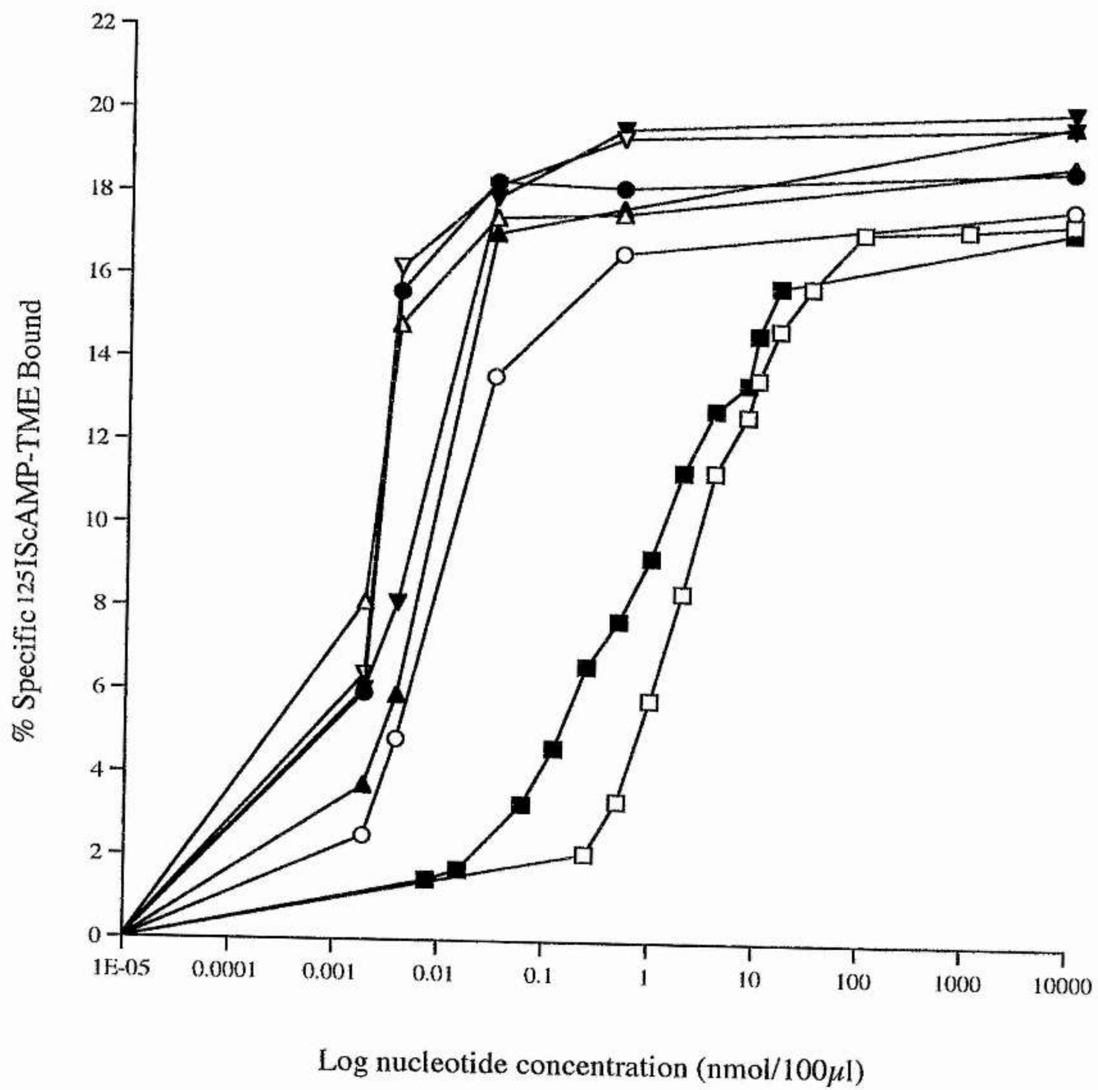
5.II a ii Preparation of $^{125}$I- succinyl-cyclic AMP tyrosyl methyl ester.

Succinyl-cyclic AMP tyrosyl methyl ester (ScAMP-TME) was iodinated with Na$^{125}$I by a modification of the method of Steiner *et al.* (1972). Succinyl cAMP-TME (2µg in 20 µl) was added to 180 µl of 0.5 M sodium phosphate buffer, pH 7.0. From this solution 20 µl was removed and placed in the iodogen treated tube along with 20 µl of 0.5 M sodium phosphate, pH 7.0 and 5 µl of Na$^{125}$I (15,000 cpm). This was mixed gently and incubated at room temperature for 10-15 minutes. The reaction was stopped by the addition of 1 ml of 0.1% TFA. The column (AmPrep C-18 ODS 100 mg reverse phase column) (Amersham Int plc) was conditioned under vacuum with three washes of 2 ml methanol and four washes with 2 ml of Milli Q water. Following addition of the reaction mixture to the column (which bound $^{125}$I-ScAMP-TME), the column was then washed with 2 x 2 ml of 0.1% trifluoro acetic acid (TFA) (to remove free $^{125}$Iodide) and the $^{125}$I-succinyl cAMP -TME was eluted from the column in 2 ml of 60% acetonitrile / 0.1% TFA. The $^{125}$I-ScAMP-TME was diluted with 18 ml of 50 mM sodium acetate buffer pH 7.4 containing 0.1 % bovine albumin serum and stored in 200 µl aliquots at -20°C. This procedure was repeated with ScGMP to produce $^{125}$I-succinyl-cyclic GMP tyrosyl methyl ester for use in the cGMP R.I.A.

## 5.II b Radioimmunoassay Procedure (using the acetylation method).

Unknowns and standard samples were acetylated by the rapid addition and mixing of 1/100 volume of 2:1 triethylamine: acetic anhydride and incubated for 30 minutes at room temperature. The radioimmunoassay comprised of 100 µl of acetylated samples, 100 µl of radiolabelled cAMP or cGMP (approximately 15000 counts per minute) and 250 µl of the appropriately diluted cAMP or cGMP antibody. This was gently mixed and incubated overnight at 4°C. Following the overnight incubation, 2 ml of ice cold 95% ethanol was added to each tube, vortexed and incubated for 30 minutes at room temperature. The precipitated proteins were then centrifuged at 1720 x $g_{max}$ for 30 minutes at 4°C in a MSE coolspin and the supernatant was decanted and the

Figure 5.4 Antibody specificity for acetylated (open symbols) and non acetylated (closed symbols) forms of cAMP (□,■), AMP (O,●), ADP (△, ▲), ATP (▽, ▼) respectively. Each point represents the means ± standard error of mean for four separate experiments.



22
20
18
16
14
12
10
8
6
4
2
0
% Specific 125IScAMP-TME Bound
1E-05
0.0001
0.001
0.01
0.1
1
10
100
1000
10000
Log nucleotide concentration (nmol/100μl)

radioactivity in the pellets determined by a gamma scintillation counter (Packard Canberra Cobra). A standard curve was prepared with known concentrations of unlabelled acetylated cAMP or acetylated cGMP and from these curves the amount of cAMP or cGMP in the extracts was determined.

For acetylated samples the standard curves ranged from 2 p moles / 100 μl to 0.0009 p moles / 100 μl for cGMP or 16 p moles / 100 μl to 0.0078 p moles / 100 μl for cAMP. Triplicate 100 μl samples of each dilution were used to construct the standard curves. The non-specific binding (NSB) was determined as the cpm bound in the presence of 400 p moles / 100 μl cGMP or cAMP. As shark Ringer (perfused gland) or $K_2CO_3$-neutralised PCA-extracted shark Ringer (cell cultures) was present in the unknown samples, all standards were made up in either shark Ringer (perfused gland) or $K_2CO_3$-neutralised PCA-extracted shark ringer (cell cultures) to ensure the unknowns and standards had identical ionic compositions. Typical cAMP and cGMP standard curves are shown in figures 5.5 and 5.6.

## 5.II c Optimisation of the Radioimmunoassay

The effect of assay buffer composition and pH profile was determined for the cAMP and cGMP assays. The different buffers and pH ranges used are indicated in table 5.1.

ASSAY BUFFER / pH (table 5.1)

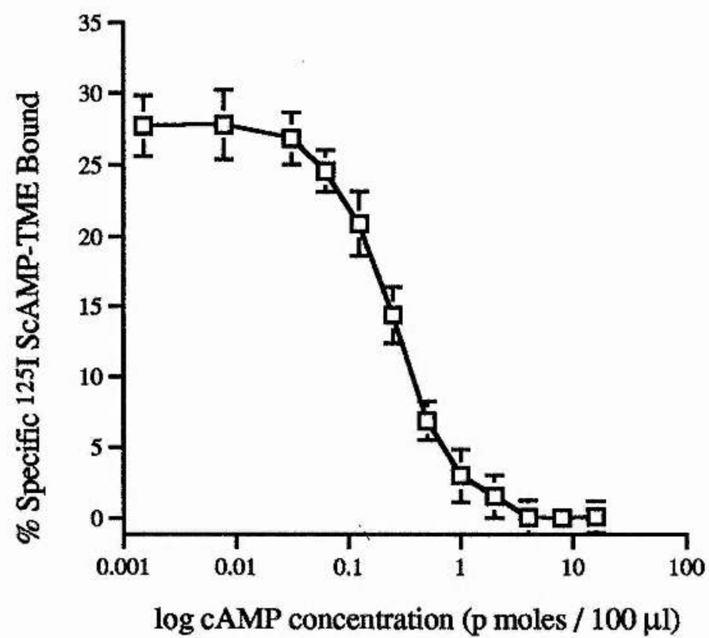
| 50 mM sodium acetate pH | 50 mM HEPES pH | 50 mM $Na_2HPO_4/NaH_2PO_4$ pH |
|---|---|---|
| 4 | 5.5 | 7 |
| 4.5 | 6 | 7.5 |
| 5 | 6.5 | 8 |
| 5.5 | 7 | 8.5 |
| 6 | 7.5 | |

In addition to buffer composition and pH (Figs 5.7 and 5.8) the effect of urea, TMAO, shark ringer and $K_2CO_3$-neutralised acid-extracted shark Ringer was also investigated in cAMP and cGMP radioimmunoassays (RIA) to determine if any of these components altered the antibody-antigen interaction. This is particularly important as

Figure 5.5 andFigure 5.6

Figure 5.5 Typical standard curve for cAMP assay. Each point represents the means ± standard error of mean for four separate experiments.

Figure 5.6 Typical standard curve for cGMP . Each point represents the means ± standard error of mean for four separate experiments.


35
30
25
20
15
10
5
0
0.001
0.01
0.1
1
10
100
% Specific 125I ScAMP-TME Bound
log cAMP concentration (p moles / 100 µl)

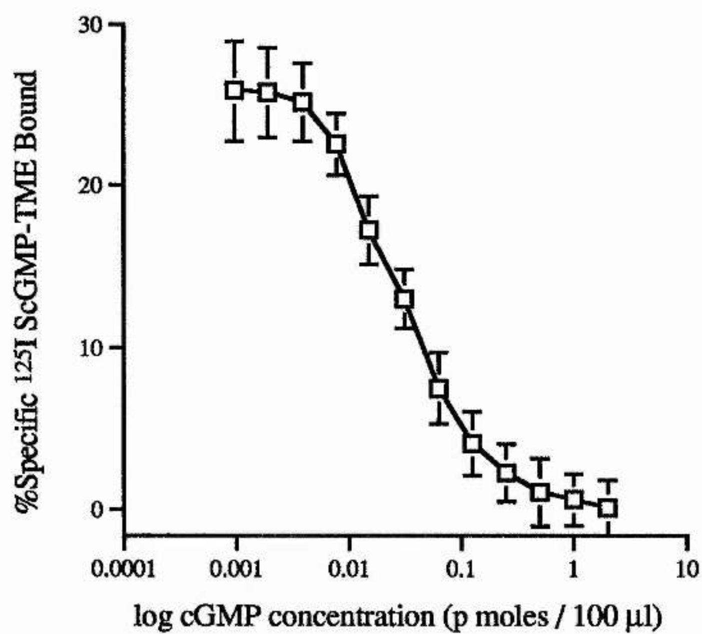
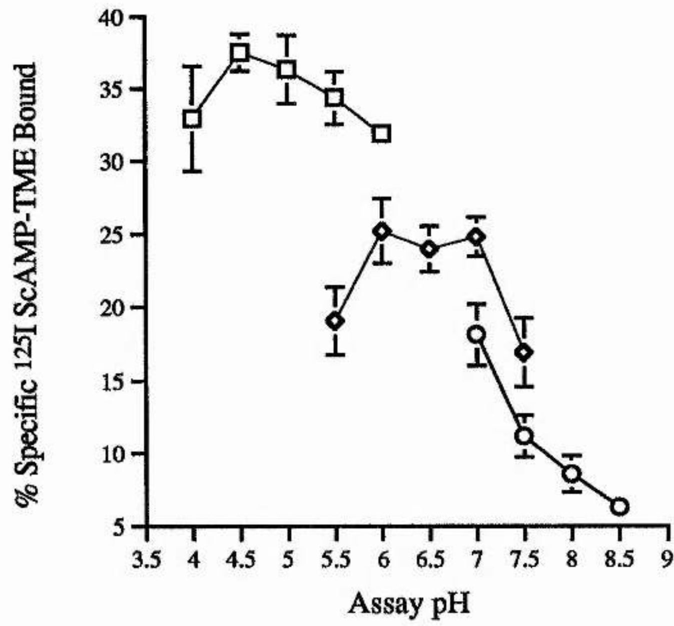
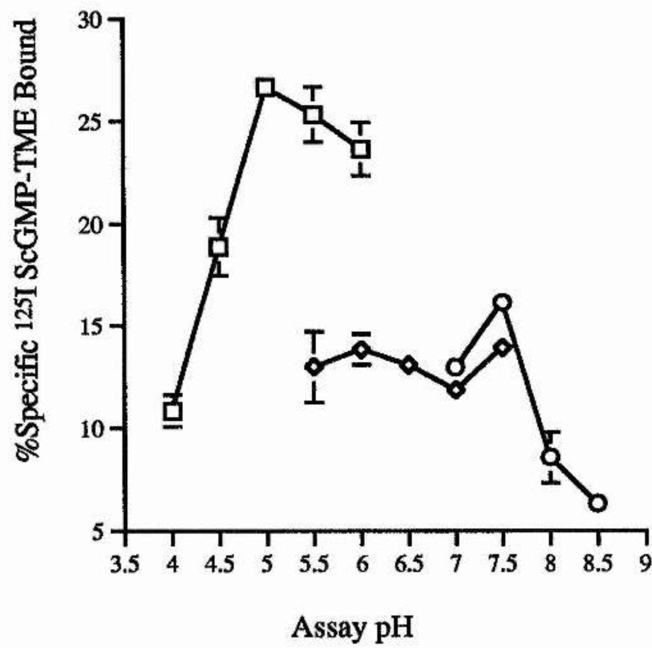

30
20
10
0
0.0001
0.001
0.01
0.1
1
10
%Specific 125I ScGMP-TME Bound
log cGMP concentration (p moles / 100 µl)

Figure 5.7 and 5.8

Figure 5.7 The effect of assay buffer and pH on maximal binding of $^{125}$IScAMP. $^{125}$I-cAMP (15000cpm) and cAMP antibody (final dilution of 1:9000) were incubated overnight at 4°C in 50 mM sodium acetate buffer (□), 50 mM HEPES (◊ ) or 50 mM sodium phosphate buffer (O) at the pH's indicated before % specific bound was determined as described in section 5.IIb. Each point represents the mean ± standard error of mean for four separate experiments.

Figure 5.8 The effect of assay buffer and pH on maximal binding of $^{125}$IScGMP. $^{125}$I-cGMP (15000cpm) and cGMP antibody (final dilution of 1:54000) were incubated overnight at 4°C in 50 mM sodium acetate buffer (□), 50 mM HEPES (◊ ) or 50 mM sodium phosphate buffer (O) at the pH's indicated before % specific bound was determined as described in section 5.IIb. Each point represents the mean ± standard error of mean for four separate experiments.


% Specific 125I ScAMP-TME Bound
40
35
30
25
20
15
10
5
3.5
4
4.5
5
5.5
6
6.5
7
7.5
8
8.5
9
Assay pH


%Specific 125I ScGMP-TME Bound
30
25
20
15
10
5
3.5
4
4.5
5
5.5
6
6.5
7
7.5
8
8.5
9
Assay pH

unknown samples extracted from shark rectal gland cells would also contain these components of shark Ringer (Figs 5.9, 5.10, 5.11 and 5.12).

## 5.II d Unknown sample preparation

### 5.II d i Cultured Cells

The cells were isolated and grown in 96 well collagen coated plates as described in Chapter 2. The growth medium was removed from the cells and the cells were washed twice with shark Ringer at room temperature, before incubating for various periods of time in shark Ringer containing various known effectors of cAMP/cGMP production either in the presence or absence of the phosphodiesterase inhibitor 3-iso butyl-1-methyl xanthine (IBMX)(1mM). The effectors used include forskolin (10 µM), $PGE_1$ (10 µM), $PGE_2$ (10 µM), CNP (10 µM) and scyliorhinin II (10 µM). All the effectors were diluted in shark ringer containing 0.2 % (w/v) BSA and the protease inhibitors 0.1 % w/v bacitracin, 1mM leupeptin and 500 µM phosphoramidon. The incubations were terminated by washing twice with ice cold shark ringer, followed by addition of 100 µl of ice cold 6% PCA. Following a 10 minute incubation at 4 °C the samples were neutralised using 3 M $K_2CO_3$ and incubated for 10 minutes at 4°C before the plates were centrifuged at 200 x $g_{max}$ 4°C, for 10 minutes in a MSE coolspin. Two 75 µl aliquots were removed from each well and diluted to 100 µl with extracted shark Ringer and acetylated as above. Radiolabelled [1] cGMP or AMP (100 µl) and cGMP or cAMP antibody (250 µl) were added as appropriate. Cyclic AMP or cGMP concentration was determined as described in section 5.IIb.

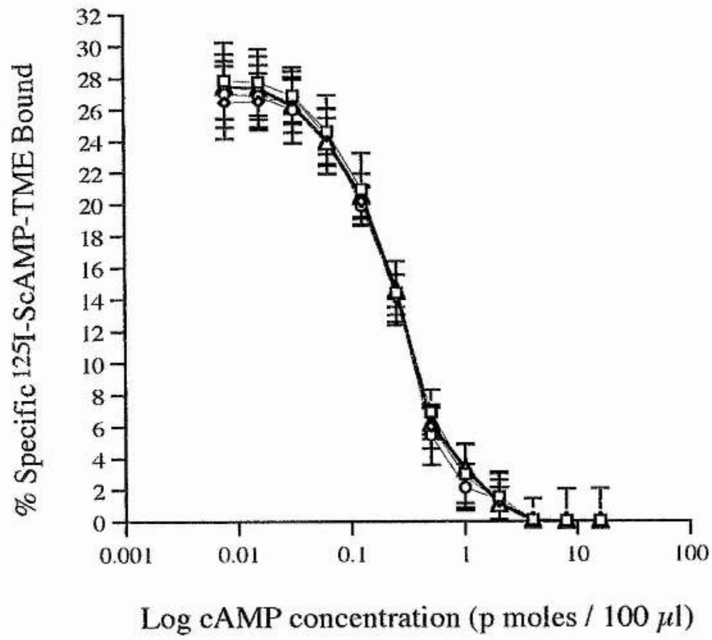
### 5.II d ii Perfused rectal gland.

The rectal gland was removed from the fish and cannulated and perfused as described in Chapter 2. The gland was initially perfused with shark Ringer for 5 minutes, then perfused with shark Ringer containing 1 mM IBMX alone or, 1 mM IBMX plus 10 µM sCNP or 10 µM scyliorhinin II. The perfusate was collected every minute starting 3 minutes before the addition of the effector, and then at the same intervals over a 10 minute period following effector administration. The collected perfusate fractions were diluted to 300 µl with shark Ringer, acetylated as described above and duplicate 100 µl aliquots of this were used in the R.I.A.

Figure 5.9 and 5.10

Figure 5.9 The effect of assay buffer composition on maximal binding of $^{125}$IScAMP. Standard curves were constructed using various assay buffers. $^{125}$I-cAMP (15000cpm) and cAMP antibody (final dilution of 1:9000) were incubated overnight at 4°C in 50 mM sodium acetate buffer (□), $K_2CO_3$ neutralised PCA extracted shark ringer (◊ ) or shark ringer with (Δ ) or without (O) urea and TMAO. Each point represents the mean ± standard error of mean for four separate experiments.

Figure 5.10 The effect of assay buffer composition on maximal binding of $^{125}$IScGMP. Standard curves were constructed using various assay buffers. $^{125}$I-cGMP (15000cpm) and cGMP antibody (final dilution of 1:54000) were incubated overnight at 4°C in 50 mM sodium acetate buffer (□), $K_2CO_3$ neutralised PCA extracted shark ringer (◊ ) or shark ringer with (Δ ) or without (O) urea and TMAO. Each point represents the mean ± standard error of mean for four separate experiments.


% Specific 125I-ScAMP-TME Bound
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
0.001
0.01
0.1
1
10
100
Log cAMP concentration (p moles / 100 μl)

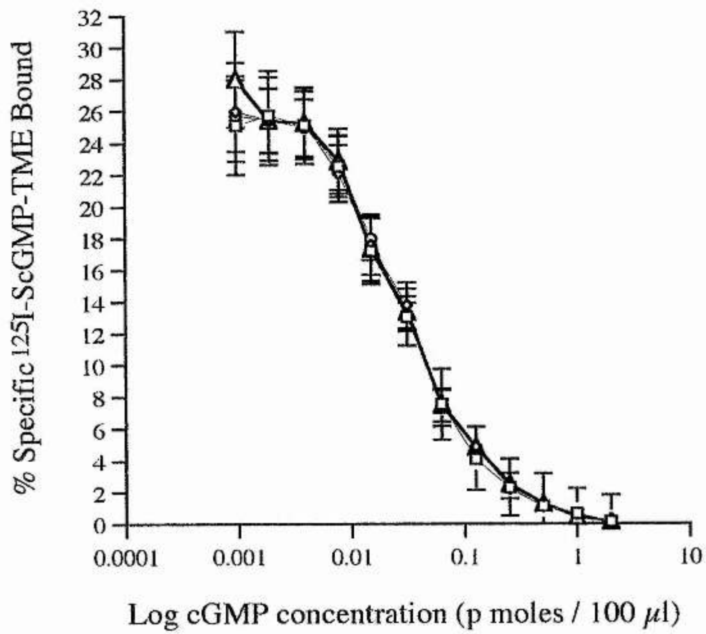
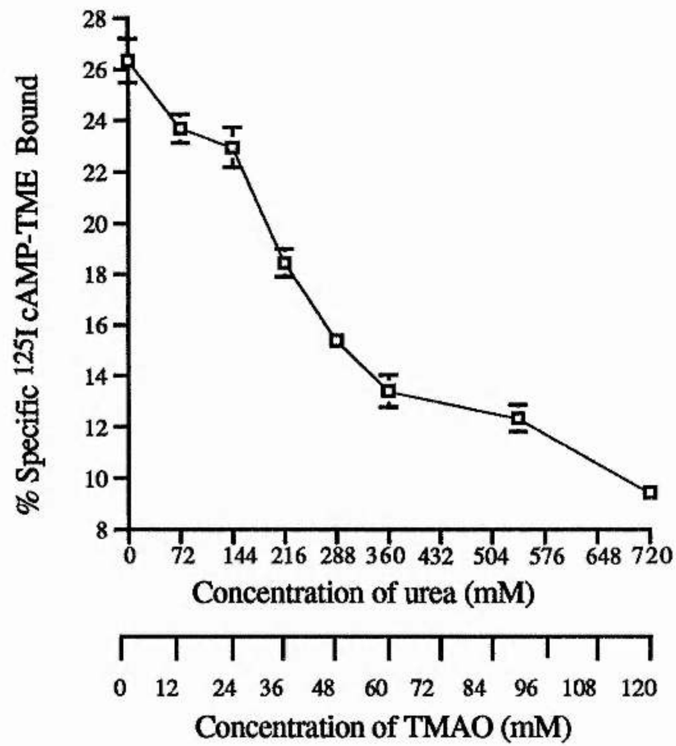
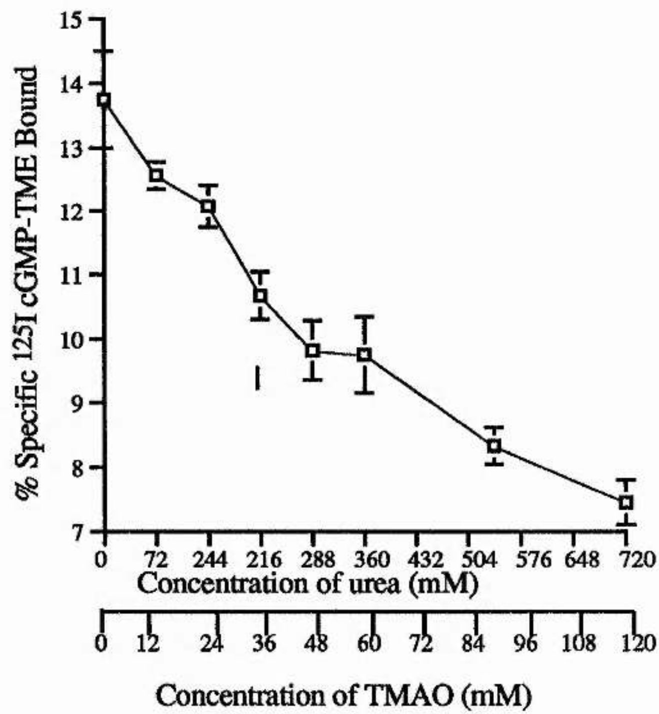

% Specific 125I-ScGMP-TME Bound
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
0.0001
0.001
0.01
0.1
1
10
Log cGMP concentration (p moles / 100 μl)

Figure 5.11 and Figure 5.12

Figure 5.11 The effect of urea and TMAO on cAMP the radioimmunoassay. Urea and TMAO at the same ratios as present in shark Ringer were added to sodium acetate buffer at the concentrations indicated, 360 mM urea and 60 mM TMAO represent the concentrations of urea and TMAO normally present in shark Ringer. $^{125}$I-cAMP and cAMP antibody at optimal dilution of 1:9000 was incubated overnight at 4°C in sodium acetate buffer containing urea and TMAO Concentrations indicated on the graph. Each point represents the mean ± standard error of mean for four separate experiments.

Figure 5.12 The effect of urea and TMAO on cGMP the radioimmunoassay. Urea and TMAO at the same ratios as present in shark Ringer were added to sodium acetate buffer at the concentrations indicated, 360 mM urea and 60 mM TMAO represent the concentrations of urea and TMAO normally present in shark Ringer. $^{125}$I-cGMP and cGMP antibody at optimal dilution of 1:54000 was incubated overnight at 4°C in sodium acetate buffer containing urea and TMAO Concentrations indicated on the graph. Each point represents the means ± standard error of mean for four separate experiments.


% Specific 125I cAMP-TME Bound
28
26
24
22
20
18
16
14
12
10
8
0 72 144 216 288 360 432 504 576 648 720
Concentration of urea (mM)
0 12 24 36 48 60 72 84 96 108 120
Concentration of TMAO (mM)


% Specific 125I cGMP-TME Bound
15
14
13
12
11
10
9
8
7
0 72 244 216 288 360 432 504 576 648 720
Concentration of urea (mM)
0 12 24 36 48 60 72 84 96 108 120
Concentration of TMAO (mM)

The standards for the perfused gland experiments were made up in regular shark Ringer and acetylated as described above.

## 5.III RESULTS

## 5.III a Optimisation of cAMP and cGMP radioimmunoassays

5.III a i Assay buffer composition and pH profile.

It was necessary to optimise the assay procedure for both the cAMP and cGMP radioimmunoassay. Buffers of known composition were used at varying pH's to establish the pH profile for the antibody-antigen binding assay. As a result of the highest percentage binding of tracer it was concluded that the optimal conditions for cAMP and cGMP assays was using a 50 mM sodium acetate buffer with pH range between 4.5 - 5 (Fig 5.7 and 5.8).

5.III a ii The effect of shark Ringer and components of shark ringer on the radioimmunoassays.

The effect of addition of shark Ringer or $K_2CO_3$ neutralised PCA extracted shark Ringer on cAMP and cGMP radioimmunoassays was determined (Figs 5.9 and 5.10). The presence of shark Ringer or $K_2CO_3$ neutralised PCA extracted shark Ringer did not appreciably affect the antibody binding, to the cyclic nucleotides and therefore the standard curves were superimposable.

Strangely enough, even although the presence of shark Ringer or $K_2CO_3$ neutralised PCA extracted shark Ringer did not appreciably affect the shape of the standard curve, the addition of urea and TMAO together, but in the absence of other shark Ringer components did appear to inhibit antibody-antigen interactions (Fig 5.11 and 5.12). The reason for this anomaly is unknown although urea, at concentrations present in shark Ringer (360 mM) may be sufficient to partially denature the antibody. It was therefore concluded that another constituent(s) of the shark Ringer suppressed the inhibitory effects of the urea/TMAO mixture. However, the sensitivity or maximum binding capacity of the assay was not increased by the removal of urea and TMAO from the shark Ringer suggesting that the constituent(s) that suppresses the inhibitory effect of the urea/TMAO mix is therefore not able to elicit an effect on its own. In all subsequent RIA the standards were either diluted in shark Ringer when determining cyclic nucleotides in the eluates from the perfused gland or $K_2CO_3$ neutralised PCA extracted shark Ringer when quantifying cyclic nucleotide concentration in the extracts

from the cell cultures. Therefore in the experiments conducted the unknown samples and standards contained identical ionic compositions.

The minimum detechtable level of cAMP in the cAMP assay was approximately 1 pmoles/100 µl, basal cAMP levels in the primary cell cultures was approximately 0.5 nmoles/100µl and 2-3 nmoles/100µl in the glands perfusate. These values were therefore within the range of the assay. The minimum detechtale level of cGMP in the cGMP assay was approximately 0.5 p moles/100µl, basal cGMP levels in the primary cell cultures was approximately 0.1-1 p moles/100µl and 1-3 p moles/100µl in the glands perfusate. The cGMP assay therefore was not sensitive enough to detech any small changes in basal cGMP levels in the primary cell cultures.

## 5.III b Cyclic AMP or cyclic GMP levels in rectal gland epithelial primary cell cultures.

5.III b i The effect of hormones on intracellular cAMP levels in rectal gland primary epithelial cell cultures.

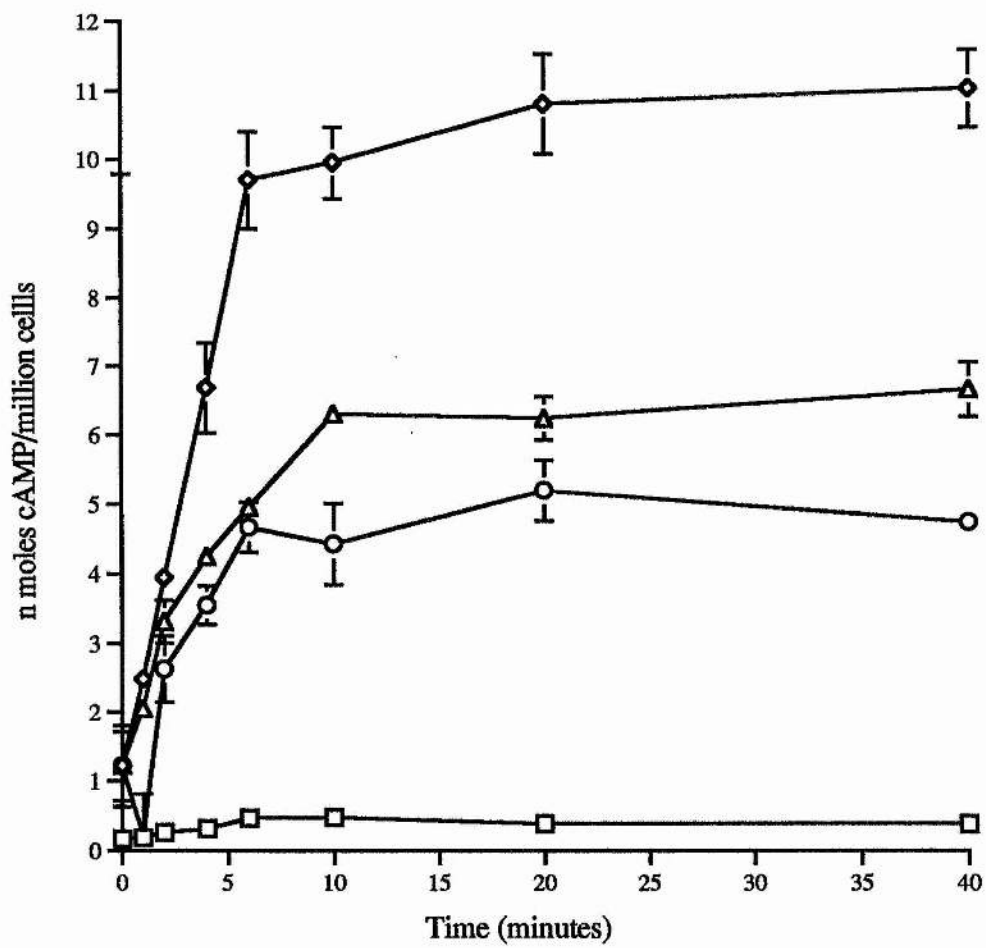
Having established the optimal radioimmunoassay conditions for samples extracted from cell cultures or for samples of perfusate, the action of various putative hormonal effectors of rectal gland secretion were investigated. Incubating cells in the presence of forskolin and IBMX, or $PGE_1$ and IBMX, or $PGE_2$ and IBMX resulted in sustained increases in intracellular cAMP concentrations (Fig 5.13). The greatest effect was seen with forskolin (10 µM) and IBMX (1 mM) (11-fold), however $PGE_1$ (10 µM) and $PGE_2$ (10 µM) both caused 5-fold and 6-fold increases in intracellular cAMP concentrations respectively after a 20 minute incubation. The effects of incubating the cells with Scyliorhinin II in the presence or absence of the phosphodiesterase inhibitor IBMX was also investigated (fig 5.14). Scyliorhinin II (10 µM), alone or in the presence of IBMX (1 mM) had no significant effect on cellular cAMP concentrations over the time period tested.

5.III b ii The effect of hormones on intracellular cGMP levels in rectal gland primary epithelial cell cultures.

The effect of various compounds on cGMP concentration was investigated in shark rectal gland primary epithelial cells cultures. Cells were incubated in the presence of sodium nitroprusside (an activator of soluble guanylate cyclase) or various

Figure 5.13

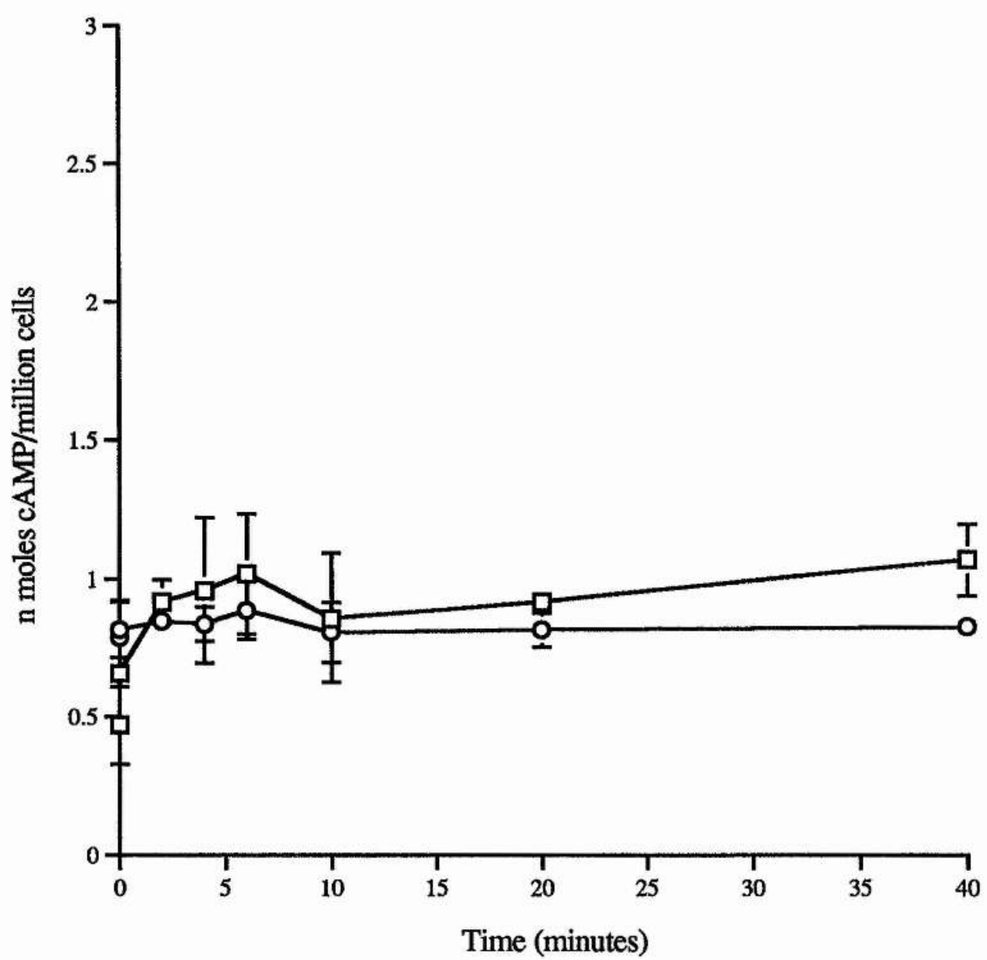
Figure 5.13 The effect of IBMX, IBMX and forskolin, IBMX and $PGE_1$ and IBMX and $PGE_2$ on intracellular cAMP concentration over an incubation period of 40 minutes. Rectal gland cell monolayers were incubated in IBMX (1 mM) and forskolin (10 μM) (◊) or $PGE_1$ (10 μM) and IBMX (1 mM) (Δ), $PGE_2$ (10 μM) and IBMX (1 mM) (O) or IBMX alone (1 mM) (□), for the times indicated. Cyclic AMP concentrations were then determined in the cell extracts as described in section 5 II d i. Each point represents the mean± standard error of mean for four separate experiments.



n moles cAMP/million cellls
Time (minutes)
0
1
2
3
4
5
6
7
8
9
10
11
12
0
5
10
15
20
25
30
35
40

Figure 5.14

Figure 5.14 The effect of various compounds on intracellular cAMP concentration over an incubation period of 40 minutes. Rectal gland cell monolayers were incubated in the presence of 1 mM IBMX (O) ± scyliorhinin II (10 μM) (□̇), for the times indicated. Cyclic AMP concentrations were then determined in the cell extracts as described in section 5 II d i. Each point represents the mean ± standard error of mean for four separate experiments.


n moles cAMP/million cells
3
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
30
35
40
Time (minutes)

concentrations of the natriuretic peptides, sCNP and rat ANP either alone or in the presence of the phosphodiesterase inhibitor IBMX and then cell extracts assayed for cGMP content. Unfortunately these experiments failed to provide any conclusive results. Reproducibility of responses were poor with the cells only occasionally responding to sCNP or sodium nitroprusside with increases in intracellular cGMP concentrations. Increases in cGMP concentrations were never consistent occurring at different time points and with highly variable concentrations of cGMP. As a result of these inconsistencies between experiments it was difficult to dissect out any real response to the effectors from the background cGMP concentrations (Fig 5.15).

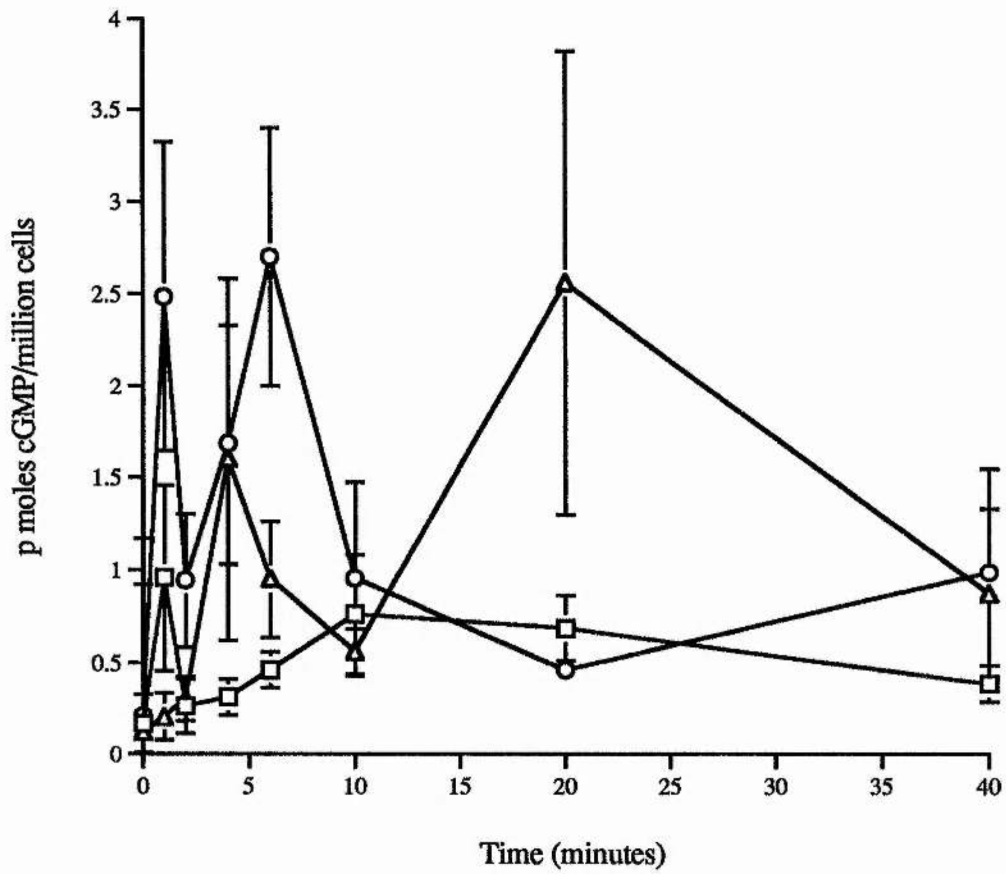
## 5.III c Hormonally elevated cAMP or cGMP levels in the perfusate of the isolated perfused rectal gland.

In the perfusion studies, glands were cannulated as described previously and then perfused with normal shark Ringer for 5 minutes. The perfusate was then collected in one minute intervals over the next 3 minutes and then effectors were added to the perfusion buffer. The gland was perfused with either the phosphodiesterase inhibitor IBMX (1mM) alone or with IBMX in the presence of sCNP (10 μM) or scyliorhinin II (10 μM). The perfusates were again collected at one minute intervals for the following 10 minutes. The nucleotides in the collected perfusates were then acetylated as described previously (Chapter 5 section II b) and assayed for either cAMP or cGMP as described (chapter 5 section IIb) (Figs 5.15 and 5.16).

From these results scyliorhinin II was observed to stimulate the release of cAMP from the perfused gland but had no measurable effect on cGMP release (Figs 5.16 and 5.17). Also sCNP stimulated the release of cGMP from the perfused gland but had no measurable effect on cAMP release (Figs 5.16 and 5.17).

Figure 5.14

Figure 5.15 The effect of various compounds on intracellular cGMP concentration over an incubation period of 40 minutes. Rectal gland cell monolayers were incubated in the presence of 1 mM IBMX (□) either alone or in the presence of CNP (10 $\mu$M) (O), or sodium nitroprusside (1 mM) ( △ ) for the times indicated. Cyclic GMP concentrations were then determined in the cell extracts as described in section 5 II d i. Each point represents the mean ± standard error of mean for four separate experiments.

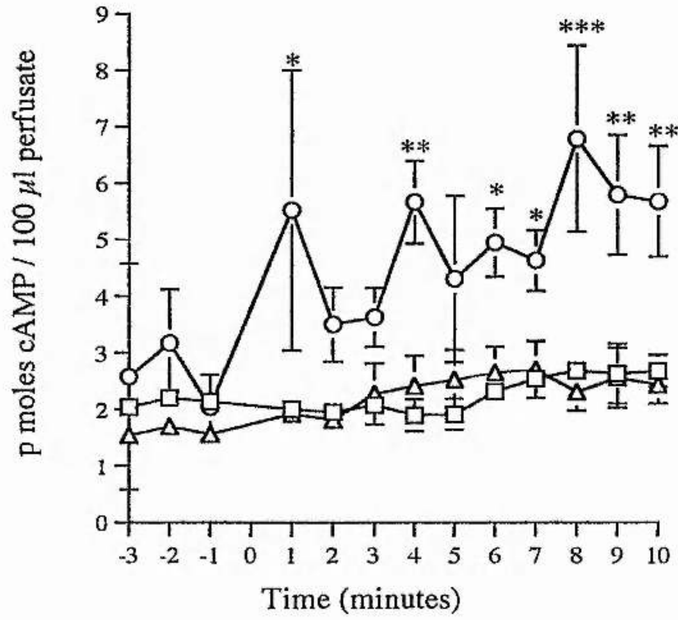


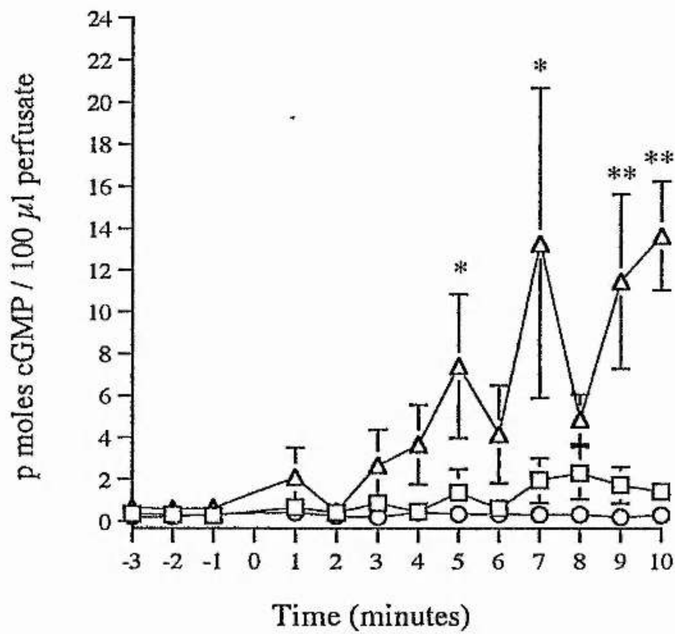
p moles cGMP/million cells
0
0.5
1
1.5
2
2.5
3
3.5
4
0
5
10
15
20
25
30
35
40
Time (minutes)

Figure 5.15 and Figure 5.16

Figure 5.16 The effect of IBMX, sCNP and IBMX, or scyliorhinin II and IBMX on cAMP released from the perfused shark rectal gland. Glands were perfused with IBMX (1 mM) alone (□) or in the presence of either 10 $\mu$M sCNP(Δ) or 10 $\mu$M scyliorhinin II (O). Perfusates were collected at 1 minute intervals for 3 minutes prior to addition of effector then at 1 minute invervals for 10 minutes following the addition of the effector. Cyclic AMP content of thefractions was then determined as described in section 5 II d ii. Each point represents the mean ± standard error of mean for eight separate experiments. Significant differences were determined by a two way ANOVA followed by Bonferroni and Dunn post hoc test. The p-value obtained was relevant to the degree of significant difference, p* ≤ 0.05 significant; p** ≤ 0.01 highly significant; p*** ≤ 0.001 very highly significant.

Figure 5.17 The effect of IBMX, sCNP and IBMX, or scyliorhinin II and IBMX on cGMP released from the perfused shark rectal gland. Glands were perfused with IBMX (1 mM) alone (□) or in the presence of either 10 $\mu$M sCNP(Δ) or 10 $\mu$M scyliorhinin II (O). Perfusates were collected at 1 minute intervals for 3 minutes prior to addition of effector then at 1 minute invervals for 10 minutes following the addition of the effector. Cyclic GMP content of the fractions was then determined as described in ection 5 II d ii. Each point represents the mean ± standard error of mean for eight separate experiments. Significant differences were determined by a two way ANOVA followed by Bonferroni and Dunn post hoc test. The p-value obtained was relevant to the degree of significant difference, p* ≤ 0.05 significant; p** ≤ 0.01 highly significant; p*** ≤ 0.001 very highly significant.


p moles cAMP / 100 μl perfusate
Time (minutes)
9
8
7
6
5
4
3
2
1
0
-3 -2 -1 0 1 2 3 4 5 6 7 8 9 10
*
**
*
*
***
**
**


p moles cGMP / 100 μl perfusate
Time (minutes)
24
22
20
18
16
14
12
10
8
6
4
2
0
-3 -2 -1 0 1 2 3 4 5 6 7 8 9 10
*
*
**
**

## 5.IV DISCUSSION

### 5.IV a Hormonally stimulated cyclic AMP in the dogfish rectal gland.

It is well documented that cAMP stimulates transepithelial sodium chloride transport in the dogfish rectal gland (Siegel *et al.* 1976, Riordan *et al.* 1994, Erlig and Rubio, 1986, Morran and Valentich, 1993, Valentich and Forrest, 1991, Forbush *et al.* 1992, Stoff *et al.* 1977). However the exact mechanism or mechanisms involved still require further investigation. Silva *et al.* (1977), reported that cAMP stimulates sodium chloride secretion in the isolated perfused rectal gland of *Squalus acanthias* and Shuttleworth and Thompson (1980), showed that the addition of cAMP and theophylline (a phosphodiesterase inhibitor) to rectal gland slices isolated from *Scyliorhinus canicula* caused a significant rise in ouabain-sensitive oxygen consumption, indicating an increase in Na,K-ATPase activity which is one of the transporters involved in sodium chloride secretion (Shuttleworth and Thompson 1980). Forskolin (which activates adenylate cyclase) has also been shown to increase sodium chloride secretion by 10 fold above basal values in the perfused rectal gland isolated from *Squalus acanthias* (Silva *et al.* 1985) and to stimulate transepithelial sodium chloride transport in cultured rectal gland epithelial cells from *Squalus acanthias* (Karnaky *et al.* 1991).

In *Squalus acanthias* VIP has been shown to stimulate chloride secretion from the rectal gland by as much as 40 fold via an increase in cAMP (Forest *et al.* 1983, Greger *et al* . 1984, Valentich, *et al.* 1991) However VIP has no effect on sodium chloride secretion in the perfused rectal gland isolated from *Scyliorhinus canicula*. Recently however the gut peptide scyliorhinin II has been shown to give dose-dependent stimulation of rectal gland secretion in the *Scyliorhinus canicula* (Anderson *et al.* 1995). In fish there is no reported evidence of tachykinins (eg scyliorhinin II) affecting second messenger systems, however, as tachykinins have been shown to stimulate cAMP concentrations in hamster ovary cells (Nakafima *et al.* 1992) this study investigated scyliorhinin II's effect on intracellular cAMP concentrations in primary epithelial cell cultures isolated from *Scyliorhinus canicula* . Scyliorhinin II (10 μM) failed to increase intracellular cAMP concentrations in the rectal gland primary cell cultures over the time period tested. However when scyliorhinin II was added to the persion buffer, an increase in cAMP concentration was found in the eluate. There are two possible

explanations for these results. The first being that scyliorhinin II may act on non-epithelial cells in the rectal gland (ie smooth muscle cells or endothelial cells), to increase cAMP levels but has no effect on the epithelial cells which are the predominant cell type present in culture. Secondly however, it is also possible that the failure to see any increase in intracellular cAMP levels in the primary cultures in response to scyliorhinin II, is due to a loss of scyliorhinin II receptors or the scyliorhinin II transduction system with cell culture. This latter possibility is unlikely as the cells retained the cAMP transduction system for both $PGE_1$ and $PGE_2$ receptors. In conclusion these results indicate that scyliorhinin II receptors coupled to the stimulation of adenylate cyclase are present in the rectal gland, possibly only on smooth muscle or endothelial cells but not on the epithelial cells directly associated with ion transport. If this is correct then it is possible that this peptide plays a role in the regulation of the extent of perfusion of the rectal gland under certain physiological conditions. Scyliorhinin II might therefore be the endogenous peptide responsible for causing vasodilatation and hence increased blood flow to the gland reported by Solomon *et al.* (1980) when the sodium chloride secretion is stimulated. If the stimulatory effect of scyliorhinin II on sodium chloride secretion is due to it's action on smooth muscle cells then there would be no increase in transepithelial sodium chloride transport when rectal gland cultures isolated from *Scyliorhinus canicula* are incubated in scyliorhinin II. This effect of scyliorhinin II has however not yet been investigated.

## 5.IV b Hormonally stimulated cyclic GMP in the dogfish rectal gland.

Although cyclic GMP has also been implicated in stimulation of sodium chloride transport in the dogfish rectal gland, the underlying signalling pathways involving ion transport and this second messenger are poorly understood (Karnaky *et al*, 1991). Monolayers of shark (*Squalus acanthias*) rectal gland cells exhibit high chloride transport rates when stimulated with mammalian ANP and this is accompanied by an increase in cGMP concentrations within the cells (Moran *et al.* 1993). However the major circulating natriuretic peptide in elasmobranchs is shark CNP (sCNP). This peptide was isolated the heart of both *Squalus acanthias* (Scholfield *et al.* 1991) and *Scyliorhinus canicula* (Suzuki *et al.* 1991), and as this peptide is known to exist in the dogfish its actions in the rectal gland are more relevant than that of mammalian ANP. Shark CNP has also been shown to stimulate sodium chloride secretion in the isolated perfused rectal gland of *Squalus acanthias* (Solomon *et al.* 1992) and *Scyliorhinus canicula* (Anderson, 1995) by up to 8-fold. However, an increase in sodium chloride

secretion was not observed following perfusion of the gland with the cell permeable analogue 8-bromo cGMP in either *Squalus acanthias* (Silva *et al.* 1987) or *Scyliorhinus canicula* (Anderson, 1995), or following the addition of 8-bromo cGMP to cell cultures of *Squalus acanthias* (Silva *et al.* 1993). These results suggest that sCNP stimulates sodium chloride secretion by a non-guanylate cyclase linked pathway. It was reported by Gunning *et al.* 1993 that two classes of natriuretic peptide receptors are expressed in shark rectal glands, the experiments in this study were conducted using rat ANP as a competing ligand. Gunning and coworkers reported that these receptors had different affinities for sCNP, the high affinity receptor was guanylate cyclase-linked and the low affinity receptor was non-guanylate cyclase linked. Little is known about the non-guanylate cyclase linked receptor.

The mechanism eliciting the effects of sCNP on sodium chloride secretion is therefore not well understood. Although the mechanism may involve increases in intracellular cGMP, this cyclic nucleotide alone is not sufficient to stimulate sodium chloride secretion in the isolated perfused gland. However as rat ANP increases sodium chloride secretion in rectal gland epithelial monolayers it seems likely that sCNP mayhave an action on rectal gland epithelial cell monolayers . This study investigated the effect that sCNP has on cGMP levels in the isolated perfused rectal gland and primary rectal gland epithelial cell monolayers from *Scyliorhinus canicula* . It was observed that sCNP increased cGMP levels in the perfusate of the perfused rectal gland but due to the low levels of endogenous cGMP and high inter-experiment variants it did not conclusively show whether sCNP had any effect on cGMP levels in the cultured epithelial cells from *Scyliorhinus canicula* . A characteristic physiological action of members of the atrial natriuretic peptide family of hormones is dilation of vascular smooth muscle, both in vivo and in vitro (Winquist and Hintze, 1990). It is therefore not surprising that sCNP was shown to produce a concentration-dependent reduction in tension of isolated vascular smooth muscle from*Squalus acanthias* ventral aorta. This suggests that one of sCNP effects in the dogfish could be to increase the blood flow to the rectal gland to enhance sodium chloride secretion. Gunning *et al.* (1993) showed that sCNP acts on the smooth muscle cells in the vasculature of *Scyliorhinus canicula* rectal gland via guanylate cyclase linked receptors to produce an increase in cGMP. He hypothesised that this action of sCNP works in conjunction with the non guanylate cyclase-linked action of sCNPs in the rectal gland epithelial cells (described above) to produce sodium chloride secretion (Gunning *et al.* 1993). If this hypothesis is true it would explain why sCNP has been shown to stimulate sodium chloride secretion in the isolated perfused rectal gland of *Squalus acanthias* (Solomon *et al.* 1992) and

*Scyliorhinus canicula* (Anderson, 1995) but why sodium chloride secretion was not observed following perfusion of the cell permeable analogue 8- bromo cGMP in the isolated perfused preparation of *Squalus acanthias* (Silva *et al.* 1987) or *Scyliorhinus canicula* (Anderson, 1995), or upon the addition of 8-bromo cGMP to cell cultures of *Squalus acanthias* (Silva *et al.* 1993). The results from this study are inconclusive. The random increases in intracellular cGMP concentrations in the cell cultures showed no reproducibility between experiments. The most likely explanation for these random results is that the increase in cGMP seen in response to sCNP was the result of variable contamiantation of epithelial monolayers with other cells eg smooth muscle or endothelial cells and was not due to increases in cGMP intracellular concentration in the epithelial cells. An alternative explanation is that the assay was not sensitive enough to detech changes in cGMP concentrations.

In conclusion many hormones may affect the rate of sodium chloride secretion by the dogfish rectal gland and these hormones may use either cAMP or cGMP as second messengers. This study did not detect any increase in intracellular cAMP concentrations in response to scyliorhinin II in rectal gland epithelial cell cultures, however it did demonstrate that the cell cultures were hormonally responsive as a 5 and 6-fold increase in intracellular cAMP concentration was seen in response to $PGE_1$ and $PGE_2$ respectively. The sCNP experiments proved inconclusive, as only occasional small increases in intracellular cGMP concentrations were found after incubation of rectal gland epithelial cells in sCNP.

# 6 General Discussion

Epithelial cells were isolated from the rectal gland of the European dogfish (*Scyliorhinus canicula* ) and maintained in primary cell culture for up to 21 days. These cultures were used to study the effects of changes in extracellular sodium concentration on the activity and expression of Na,K-ATPase, a key enzyme involved in epithelial sodium chloride transport. Dogfish rectal gland epithelial cells are a particularly good model for such a study since their primary function in vivo is the excretion of excess absorbed sodium chloride, therefore helping maintain plasma sodium and chloride homeostasis (Burger and Hess, 1960). The rectal gland has a particularly important role in sodium chloride homeostasis following acute feeding episodes, when there is a marked increase in sodium chloride concentration in the plasma. However, how the rectal gland increases its rate of sodium chloride secretion in response to this ionic challenge is uncertain and requires further investigation. One theory suggested that the stimuli for sodium chloride secretion by the rectal gland in response to sodium chloride loading was blood volume expansion (Burger, 1962) and the discovery by Stoff *et al* 1977, that sodium chloride secretion from the rectal gland is stimulated by cAMP laid the foundation for the study of hormonal regulation of sodium chloride transport in the shark rectal gland. The mechanisms responsible for stimulation of this process is still under investigation. Several hormones have been implicated as potential stimulants of sodium chloride secretion and VIP (in spiny dogfish), scyliorhinin II (in European dogfish), adenosine and sCNP (Stoff *et al.* 1977, Anderson, 1996, Silva *et al.* 1996) have been shown to increase sodium chloride secretion from the isolated perfused gland, however the mechanisms of action of these hormones have not yet been established. The present study investigated the effect of sCNP and scyliorhinin II on cAMP and cGMP levels in dogfish rectal gland primary cell cultures and the perfusate of the isolated perfused rectal gland of the European dogfish.

## 6.I Tissue Culture

Valentich (1991) successfully isolated cells from the rectal gland of the spiny dogfish (*Squalus acanthias* ) which, when cultured, formed a transporting epithelial monolayer exhibiting hormone-sensitive chloride secretion (Valentich, 1991; Valentich *et al* . 1991). In the present study the European dogfish (*Scyliorhinus canicula* ) was used, however Valentichs method resulted in very low yields of rectal gland tubules. As a

result of this a perfusion technique was developed. The cell yield using the perfusion technique was estimated to be 3-4 times greater compared to Valentich's method. As the perfusion method gave a larger cell yield and the viability of cells (as ascertained by ethidium bromide/acridine orange staining) did not appear compromised it was concluded that the collagenase perfusion technique was the optimal method of choice.

The ultrastucture and morphology of cultured shark rectal gland epithelial cells grown either on collagen coated plates or in suspension was investigated using light and transmission electron microscopy. When rectal gland cells were cultured in suspension the cells tended to adhere to each other and grow very rapidly forming large groups or clumps of cells with distinct intercellular vacuoles being visible. The cells grown as monolayers on collagen coated plates or membranes adopted a flattened morphology (approximately 0.1-1 μm thick), hence covering a vast surface area with respect to cell volume. Some of the cells on the periphery of the monolayer contained large vacuoles which make up as much as 90 % of the cell volume, therefore the cell cytoplasm only makes up approximately 10 % of the cell volume. The area of cytoplasm present in the cell is further reduced due to the large number of mitochondria present, leading to the cell cytoplasm contributing to less than 10% of the total cell volume. In both monolayers and cells grown in suspension the plasma membranes of adjacent cells were highly interdigitated and the plasma membranes between vacuoles exhibited extensive folding. The prominent cytoplasmic organelles in both cells grown on collagen and cells in suspension included large numbers of mitochondria which is consistent with transport epithelia (Valentich, 1991). Adjacent epithelial cells were joined by junctional complexes, both tight junctions and desmosomes made up the junctional complexes. The most significant difference in the morphology of cells grown in suspension compared to cell monolayers is the flattened morphology of the cells grown on collagen compared to the spherical morphology of the cells grown in suspension. The vacuoles observed at the periphery of the monolayer and in cells grown in suspension remained present in cells after treatment with trypsin, it was concluded from this observation that the vacuoles existed inside the cell and were not due to fluid filled compartments between the basolateral surface and the impermanent substrate as observed in MDCK cells due to transcellular ion transport (Taub, 1985). Cells grown in suspension also exhibited extracellular vacuoles in the centre of their tubular like structures, these vacuoles were thought to be fluid filled cavities due to ion transport and therefore are similar to those observed in MDCK cells.

Although the yield of cells isolated using the perfusion technique was greatly improved compared to that of Valentich's method therefore allowing a limit number of experiments to be conducted, an immortalised cell line would be advantageous in a number of respects. If successful immortalisation of the dogfish rectal gland epithelial cells was achieved, preparation of cell cultures would become much simpler and large scale experiments could be carried out. It is possible that spontaneous immortalisation may occur when primary cell cultures are maintained for prolonged periods . However although numerous dogfish epithelial cells isolated during the three years of study were cultured for up to 34 days all reached a crisis point, this resulted in death of all cells and no spontaneous immortalisation of any cell line was found. Establishment of an immortal epithelial cell line from the rectal gland primary cell cultures was also attempted using a number of experimental techniques including transformation of cells with the plasmid, pSVori. Cationic liposomes and poration of the cell membrane using Streptolysin O were both methods used in an attempt to achieve transfection of cells with the plasmid. However different attempts to immortalise rectal gland epithelial cells were unsuccessful. The primary cultures were therefore used to investigate some of the factors which may regulate transepithelial sodium chloride secretion in the intact rectal gland. Studies were limited to studying the effects of putative hormonal secretagogues in cAMP, cGMP second messenger systems and also how changes in the extracellular sodium chloride concentration affected the activity and expression of Na,K-ATPase, an essential component of the secretory pathway in these cells.

## 6.II The effect of sodium chloride loading on dogfish rectal gland primary cell cultures.

The shark rectal gland is a sodium chloride transporting epithelium, and has a particular role in sodium chloride homeostasis after the dogfish feeds when there is an increase in sodium chloride concentration in the plasma. However, the mechanism(s) by which the rectal gland increases its rate of sodium chloride secretion in response to this increased sodium chloride load is still uncertain.

The objective of this study was to investigate if changes in sodium chloride concentrations in the external environment (thus mimicking an increase in plasma sodium chloride concentration following feeding) affects the expression and activity of the Na,K-ATPase. Work from our laboratory has previously shown that 9 hours following a feeding event of 6% (w/w) sodium chloride, Na,K-ATPase activities in rectal gland homogenates transiently increase by 44-fold (MacKenzie, 1996). The

increase in Na,K-ATPase activity was not paralleled by a concomitant rise in the levels of mRNA for the $\alpha_1$ or $\beta_1$ Na,K-ATPase subunits. The mechanism responsible for this increase in Na,K-ATPase activity was not investigated, however it was shown that the increases in dietary sodium chloride caused a transient increase in plasma sodium chloride concentration, which suggests the possibility that the extracellular availability of one or either of these ions was responsible for the increase in enzyme activity. The object of this study was to investigate the possibility that increased extracellular sodium chloride concentration may have a direct influence on Na,K-ATPase activity in rectal gland epithelial cells.

Incubation of cultured rectal gland cells in the presence of increased concentrations of sodium chloride resulted in transient increases in cell homogenate Na,K-ATPase activity. Activities were increased by more than 3-fold some 12 hours after a 50 % increase in extracellular sodium chloride concentration. It was confirmed that the sodium chloride-stimulated increase in Na,K-ATPase activity was due to an increased concentration of both of these ions in the incubation medium and was not the result of any osmotic effect as addition of equivalent tonicities of choline chloride or sodium sulphate to the growth medium were without effect. Epithelial cell Na,K-ATPase activities show a similar delayed increase in response to monensin treatment, a sodium-proton ionophore which increases intracellular sodium concentrations indicating that increased intracellular sodium concentrations alone are able to induce the effect. The presence of chloride is however necessary to allow entry of sodium to the cell as the observed response of Na,K-ATPase to an increase in extracellular sodium chloride is linked to the bumetanide sensitive Na, K, Cl cotransporter. When the cells were treated with bumetanide the normal sodium chloride mediated increase in Na,K-ATPase activity was not seen. These results suggest that the sodium and chloride ions enter the cell down their concentration gradient via the cotransporter, increasing intracellular sodium concentrations resulting in increases in Na,K-ATPase activity. To confirm this hypothesis it would have been advantageous if intracellular sodium and chloride concentrations could have been measured, however due to the large amount of intracellular vacuoles present in the cells this was not possible using flame cytometry, and alternative methods such as electron probe analysis were not possible due to lack of equipment.

Treatment of the cells with bumetanide did not only inhibit the increase in Na,K-ATPase activity seen in response to increased extracellular sodium chloride concentrations but it also resulted in a rapid (within 30 minutes) and significant

(approximately 65 %) decrease in basal Na,K-ATPase activities. However, if the bumetanide was removed within 60 minutes, of initial application the Na,K-ATPase activities in cell homogenates returned to the original values within 3 hours. If bumetanide treatment however exceeded 4 hours the Na,K-ATPase activity did not return to the original values within the time period of the experiment. Pre-treatment of the cells with colchicine, an anti-microtubular agent, prevented the inhibitory effect of bumetanide on Na,K-ATPase activities. Colchicine was also able to inhibit Na,K-ATPase recovery following bumetanide removal. These results implicate the cytoskeleton in the regulation of Na,K-ATPase activity in response to changes in extracellular and presumably intracellular sodium concentrations. One possible explanation is that bumetanide treatment reduces the influx of sodium ions into the cell and therefore decreases the intracellular sodium concentration. This decrease in intracellular sodium by some unknown way induces the removal of Na,K-ATPase units from the plasma membrane to some intracellular store. Sequestered Na,K-ATPase units may then be reinserted back into the membrane when the intracellular sodium concentration is raised. It was therefore hypothesised that on bumetanide treatment the sodium pumps were removed from the membrane and stored in some unknown intracellular membrane compartment for a limited period of time. During this period they could be reinserted into the cell membrane if intracellular sodium concentrations increased. If however the requirement for Na,K-ATPase activity remained low for a long period of time (half life is 4 hours) the stored pumps were degraded or at least could not be immediately activated again. It is also unlikely that pre-formed stores of inactive pumps are normally present in shark rectal gland cells as an increase in intracellular sodium via an increase in extracellular sodium chloride or via monensin shows no increase in Na,K-ATPase activity within the first 30 - 60 minutes the time taken for recovery in the bumetanide experiments. Therefore these results would suggest that this intracellular pool is not present in normal rectal gland epithelial cell cultures. The sodium mediated increases in Na,K-ATPase activities were dependent on protein synthesis as the protein synthesis inhibitor cycloheximide totally suppressed the response. When cycloheximide was present in the cell culture medium for the first 12 hours of sodium chloride treatment, not only was sodium chloride-stimulated increase in Na,K-ATPase activity totally suppressed but activities were reduced to below basal levels. It was concluded from these results that protein synthesis was involved in the response of Na,K-ATPase to increased extracellular sodium chloride concentrations.

Although the sodium chloride-stimulated increase in epithelial cell Na,K-ATPase activity was dependant on de novo protein synthesis, inclusion of the transcription

inhibitor actinomycin D in the medium had no measurable effect, indicating that transcription of new mRNAs was not associated with the increase in Na,K-ATPase activity. Unfortunately this result could not be conclusively confirmed by Northern or dot blot analyses due to difficulties in isolating workable quantities of viable RNA from cell culture. Although RNA samples were badly degraded analysis of the dot blots obtained indicated there was no increase in Na,K-ATPase $\alpha$-subunit mRNA in response to a 9 hour incubation of cells in medium containing elevated sodium chloride concentrations. These results suggest that the up regulation of the Na,K-ATPase in response to elevated sodium chloride concentration occurred downstream of transcriptional regulation. This is in agreement with the earlier in vivo studies carried out by MacKenzie, (1996). The increase in Na,K-ATPase activity observed in response to increased extracellular sodium chloride concentration could be caused by translation of a protein which activates pre-formed sodium pumps already present in the cell membrane or present in intracellular stores which when activated move to the cell membrane or the increase in intracellular sodium could activate translation of new sodium pump proteins. If time had allowed the use of ribonuclease protection assay might have given more conclusive results and this technique might also have allowed us to measure levels of Na,K-ATPase $\beta$-subunit mRNA.

The effects of calcium on the sodium mediated increase in Na,K-ATPase activities were investigated as calcium is involved in regulation of the cytoskeleton and hence would affect any mechanism involving movement of sodium pumps to and from intracellular stores. This study demonstrated that calcium was necessary to elicit the sodium chloride-stimulated increase in Na,K-ATPase activity, however increases in intracellular calcium using the calcium ionophore A23187 did not significantly change basal Na,K-ATPase activities in cell homogenates over a 12 hour incubation period. These results suggest that the transient increase in Na,K-ATPase activity observed in response to an increase in extracellular sodium chloride concentration is dependant on the presence of calcium, but cannot be induced by raising intracellular calcium alone.

It was also observed that attachment of the cells to a substrate played an important part in the sodium chloride-stimulated increased in Na,K-ATPase activity as cells grown in suspension did not show similar increases in activity following increases in extracellular sodium chloride. The reason for this is unknown but may relate in some way to the differences in cell morphology and membrane polarity seen when cells were grown in suspension.

## 6.III Hormonal regulation of sodium chloride secretion by the dogfish rectal gland.

Following the studies involving Na,K-ATPase regulation in response to changes in extracellular sodium chloride, the actions of two elasmobranch peptides on cyclic nucleotide production was investigated in primary cultures of dogfish rectal gland cells. It is well documented that cAMP and cGMP stimulate transepithelial sodium chloride transport in the dogfish rectal gland (Siegel *et al.* 1976, Riordan *et al.* 1994, Erlig and Rubio, 1986, Moran and Valentich 1993, Valentich and Forrest, 1991, Moran *et al.* 1993; Stoff *et al.* 1977), however the endogenous hormones responsible for elevating these cyclic messengers are still unknown. This study investigated the effect of scyliorhinin II and sCNP on intracellular concentrations of cAMP and cGMP as scyliorhinin II and sCNP have been shown to stimulate sodium chloride secretion in the isolated perfused dogfish rectal gland (Anderson, 1995; Moran *et al.* 1993). The rectal gland primary cell cultures were shown be hormonally active as they responded with an intracellular increase in cAMP concentration to forskolin, $PGE_1$ and $PGE_2$ .

The present study found no increase in intracellular cAMP concentration after incubating the rectal gland cell cultures in scyliorhinin II. However when scyliorhinin II was added to buffers and perfused through the isolated rectal gland an increase in cAMP concentration was found in the perfusate. An explanation for this is that scyliorhinin II acts on the vascular smooth muscle or endothelial cells of the rectal gland to increase cAMP levels but had no effect on the rectal gland epithelial cells.

Shark CNP increased cGMP concentrations in the perfusate of the isolated perfused rectal gland by up to four fold after seven minutes, however there was no significant effect on cGMP concentrations in the cultured cell monolayer. However occasionally during this study sCNP induced variable increases in cGMP concentrations in cell culture extracts were reported. These increases in intracellular cGMP concentrations in the cell cultures were random and showed no reproducibility between experiments. The reason for this discrepancy between the perfused rectal gland and the isolated cell systems is not known however it is possible that sCNP is stimulating cGMP production in other cell types in the whole gland (e.g. vascular smooth muscle cells or endothelial cells) which are present in variable amounts in the cultured cell monolayer as a result of inconsistencies in the isolation procedures. It seems entirely possible,

given the known actions of CNP in other species that a vascular action of this peptide is responsible for the observed increases in cGMP concentrations.

In conclusion this study demonstrated that high salt levels in the medium of shark rectal gland cell monolayers increase Na,K-ATPase activity and that this response is dependent on protein synthesis but not transcription. It also showed that the response is inhibited by the loop diuretic bumetanide, indicating that entry of the ions into the cell is via the Na,K,Cl cotransporter and that the increase in Na,K-ATPase activity is presumably due to an increase in intracellular sodium concentration. It also demonstrated that scyliorhinin II and sCNP increased concentrations of cAMP and cGMP respectively in the perfusate of the isolated perfused rectal gland but not in the epithelial cell monolayer. In conclusion although sodium chloride transport in the dogfish rectal gland requires much more investigation, this study has hopefully proved that dogfish epithelial cell cultures provide a good model for further investigations involving the regulation of activity and expression of the sodium pump. The information obtained form experiments using this model combined with information from classical methods such as in vivo studies and isolated perfused gland experiments will in the future hopefully solve the mystery of shark rectal gland regulation.

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