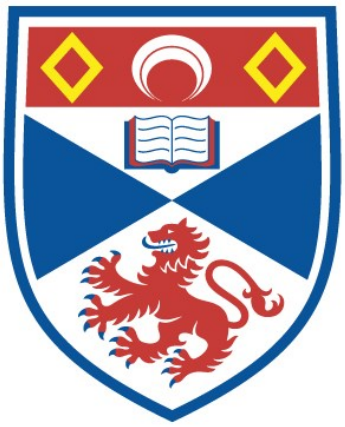
EXCITATION-CONTRACTION COUPLING IN THE RAT ANOCOCCYGEUS MUSCLE

David Albert Saint

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



1982

EXCITATION - CONTRACTION COUPLING IN THE RAT ANOCOCCYGEUS MUSCLE

D.A. SAINT

A thesis submitted for the degree of Doctor of Philosophy to the University of St Andrews, May 1982.

I, David A. Saint, hereby certify that this thesis has been written by me, that it is the record of work carried out by me, and that it has not been submitted in any previous application for a higher degree.

28/6/82
Date

Candidate

I was admitted as a research student under Ordinance No. 12 on 1/10/78 and a candidate for the degree of Ph.D. on _____; the higher study for which this is a record was carried out in the University of St. Andrews between 1978 and 1981.

28/6/82
Date

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I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the degree of Ph.D. of the University of St. Andrews and that he is qualified to submit the thesis in application for that degree.

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Acknowledgements;

I would like to thank Dr. C.J.M Nicol, my supervisor, for his guidance, Dr. F.W Flitney for his helpful dissussion and suggestions, Mr. M. Coutts for his invaluable photographic help, and last but not least, the M.R.C. for their financial help during the course of this work.

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SUMMARY

Smooth muscles as a group exibit great diversity of pharmacological and physiological properties. This makes it impossible to produce any but extremely generalised schemes for smooth muscle contractile mechanisms. However, knowledge of the detailed physiology and pharmacology of specific types of smooth muscle has been growing at an increasing rate, especially regarding vascular and visceral muscles. The rat anococcygeus muscle has, however, been investigated little. This work describes the excitation- contraction coupling mechanism in this preparation.

The rat anococcygeus muscle was found to contract to all three of the agonists used (noradrenaline, acetylcholine and potassium chloride). In the first section of this work the properties of these contractions were investigated. It was found that the contractions induced by each agonist exibited different pharmacological properties, (with regard to low calcium, sodium nitroprusside, verapamil, Stellazine and theophylline). This can be taken as an indication that the different agonists use different activation pathways.

Electrophysiological studies showed that the membrane potential per se is not important in the regulation of contraction. (ie. the depolarisation produced by an agonist is not simply related to the tension produced). KCl produces the greatest change in membrane potential (from -55mV to -20mV for a maximal dose), but produces the least rise in tension of the three agonists.

Evidence from other preparations and the results of the experiments with Stellazine suggest that the rise in tension produced by the agonists is not simply related to the rise in intracellular calcium concentration, but that some amplification of the response ocurrs.

The way in which the agonists produce this amplification of the response is suggested as being related to changes in the levels of the cyclic nucleotides, cAMP and cGMP within the cells. It was found that the agonists did not substantially affect cAMP levels, but that all three reduced cGMP levels by varying amounts. The ratio of the levels of cAMP/cGMP produced by activation with each agonist correllates very well with the tension produced. This suggests that the ratio cAMP/cGMP is important in the regulation of contractility in this muscle.

However, doubt is cast upon this theory by the results of experiments using sodium nitroprusside (NP). It was found that NP (2×10^{-7}M) caused a pronounced change in the ratio cAMP/cGMP (by increasing cGMP levels), but only a small change in tension (so that the tension in these experiments does not correllate well with the ratio cAMP/cGMP).

In order to retain the hypothesis that cyclic nucleotides are important in the regulation of contractility in this preparation, it is proposed that some form of compartmentalisation of the cyclic nucleotide changes occurs within the cells.

INTRODUCTION

There is at pesent a great disparity between the knowledge of the mechanisms of contraction of striated muscle and smooth muscle. The excitation- contraction coupling process in striated muscles has been intensively investigated and now it is thought that the fundamental processes are largely understood. However, the fundamental processes which regulate smooth muscle contraction are still only poorly understood at present. This is due in no small part to the great diversity of physiological and pharmacological properties found amongst smooth muscles as a group.

The sliding filament theory proposed by Huxley (1971) as a model of striated muscle contraction was aided by the well defined structure of striated muscles Such a model was not feasible in smooth muscle for many years, due to the lack of this ordered structure. With the demonstration of actin (Hanson and Lowry 1963) and, much later, myosin filaments in smooth muscles (Kelly and Rice 1968) such a model was proposed for smooth muscle also (Small and Squire, 1972). However, although this clarified the basic contractile mechanism, the processes involved in the control of contraction were still poorly understood. The major factor in the control of the contractile proteins was apparently the intracellular concentration of calcium, in the same way as for striated muscles. (Bianchi 1969, Hurwitz and Joiner 1969) , but how this in turn was regulated during excitation and relaxation was unclear. Whereas in striated muscle the source of activator calcium is the sarcoplasmic reticulum, no (or very little) of this is present in smooth muscles. It became clear that, in many

muscles, an influx of extracellular calcium provided much of the calcium necessary for activation. However, this cannot account for the observed properties of many smooth muscles, so intracellular release of calcium must play an important part in many situations (Freeman and Daniel 1973, Greenberg, Long and Dieke 1973). This intracellular release of calcium may be linked to the small rise in intracellular calcium produced by the influx of a small amount of extracellular calcium by a process of calcium induced calcium release, in a similar way as that described in skeletal muscle. Such a process has been described in smooth muscle by Cheng (1976). It was found that many of the pharmacological properties of different types of smooth muscles could be explained in terms of the different relative importance of these two sources of activator calcium (Deth and Van Breemen 1974). In addition, the dependency of any particular smooth muscle on extracellular calcium seems to correllate well with a lack of developement of structures analogous to the sarcoplasmic reticulum (Devine, Somlyo and Somlyo 1972).

Relaxation of smooth muscles is achieved by lowering of the cytoplasmic calcium concentration. This is produced by either calcium extrusion from the cell, across the cell membrane, or by calcium sequestration by intracellular stores. (Casteels, Goffin, Raeymakers and Wuytack 1973). Those muscles which utilise largely extracellular calcium appear to mainly pump calcium out of the cell, while those dependent upon intracellular calcium have calcium pumps located primarily on intracellular structures (Hurwitz et al 1973).

While it became clear that calcium and calcium handling processes were important in the control of smooth muscle contraction, the means by which these in turn were controlled was still unclear. In those smooth muscles which generate action potentials (APs), it was found that one of the main current carriers involved in the AP is calcium (Kuriyama 1970, Tomita 1970, Job 1969). This was proposed as being the major source of activator calcium in these muscles. It should be remembered, however, that many smooth muscles show a slow cyclical variation in the resting membrane potential, called the slow wave (wave duration .5 to 5 seconds), upon which the action potentials are superimposed. The slow wave is sodium dependent and may reflect cyclical variations in the rate of electrogenic sodium/calcium exchange (Huddart and Hunt 1975). In those muscles which do not generate APs, a process of graded membrane depolarisation could still be responsible for initiating an influx of calcium which acts in the same manner (Droogmans, Raemakers and Casteels 1977). However, it is still possible in some circumstances for activation of the muscle to occur without apparent depolarisation of the membrane (Su, Bevan and Ursillo 1964, Casteels, Kityamura, Kuriyama and Suzuki 1977). This process was called pharmacomechanical coupling by Somlyo and Somlyo (1968). An example of this type of direct activation was shown in frog skeletal muscle by Saki, Geffner and Sandow (1970). They showed that muscles with the T tubule system disrupted by osmotic shock could still be activated by caffeine, probably by a direct action on the intracellular calcium stores. In addition to this, the effects of many excitatory and relaxant drugs are difficult to

explain on this basic model of activation (eg. phosphodiesterase inhibitors etc.)

These difficulties led to the concept that smooth muscle contraction could be controlled, at least in part, by some "second messenger" within the cell. The first substance to be proposed for this role was cAMP, which was first suggested as being involved in muscle relaxation (Bar 1974, Anderson 1972). It was proposed that cAMP could act to stimulate the calcium removal processes responsible for lowering the cytoplasmic calcium concentration and so cause relaxation. This hypothesis could account for the action of relaxant drugs such as phosphodiesterase inhibitors, which raise cAMP levels (see Bar 1974). Only a few inconsistent results have been quoted, for example caffeine has been shown to induce relaxation in mammalian smooth muscles, but this is preceded by a contraction in guinea pig taenia coli (Ito and Kuriyama 1971).

This concept was soon extended to include the involvement of second messenger substances in the control of contraction also, although cAMP seems to be unimportant in this respect, it appeared that another cyclic nucleotide, cGMP, was involved in the control of contraction. cGMP changes induced by agonists and many drugs were found to occur in many instances, usually a rise associated with contraction. Thus it appeared that smooth muscle contraction could be controlled by the changes in either cAMP or cGMP. A rise in cAMP would be associated with relaxation and a rise in cGMP with contraction. This idea seemed to fit well with the proposed actions of these cyclic nucleotides in many situations, the so

called Ying/Yang hypothesis proposed' by Goldberg et al. (Goldberg, Haddox, Nicol, Glass, Sanford, Keuhl and Estensen 1975).

It soon became apparent that this idea was an over simplification of the situation, however. In many cases it could be shown that the changes in cAMP and/or cGMP could be dissociated from the changes in contractility (Diamond and Janis 1978,1980, Diamond and Hartle 1974). For example, in many muscles contraction is associated with a rise in cGMP levels. However, many relaxant drugs were shown to cause a rise in cGMP levels also, eg. sodium nitroprusside, (NP). A further complication arose when it was shown that, although the changes in cGMP induced by agonists are dependent upon extracellular calcium, the changes induced by NP are not (Schultz, Hardman, Hurwitz and Sutherland 1973, Schultz, Schultz and Schultz 1977).

In order to circumvent these difficulties a modification of this theory has been proposed. In this it is assumed that the important regulatory changes in cyclic nucleotides take place in some small compartment within the cell. These changes are thought to be impossible to detect against the background of the gross changes occurring in the cell as a whole. Such a compartmentalisation has been proposed by various workers.

It has been shown that cyclic nucleodides are important in the regulation of calcium handling mechanisms (Nilsson and Andersson 1977). A role proposed for cyclic nucleotides on this basis is

that cGMP, at least, could act as a negative feedback signal, to regulate cytoplasmic calcium concentration (Schultz, Hardman, Schultz, Baird and Sutherland 1973).

Cyclic nucleotides have also been implicated in the control of many metabolic processes in smooth muscle. In particular, it has been demonstrated that there is a cAMP dependent protein kinase which can phosphorylate Myosin Light Chain Kinase (MLCK), thus rendering it inactive (Adelstein, Conti, Hathaway and Klee 1978). This data supports the view that cAMP is important in the relaxation of at least some smooth muscles. Another possibility is that cyclic nucleotide changes are responsible for ensuring that the appropriate metabolic changes are stimulated concurrently with contraction.

Recently much work has been done on the biophysics and biochemistry of smooth muscle contraction, which has led to a more complete understanding of this process. The contractile proteins are unlike those of striated muscle, in that there is no troponin C like component in smooth muscle (Driska and Hartshorne 1975). Instead, contraction is initiated by phosphorylation of Myosin light chains, by MLCK. MLCK activity is regulated in turn by a calcium dependent modulator protein, called calmodulin. It is this calcium dependency which conferes calcium sensitivity on the contractile mechamism as a whole. Many drugs have been found to act at this stage in the activation process, eg. triflouroperazine (Stelazine) is thought to prevent the activation of MLCK by calmodulin (Cassidy, Hoar and Kerrick 1980).

The aim of this work was to investigate some aspects of the excitation- contraction coupling process in the rat anococcygeus muscle.

The rat anococcygeus muscle was introduced as a preparation for the study of smooth muscle physiology and pharmacology by Gillespie, (Gillespie 1972). It has several properties which act in its favour in studies of this kind. The muscles contain little connective tissue and are in the form of thin (300um), flat sheets, with an in situ length of approx 3cm. and width of approx 5mm. This mimimises the geometrical problems associated with the use of smooth muscles from the walls of hollow viscera. In addition, the diffusion pathway for drugs and oxygen, etc. is minimised. The muscles are easily isolated.

Although it is associated with the alimentary tract, the pharmacology of the rat anococcygeus differs from that of visceral smooth muscle, in that it will contract in response to noradrenaline, acetyl choline, 5 HT and KCl depolarisation. The muscle has a dense adrenergic innervation, demonstrated by Falck/Hillarp staining, (Gillespie and Lullman- Rauch 1974), which, upon stimulation of the extrinsic nerves, causes a contraction. In addition, there is a nerve supply which will produce relaxation when the muscle tone has previously been raised. The transmitter responsible for this has not been identified.

Electrophysiological studies have shown that the rat anococcygeus does not produce spontaneous activity and does not

normally generate action potentials (APs) (Creed 1975). Stimulation of the extrinsic, excitatory nerves, or the addition of stimulating agents produces a graded depolarisation of the membrane (Creed, Gillespie and Muir 1975). The muscle is classified as multi unit in the classification proposed by Bozler. (Bozler 1948).

The excitation contraction coupling mechanism was investigated in this study from three main standpionts.

a) The mechanical responses of the tissue to three agonists, NA, ACh and KCl, under different conditions and the effects of some antagonists on these.

b) The changes in cyclic nucleotide levels in the muscles associated with contraction induced by each of the three agonists and the influence of various proceedures on these.

c) The electrophysiological response of the muscle to various agents, measured with intracellular microelectrodes.

The data from all three aspects of this study were gathered together in the hope of obtaining a more complete picture of the mechanism of excitation contraction coupling in the rat anococcygeus, and how this might relate to the results of similar studies on different muscles.

METHODS

1.0 MECHANICAL STUDIES

1.1 General

Throughout this study male Wistar rats of weight 350-400g were used. The anococcygeus muscles were dissected out using a method described by Gillespie (1972). The animal was stunned and killed by exsanguination. The abdomen was opened in the midline and the pelvis split. The bladder was moved to one side to allow access to the colon and anococcygeus muscles. The two anococcygeus muscles arise from the upper coccygeal vertebrae close to one another, dorsal to the colon. They then pass caudally, lying first behind, then alongside, the colon. The muscles finally join to form a ventral bar in front of the colon, a few mm. from the anus. The colon was raised and the anococcygeus muscles located in the connective tissue on each side. Each muscle was then freed from this and a loop of thead passed round it near to the ventral bar. This was used to secure a silver chain to the muscle. The ventral bar was then cut, so freeing the ventral end of the muscle. A further loop of thread was used to secure a silver wire loop to the muscle, as near to the coccygeal origin as possible. The muscle was then cut just dorsal to this and lifted free. Both muscles were similarly prepared and suspended vertically in a single organ bath between hooks formed on the side of the organ bath and the tension transducers (single bridge isometric tranducers, 0-100g,

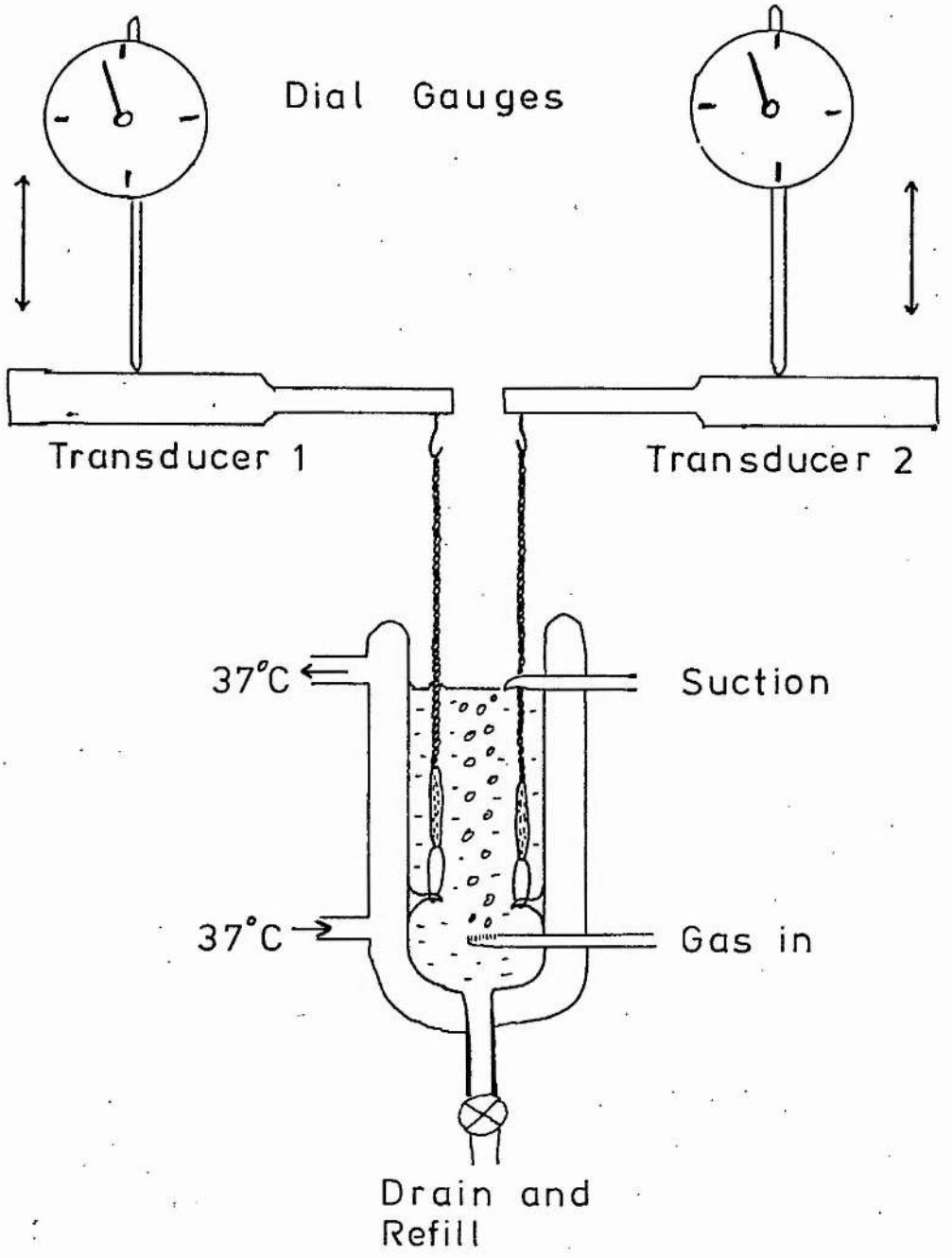

Dial Gauges
Transducer 1
Transducer 2
37°C
Suction
37°C
Gas in
Drain and
Refill

Fig M1 Apparatus.

Diagramatic representation of the apparatus for mechanical studies. The muscles are suspended in pairs between two fixed hooks on the sides of the bath and two isometric tension transducers. These are mounted on a rack and pinion arrangement (not shown) which allows them to be moved vertically, in order to maintain a constant resting tension on the muscles. The increments of length are monitored by the dial guages.

supplied by Devices) (see fig. M1). The bathing medium was Krebs solution, gassed with 95% O_2/ 5% CO_2. The composition of the Krebs solution varied according to the experimental protocol followed. The composition of the various solutions used are shown in table (M1). In all experiments using Ach or KCl as the agonist, phentolamine (10^{-6}M) was routinely added to the Krebs solution. This is necessary to prevent the effects of any noradrenaline which may be released from nerve terminals by either of these agents. Isometric tension changes were recorded by the transducers and displayed on a pen chart recorder. The system was calibrated by hanging weights from the transducer in the range 1-10g. In all experiments the muscles were maintained at a resting tension of 1g. Generally the muscles exibited stress relaxation throughout the course of the experiment, so that the length had to be increased to maintain this resting tension. Typically this change in length was of the order of 1.5 to 2 mm. in a muscle of overall length 10 mm. (ie. about 15 to 20 %). To facilitate this, each transducer was mounted in a vertical rack and pinion arrangement. The incremental changes in length were monitored by dial gauges mounted as in fig. M1. The length of each muscle was measured to +/- 0.1mm. using a vernier microscope, sighting through the side of the organ bath. At the conclusion of the experiment the muscles were weighed. The silver chain and loop were removed by cutting the muscle as near to the knot as possible with a new scalpel blade. They were then dried of surplus surface moisture by dabbing gently onto filter paper and weighed to +/- 0.01mg. This process was repeated three times and the weight taken as the mean of these.

Assuming a density of $1.0gcm^{-3}$ for the muscle, (values

Table M1 Krebs solutions.

Composition of the modified Krebs solutions used.

		Normal Krebs	Ca^{++} free	Depolarising (high K^+)
	Mol. wt.	m.mol.	m.mol.	m.mol.
$NaHCO_3$	84·01	25·0	25·0	25·0
NaCl	58·44	118·4	118·4	43·1
KCl	74·55	4·7	4·7	80·0
$NaHPO_4$	156·01	1·15	1·15	1·15
$MgCl_2$	203·31	1·13	2·26	1·13
$CaCl_2$	147·02	2·56	—	2·56
Glucose	180·16	11·1	11·1	11·1

quoted by Carlson and Wilkie (1974) are 1.05 to 1.06 for typical muscle), (Carlson and Wilkie, 1974), the volume of each muscle can then be calculated from the weight. Since the muscle is fairly uniform in thickness and width, the cross sectional area is obtained, to a good approximation, by division of the volume by the length.

1.2 Log dose response curves of Agonists.

Paired muscles were dissected out and set up as above. When a stable resting tension of 1g. had been obtained the agonist (either noradrenaline, (NA.), acetyl choline, (Ach), or potassium chloride, (KCl) was added to the bath, starting at the lowest dose. After each dose the bath was washed three times (over a period of 10 minutes). The drugs were added in 0.9% saline, the added volume never exceeding 1ml. (in order to minimise changes in the ionic composition of the Krebs solution.) The bath volume was 20ml. Log dose response curves were constructed using the data from several muscles. The -log ED50 was calculated for each curve using probit analysis.

The effect of adding 1ml. of 0.9% saline to the bath was checked and found to produce no significant response. The addition of hyperosmolar solutions to the bath can affect the response of the muscle. This phenomenon has been investigated by McKnight. (personal communication). He found that the addition of hyperosmolar solutions of either sucrose or NaCl produced a slight rise in tone in resting muscles. The threshold for this effect was 25mOsmol, with a maximum of 1.5g rise in tone produced by 400mOsmol

solutions. The only hyperosmolar solution used in this study was KCl solution, usually 80mM. This solution would therefore be expected to produce a rise in tone of around 0.5g (equal to about 6 to 7 Nm^{-2} for typical muscles). The addition of 80mM KCl produces an active tension increase of about 80 Nm^{-2}, so the error introduced by the effect of the hyperosmolarity of the solution is approximatly 7-8 %.

1.3 Antagonist dose response curves

The muscles were set up and stabilised in normal Krebs as before. The maximal dose of agonist was repeated until a reproducible response was obtained. An increasing dose of antagonist was then added 10 minutes prior to each maximal dose of agonist. This process was repeated for each of the three agonists in combination with four antagonists, (Nitroprusside, Verapamil, Stelazine, and Theophylline). The results of several experiments were combined to produce a series of antagonist dose response curves. The results of these experiments were used to determine the concentrations of antagonists used in later experiments.

1.4 Separation of the phases of contraction

It was noticed that contractions produced by NA and Ach, which are normally a single contraction, become bi-phasic at low calcium concentrations. In contrast, the contraction produced by the addition of KCl to muscles in normal Krebs is always bi-phasic.

Calcium 45 studies with both single unit and multi unit muscles have shown that the two phases of this type of contraction in other muscles may utilise different sources of activator calcium (Saad and Huddart 1981). Possible differences in the pharmacology of the two phases of these bi-phasic contractions were investigated therefore, in order to determine if they are produced by different mechanisms in the rat anococcygeus.

The muscles were set up and stabilised in calcium free Krebs as for the calcium dose response curves. The Krebs solution was then changed for one containing low calcium (0.12 mM) This concentration was chosen on the basis of the calcium dose response curves as the highest in which Ach and NA produced distinctly bi-phasic contractions. The maximal dose of the agonist was repeated until the response was stable in this low calcium concentration. The effect of increasing concentrations of antagonists on this contraction was then investigated (An "antagonist dose response curve"). An increasing concentration of the antagonist was added 10 min prior to each maximal dose of agonist. Curves so produced were plotted seperately for the primary and secondary components of the contraction.

As before, in experiments using Ach or KCl, Phentolamine (10^{-6}M) was added to the Krebs solution.

1.5 Calcium Dose response curves and Calcium washout curves.

A dose of each agonist was selected , on the basis of the Log dose response curves for the agonists, which gave a "just maximal" response. These doses were;

NA 2.5×10^{-5} M

Ach 2×10^{-3} M

KCl 80 mM.

This dose of agonist was added repeatedly until a reproducible response was obtained, (typically three additions), with the muscles in normal Krebs solution. This was then replaced with nominally calcium free Krebs, produced by omitting calcium from the solution. EDTA. or other chelating agents were not used. The dose of agonist was then repeated at intervals of 2 min, 10 min, 18 min and 26 min after the change, with two washes between each. The results obtained were used to produce calcium washout curves for each agonist.

When the response of the muscles was reduced to the minimum possible by the calcium free Krebs, (shown by repeated doses of agonist), the calcium dose response curve was started. Calcium chloride was added in solution using micropipettes to give a bath concentration in the range 15 uM to 6.4 mM. The calcium was added 10 minutes prior to the addition of the agonist.

Where the contractions were distinctly bi-phasic (eg. the contraction produced by NA in low calcium concentrations), the two phases were measured seperately and curves produced for both primary and secondary phases.

These curves were also produced against a background concentration of verapamil or nitroprusside in order to assess the effects of these agonists. The antagonists were added to the Krebs solution before the experiment to give concentrations of 2×10^{-7}M for nitroprusside and 5×10^{-7}M for verapamil. These concentrations were chosen on the basis of the antagonist dose

response curves (see section 1.3).

1.6 Drugs added during the Plateau Phase of contraction.

Some pilot experiments were performed in order to asses the effects of antagonists added during the plateau phase of the contraction produced by each agonist. The muscles were set up and stabilised in normal Krebs as above. The maximal dose of the agonist was then added and the contraction allowed to continue for an extended period (30 min.). This was to assess whether the contraction was well maintained. The bath was then washed out three times and the dose repeated. During the plateau phase of this contraction, (after about 15 min), the antagonist under investigation was added to the bath. Antagonists used were Nitroprusside (NP) and Verapamil (V).

1.7 Addition of Agonists

Some experiments were done in which two or more agonists were added to the bath concecutively. In this case the response to the maximal dose of the first agonist was stabilised as before. The dose was then repeated and at the desired time during the contraction (usually at the peak of the plateau phase) the maximal dose of the second agonist was added .

2.0 CYCLIC NUCLEOTIDE STUDIES

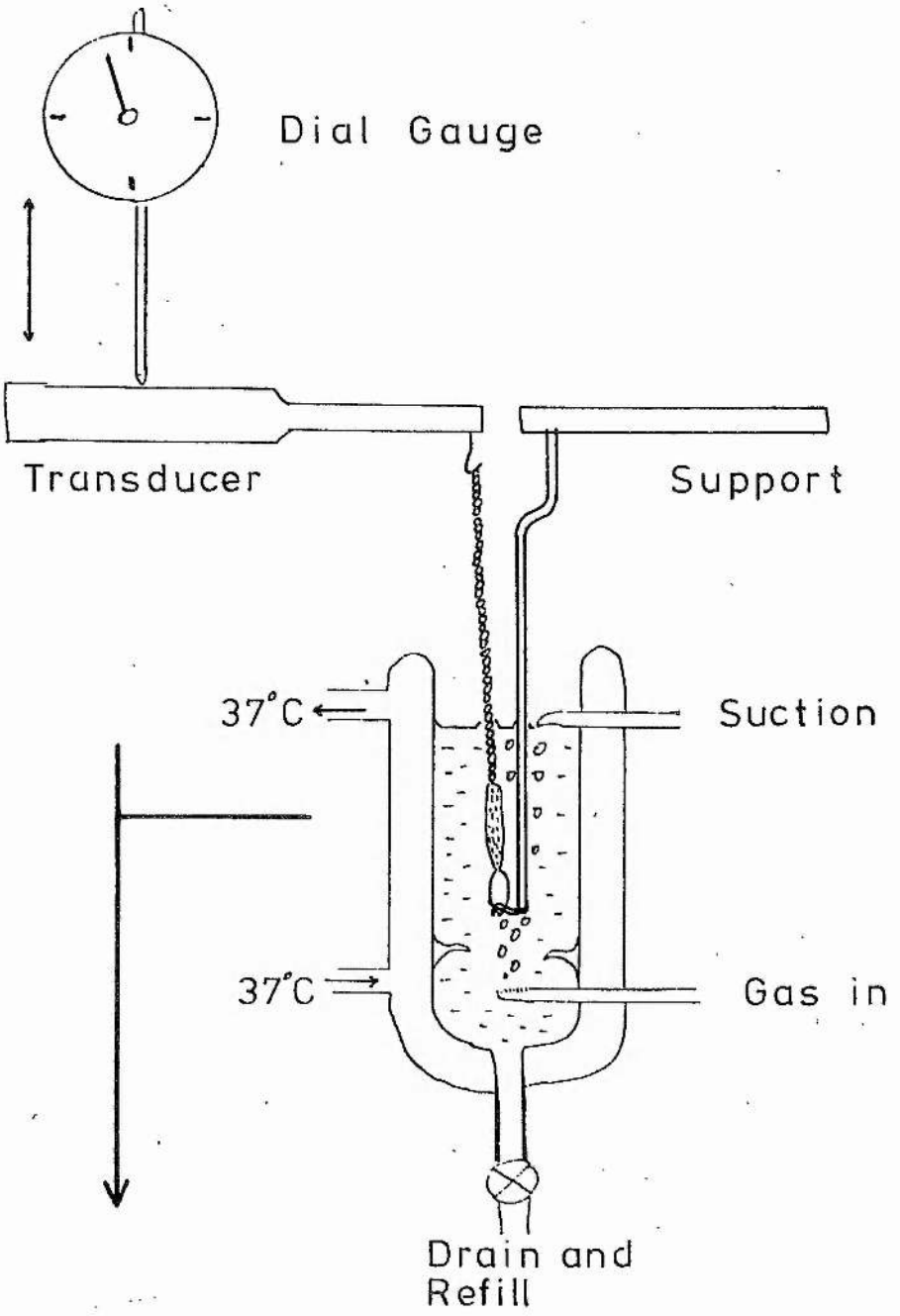
2.1 General

The changes in cyclic AMP (cAMP) and cyclic GMP (cGMP) during contractions produced by each of the three agonists, (NA), (Ach) and (KCl), were investigated. The apparatus was modified as in Fig. M2. Muscles were set up singly and the maximal dose of the appropriate agonist added to the bath. The progress of the contraction was monitored as before on the pen chart recorder. When the maximum tension had been reached, the whole bath was lowered to expose the muscle. This was frozen as quickly as possible by freeze clamping with forceps cooled in liquid nitrogen. The silver chain and silver wire loop were detatched and the muscles stored in liquid nitrogen. In order to obtain enough tissue for the cyclic nucleotide assays, it was necessary to pool 6 to 8 muscles for each determination. Control levels of cyclic nucleotides were determined by setting up the muscles at a resting tension of 1g. for 15 minutes before freezing, (approximately the length of time taken for the stimulated muscles to reach maximum tension).

The time taken to actually freeze the muscles was usually about 30 seconds. This was the time taken to lower the bath from the muscle, freeze clamp the muscle with pre-cooled forceps, and then detatch it from the tranrsducer and the support. The muscle has then to be freed from the silver chain and loop and placed in small plastic vials for storage in liquid nitrogen. The main

Fig M2 Modified apparatus for Cyclic Nucleotide studies.

The apparatus is arranged as in Fig M1, except that only one muscle is used. The fixed hook on the side of the organ bath is replaced by one which is supported outside the bath, so that the bath as a whole can be lowered away from the muscle, which can then be freeze clamped.


Dial Gauge
Transducer
Support
37°C
Suction
37°C
Gas in
Drain and
Refill

criticism of the procedure is that the time taken to actually lower the bath and freeze the muscle is too long and may be quite variable. However, it was felt that the time-constraints on this stage of the experiment were not too severe. The muscle contracts only very slowly, and maximum tension is maintained for a considerable length of time. If a time-course study were attempted, in which the changes in cyclic nucleotide levels on a minute by minute basis were important, the method would probably not be satisfactory as it stands. However, the present study attempted to measure the levels of cyclic nucleotides at the time of maximum tension production, which is generally 15 to 20 minutes after stimulation, and is maintained for an extended period of time. Under these conditions the above procedure was felt to be adequate.

2.2 Extraction

Each group of muscles was crushed in a brass motar and pestle cooled in liquid nitrogen. The crushed tissue was then extracted with about 2ml. of acidified ethanol (100ml. ethanol + 0.1ml. 10M. HCl) for 5 minutes at 0°C. During this time the extract was vortex mixed three times. The extract was then centrifuged (2,000rpm. for 5 min.) and the supernatant decanted off. The pellet was then re-extracted in the same way. The supernatants were combined, warmed to 45°C in a water bath and the ethanol blown off in a stream of nitrogen. The dry extract was then re-dissolved in 0.5 ml. of Tris/EDTA buffer (supplied with the kit -see later) This solution was then assayed for cyclic nucleotides.

The pellet produced was assayed for protein content using the Biuret method (see later -- section 2.5).

In each extraction performed a recovery check was included. In this a mixture of 10 mg. Albumin and 10 pmol of cAMP was extracted in the same way as the muscles.

The results of these recovery checks were usually 51 to 56% protein recovery and 75 to 80% cyclic nucleotide recovery. If in any assay the results of the recovery checks were not consistent with those usually obtained, the results of the assay were discarded. No correction for variations in the results of the recovery checks was attempted.

The extraction stage produced unsatisfactory results initially. At first the extraction medium used was 100ml ethanol + 1.0ml of 1M HCl. This was found to produce very low protein recoveries (around 30% for albumin) and that the results for the cAMP assays in these experiments was very variable, although the cGMP assays were fairly reproducible. The extraction medium was changed to 100ml ethanol + 0.1ml of 10M HCl. It was found that this gave much higher protein recoveries (around 55%), and the cAMP results became much more reproducible. It is suggested that the more aqueous extraction medium was producing greater "carry over" of proteins into the cyclic nucleotide fraction. Since the cAMP assay is a protein binding assay, it seems reasonable to suggest that this extra protein may, in some way, have interfered with the binding of cAMP in the assay. cGMP would not be affected so much, since it relies on the much more selective binding to a specific antibody. In connection with this problem Theobold, Syson

and Burrin (1978) used trypsin to remove contaminant peptide factors from the assay. However, they used a different extraction proceedure (boiling KH_2PO_4 solution). In view of the reproducible results obtained in this study using ethanol extraction, this was felt to be uneccessary.

2.3 Cyclic Nucleotide Assay

Cyclic nucleotides were assayed using kits supplied by Amersham Ltd. cGMP was assayed by Radio-immune assay. This assay is based on the competition between unlabelled cGMP and a fixed quantity of tritium labelled cGMP for binding to an antiserum which has a high specificity and affinity for cGMP. The amount of labelled cGMP bound to the antiserum is inversely related to the amount of cGMP present in the assay sample. The assay is carried out by incubating a fixed amount of the solution to be assayed (100ul) with a known amount of tritium labelled cGMP and a fixed amount of the specific antibody (Incubation is for 2 hours at $0^{o}C$).

Zero dose incubations were carried out by substituting 100ul of Tris-EDTA buffer for the assay sample. Blank reagent is supplied with the kit. This has a very high affinity for the specific binding sites of the antibodies and displaces cGMP completely from them. This can therefore be used to produce blank (ie. no binding of cGMP) incubations. Standard solutions were used to produce a calibration curve in the range 0.5 to 8 pmol. After incubation the protein was separated out by precipitation by

the addition of ammonium sulphate solution (60% saturated), followed by centrifugation in a refrigerated centrifuge. (MSE coolspin) The supernatant was then decanted off and the protein re-dissolved in 1.1 ml of water. 1 ml. samples of this were taken for liquid scintillation counting. The counts for the blank, zero dose and standard solutions were used to produce a calibration curve from which the amount of cGMP in the assay sample can be read. All the incubations were performed in duplicate.

Specificity; in view of the specificity quoted by Amersham as shown below, cross reactivity with other nucleotides was not considered to be a problem in this study.

Nucleotide	cGMP	cAMP	AMP,ADP,ATP	GMP,GDP,GTP
pmol per assay tube to give 50% inhibition	0.8	120,000	>10 6	approx. 25,000

cAMP was assayed using a specific protein binding assay. This is similar to the cGMP assay, except that the competition between unknown amount of unlabelled cAMP and fixed amount of tritium labelled cAMP is for binding to a specific binding protein, rather than an antibody. The amount of labelled cAMP bound is inversely related to the amount of unlabelled cAMP present.

The method suggested in the kit was modified slightly to improve the sensitivity of the assay. 100ul samples (instead of

the 50ul suggested) of the soulution to be assayed were incubated with a known amount of tritium labelled cAMP and a fixed amount of specific binding protein. Blank determinations were made by substituting buffer for both the unknown solution and the labelled cAMP solution. All tubes other than those with assay sample had an extra 50ul of buffer added to them to compensate for the increase in overall volume (due to adding 100ul of assay sample rather than the 50ul suggested). After incubation at $0^{o}C$ for 2 hours the free nucleotide was removed by the addition of 100ul of ice cold charcoal suspension, vortex mixing briefly and centrifuging in a refrigerated centrifuge. 250ul samples (as opposed to the 200ul in the unmodified procedure) of the supernatant were then taken for liquid scintillation counting. The counts for the standards and the blank were then used to produce a calibration curve, from which the amount of CAMP in the assay samples was read.

The results of the assay of a solution of known concentration by the standard and by the modified procedure were compared. It was found that the modification did not affect the calibration of the assay.

Specificity; the data quoted by Amersham ltd. give the specificity of the assay as follows; ATP does not cross react at concentrations up to 5mM. This is approximatly a million fold excess over the amount of labelled cAMP present. An approximatly 200 fold excess of cGMP is required to give 50% inhibition of binding. Although this assay is therefore not as specific as the cGMP assay, cross-reactivity with other nucleotides was not considered to be a problem.

2.4 Liquid scintillation counting

Samples from the cyclic nucleotide assays were counted in a Packard Model 3320 liquid scintillation counter. The counting time was 10 minutes. The samples from the assays were counted in 10ml of scintillant made up as follows;

Triton X 100 = 666 mls
Scintol 2 = 100 mls
Toluene to 2 litres.

Scintol 2 (a toluene based scintillator, E max 430nm.) was obtained from Koch-Light. The samples taken from the cAMP assay were made up to 1ml by the addition of 750ul of water, to ensure that, as with the cGMP assay, the total aqueous content of each vial was approximatly 9% of the total volume. This has been shown to produce a clear homogeneous mixture which remains stable at both room temperature and at $4^{o}C$, the temperature at which the vials were counted. A futher advantage of using this scintillant, rather than the more commonly used 1:1 Toluene:Triton X mixture, is that the 2:1 mixture has a much lower vicosity. This allows the more rapid clearing of trapped air from the mixture. (trapped air can lead to unpredictable variations in counting efficiency) To minimise the effects of changes in counting efficiency produced by changes in temperature, the vials were left to equilibrate in the counter for 60 minutes before counting. This period of dark adaptation also serves to reduce the residual photo-excitation of the scintillant due to exposure to sunlight or flourescent lights.

2.5 Protein Assay

The pellet left from each extraction was assayed for protein using the Biuret test. Biuret reagent was made up as in Appendix A.

1 ml. of water was added to each assay tube and the pellet broken up by vortex mixing. To this was then added 4 ml. of Biuret reagent and the tube vortex mixed again. The tubes were then allowed to stand for 30 min. at room temperature, during which time they were vortex mixed a further three times. A calibration curve was constructed using serial dilutions of a solution of Bovine serum albumin, over the range 0.5 to 10mg. protein per ml. The absorbance of the solutions was read at 550 nm. in a Unicam SP 1800 spectrophotometer. Each result was taken as the mean of three readings.

3.0 ELECTROPHYSIOLOGY

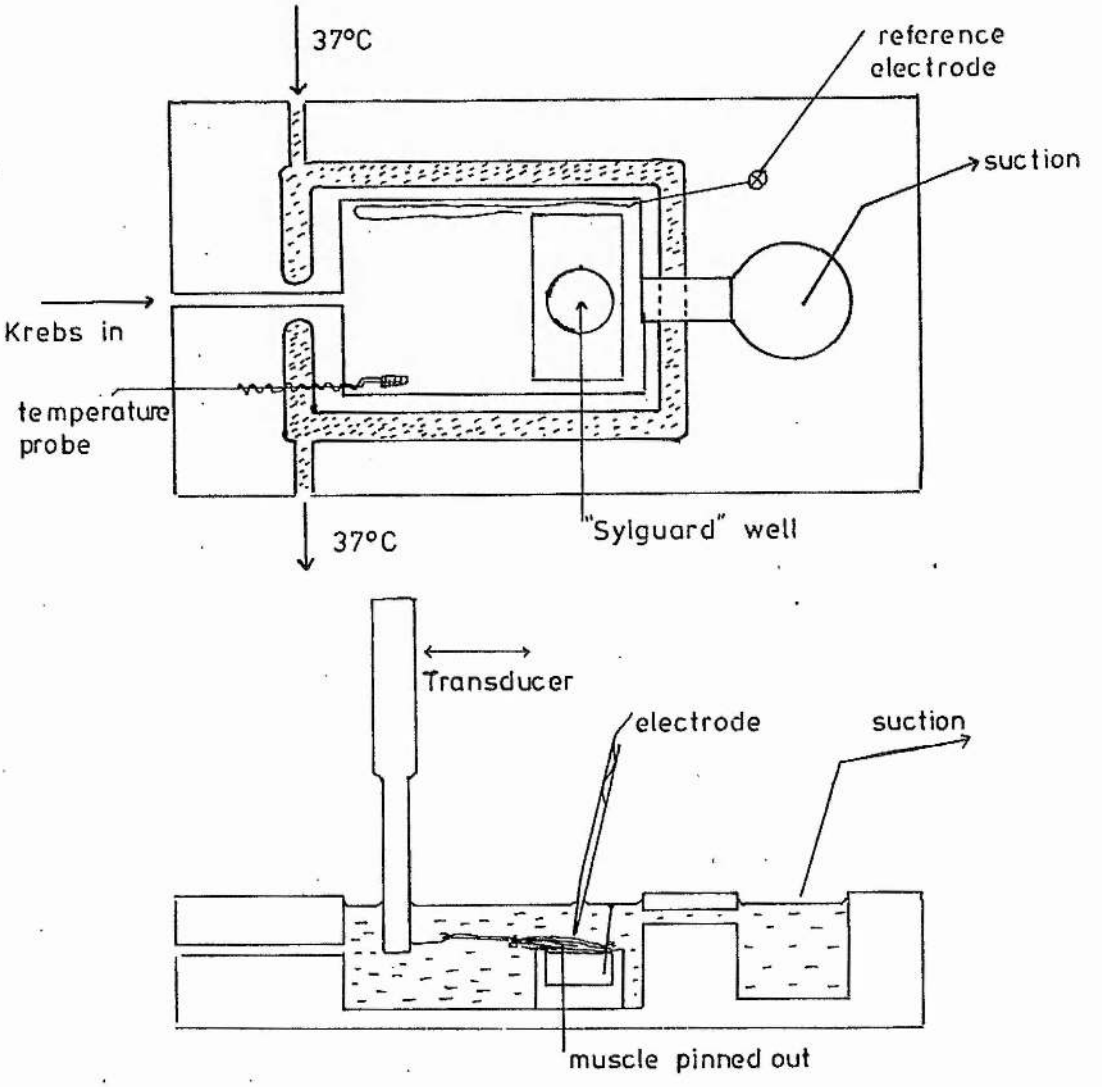
3.1 General

Intracellular recordings of membrane potential of the rat anoccocygeus muscles were obtained using glass micro-electrodes. The muscles were dissected out as before, except that the silver chain and wire loop were not attatched. Instead the ventral end of the muscle was left free, while the dorsal end had a small loop of thread attatched to it. The muscles were mounted singly in the bath as shown in fig. M3. The ventral end of the muscle was pinned flat to a Sylguard base and the dorsal end attatched to a tension transducer by the loop of thread. Tension was not monitored accurately in these experiments, so the compliance of the thread as compared to silver chain did not create a problem. Rather, tension was monitored as a means of assesing tissue activity and to indicate when maximum tension had been reached.

In order to minimise movement of the muscles when stimulated (which creates problems of eletrode breakage etc.), the muscles were set up at a resting tension of 5g. in these experiments. Krebs solution entered the bath after being pre-warmed to 37°C by a heat exchanger. The bath itself was also warmed to 37°C by circulating water. The temperature of the bath was monitored using a digital thermometer. Krebs solution was continously removed from the bath by suction from a well at the opposite end to the inflow. The surface of the solution in the bath does not comunicate with this well, so greatly reducing electrode movement due to ripples

Fig M3 The Electrophysiology Bath.

Muscles are pinned out singly on the Sylguard well as shown. The temperature probe is a small diode which signals temperature changes as changes in junction potential. The bath is maintained at 37° C by a water jacket.


37°C
reference electrode
suction
Krebs in
temperature probe
37°C
"Sylguard" well
Transducer
electrode
suction
muscle pinned out

produced by the suction. In this way a continous perfusion of the bath is maintained. Doses of agonists were injected into the inflow end of the bath by syringe. The actual concentration of the agonist over the muscle is therefore a complex product of diffusion and the flow through the bath. The dose of each agonist used was therefore decided on an empirical basis, as the minimum dose necessary to give a well maintained, maximal contraction. The bath volume was 10 ml. The Krebs solution was gased with 95% O_2 / 5% CO_2, both in the resevoir and in the bath.

3.2 Electrodes

Electrodes were pulled using thin walled capillary glass tubing obtained from Clarke Electromedical Instruments ltd. These were filled with 1 M. potassium acetate. Electrodes in the correct resistance range (20 to 35 megohm) could be obtained routinely. The main problem was in filling the electrodes properly. The technique adopted was to fill the electrodes as well as posible from a syringe, with a millipore filter attatched. The electrodes were then placed vertically in a covered beaker, with their tips immersed in 1M potassium acetate overnight. Before use each electrode was examined microscopically for bubbles, dirt or damage. Each one was then measured electrically and discarded if the resistance was not in the range 20 to 35 megohm. Usually about 1/3 of the electrodes proved to be servicable.

3.3 Recording System

Tension and membrane potential were recorded simultaneosly. Tension changes were recorded by the transducer and displayed on one channel of a dual beam storage oscilloscope and also on a Devices heated pen chart recorder. Potential changes from the electrode were recorded using a Neurolog pre-amplifier (gain 1:1) and the output of this fed to the other channel of the oscilloscope. Photographs were taken of both membrane potential and associated tension changes directly from the oscilloscope, while the continuous record of tension changes on the chart recorder provided a useful guide to the tissue viability throughout the experiment. Calibration of both recording systems was carried out before each experiment. The tension transducer was calibrated by hanging weights vertically from it in the range 1 to 10g. The potential recording system was calibrated using a 10mV signal, which can be generated by the pre-amplifier for this purpose.

Due to the small size of the smooth muscle cells in this tissue (approx. 10 um. diameter x 100 um. length) (Gillespie and Lullman- Rauch 1974) it was found to be extremely difficult to maintain intracellular recording for any length of time (usual time in one cell being of the order of 10-30 seconds) This made it impossible in most cases to obtain recordings of the change in membrane potential caused by the action of an agonist, especially since the movement of the tissue when stimulated usually caused the electrode to be dislodged. The tecnique adopted, therefore, was to record membrane potentials from a sample of cells in the control

situation, then to add the agonist and, when the tension was near maximal, to record from another sample of cells. To enable the recordings to be as objective as possible, certain criteria were adopted as to which penetrations should be included in the sample. Penetration of the cells could only be judged by the potential recorded as the electrode was advanced through the tissue, not by visual means. A good penetration was judged by the following criteria;

(1) On advancing the electrode the recorded potential drops suddenly upon penetration of a cell (a vertical step in the trace).

(2)The potential recorded remains reasonably steady and noise free for at least 10 seconds.

A trace showing a cell penetration which meets these criteria is shown in Fig R9-1.

The electrode resistance was checked after each attempted penetration, sucessful or otherwise. The electrode was discarded if the resistance had changed significantly (indicating a blocked or broken electrode).

RESULTS

1.0 MECHANICAL STUDIES.

1.1 Log Dose Response Curves of Agonists.

The log dose response curves of the muscle to each of the three agonists are shown in Fig R1. It can be seen that the maximum response to each of the agonists is very different , being 165.6 [SE=8.5], 85.6 (SE=6.8) and 65.7 (SE=6.3) Nm^{-2} for NA, ACh and KCl respectively. The corresponding -log ED50 values are 5.6 , 4.1 and 3.1.

1.2 Antagonist Dose Response Curves.

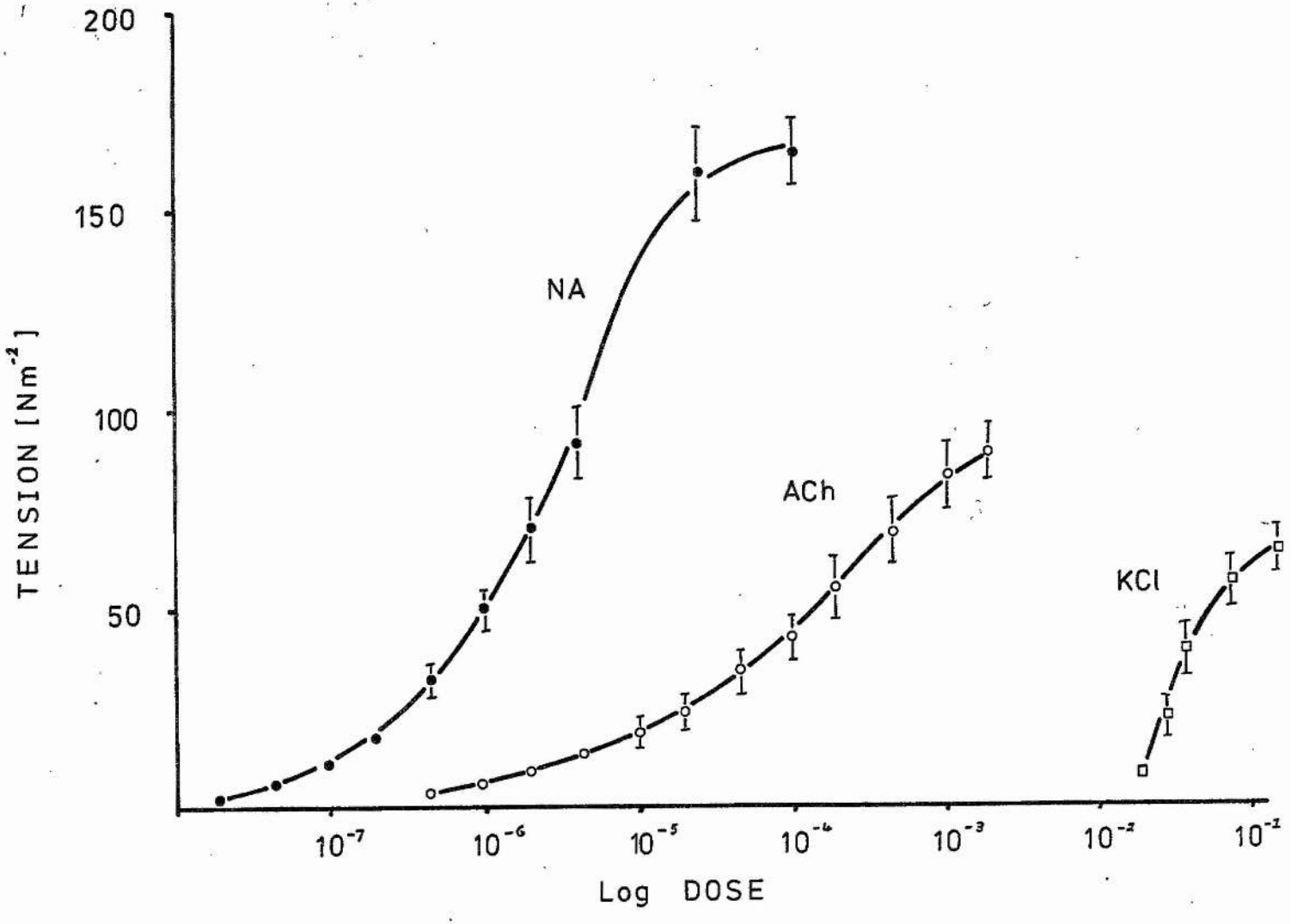
Antagonist dose response curves are shown in Figs. R2-1 to R2-12. The rational behind obtaining these curves was;

a) To investigate possible differences in the sensitivity of each of the three agonists to sodium nitroprusside, Verapamil, Stelazine and theophylline, thus clarifying the ways in which the three agonists produce a response.

b) To investigate possible differences between the different phases of contractions produced by the three agonists. The contraction produced by KCl is bi-phasic in normal Krebs, but the contractions produced by NA and ACh are only bi-phasic in low calcium Krebs. The curves for the primary phases of NA and ACh

Fig R1 Log Dose Response Curves for Each Agonist.

Curves for each agonist are shown on the same scale. Error bars = 1SE. No error bars are shown where they are too small to represent accurately.


200
150
100
50
TENSION [Nm^{-2}]
NA
ACh
KCl
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
Log DOSE

contractions were therefore obtained in low calcium (0.12mM).

1.2.1 Curves in normal calcium solution; Sodium nitroprusside and Verapamil -

It can be seen that the dose response curves obtained in normal calcium (Figs R2-1 to R2-4), show marked differences between the three agonists. The contraction produced by NA appears to be largely unaffected by concentrations of V or NP up to 10^{-5}M (no significant antagonism compared to control levels) The contractions produced by KCl and ACh, however, show marked antagonism by both NP and V. In the case of KCl both phases are inhibited by both NP and V, the greatest inhibition being produced by V in both cases. V (10^{-5}M) produces an inhibition of about 93% on the primary contraction, and about the same on the secondary. NP (10^{-5}M) has different effects on the two phases, producing about 30% inhibition of the primary and a slightly greater inhibition of the secondary (about 45%).

Ach induced contractions are also markedly inhibited by both NP and V, but in this case the greater antagonistic effect is produced by NP, V (10^{-5}M) producing about 60% inhibition and NP (10^{-5}M) producing about 75% inhibition (although in this case the significant difference between the two curves is less pronounced, due to the greater errors in the curves themselves, since the contractions produced by ACh tend to be more variable than those produced by the other agonists).

Fig R2-1 Antagonist Dose Response Curves; NA (NP and V).

The figure shows the effect of an increasing dose of Verapamil (open circles) and Nitroprusside (filled circles) on a contraction induced by a constant, maximal dose of NA (2.5×10^{-5}M). Point labelled "Control" is the response with no antagonist present. Error bars = 1SE. n = 8 for each point.

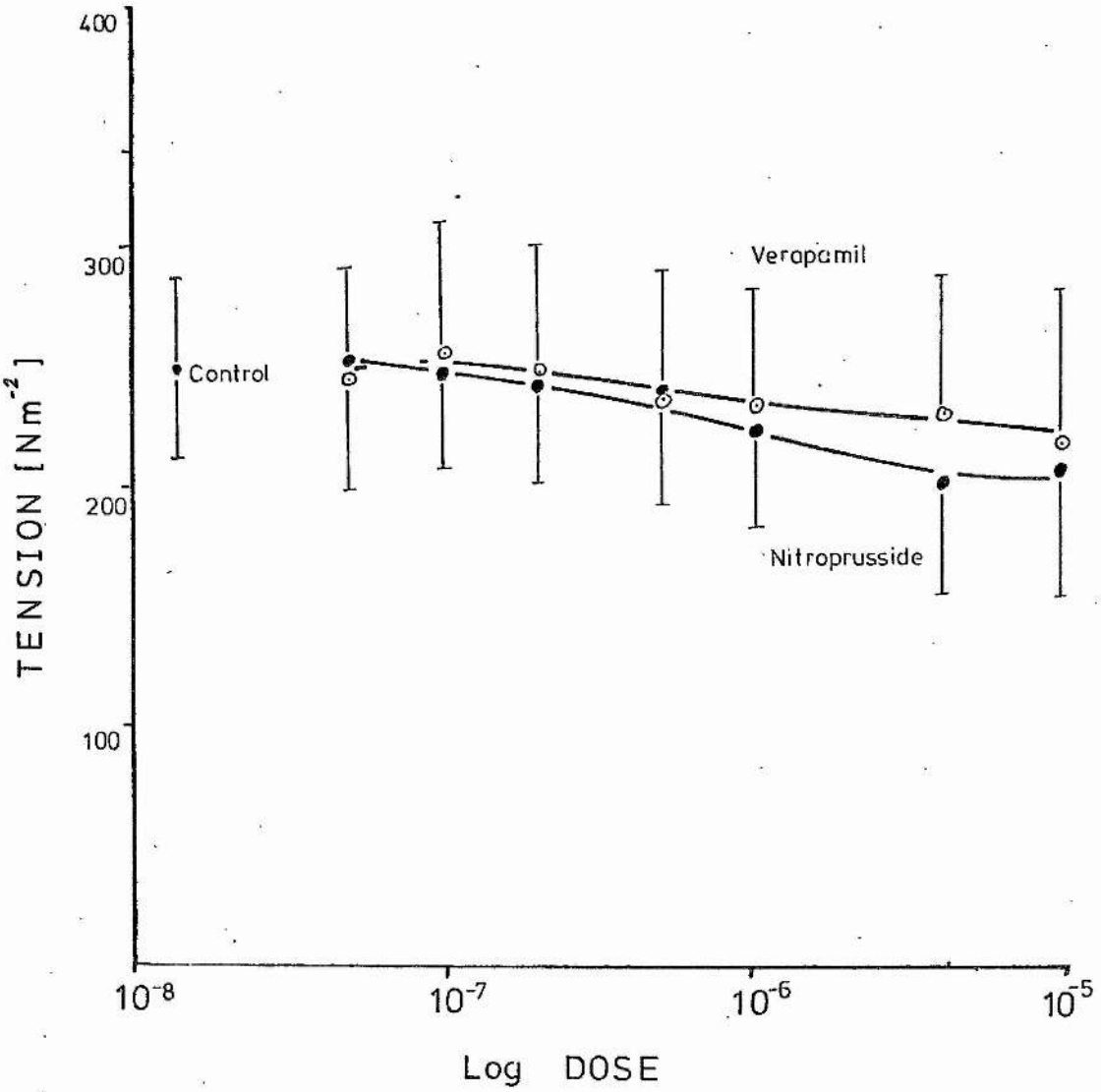

400
300
200
100
TENSION [Nm^{-2}]
Control
Verapamil
Nitroprusside
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Log DOSE

Fig R2-2 Antagonist Dose Response Curves; KCl (primary), (NP and V).

As for Fig R2-1, except that the response measured is the primary phase of a contraction induced by KCl (80mM).

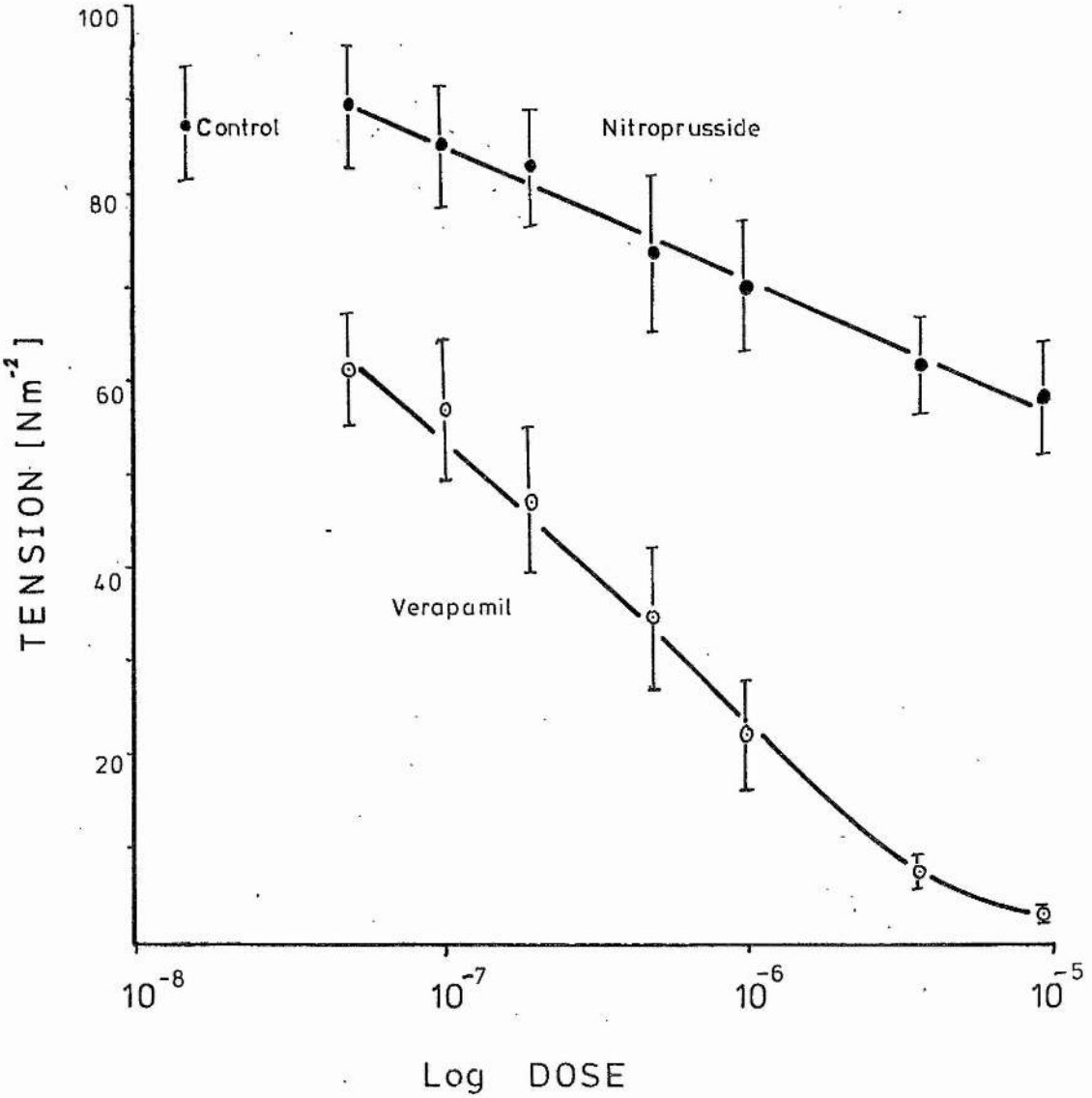

100
80
60
40
20
TENSION [Nm^{-2}]
Control
Nitroprusside
Verapamil
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Log DOSE

Fig R2-3 Antagonist Dose Response Curves; KCl (secondary), (NP and V).

As for Fig R2-1, except that the response measured is the secondary phase of a contraction induced by KCl (80mM).

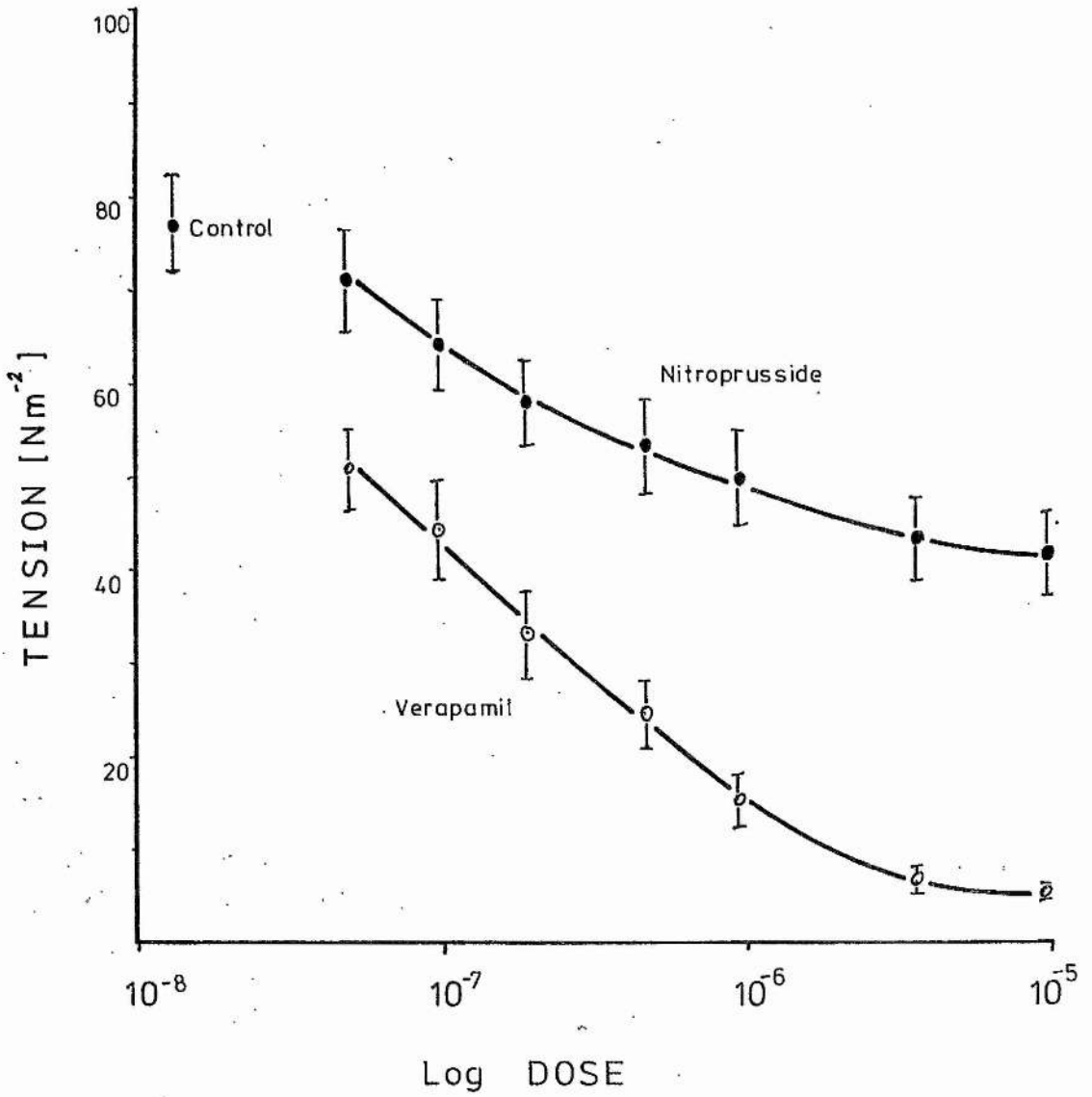
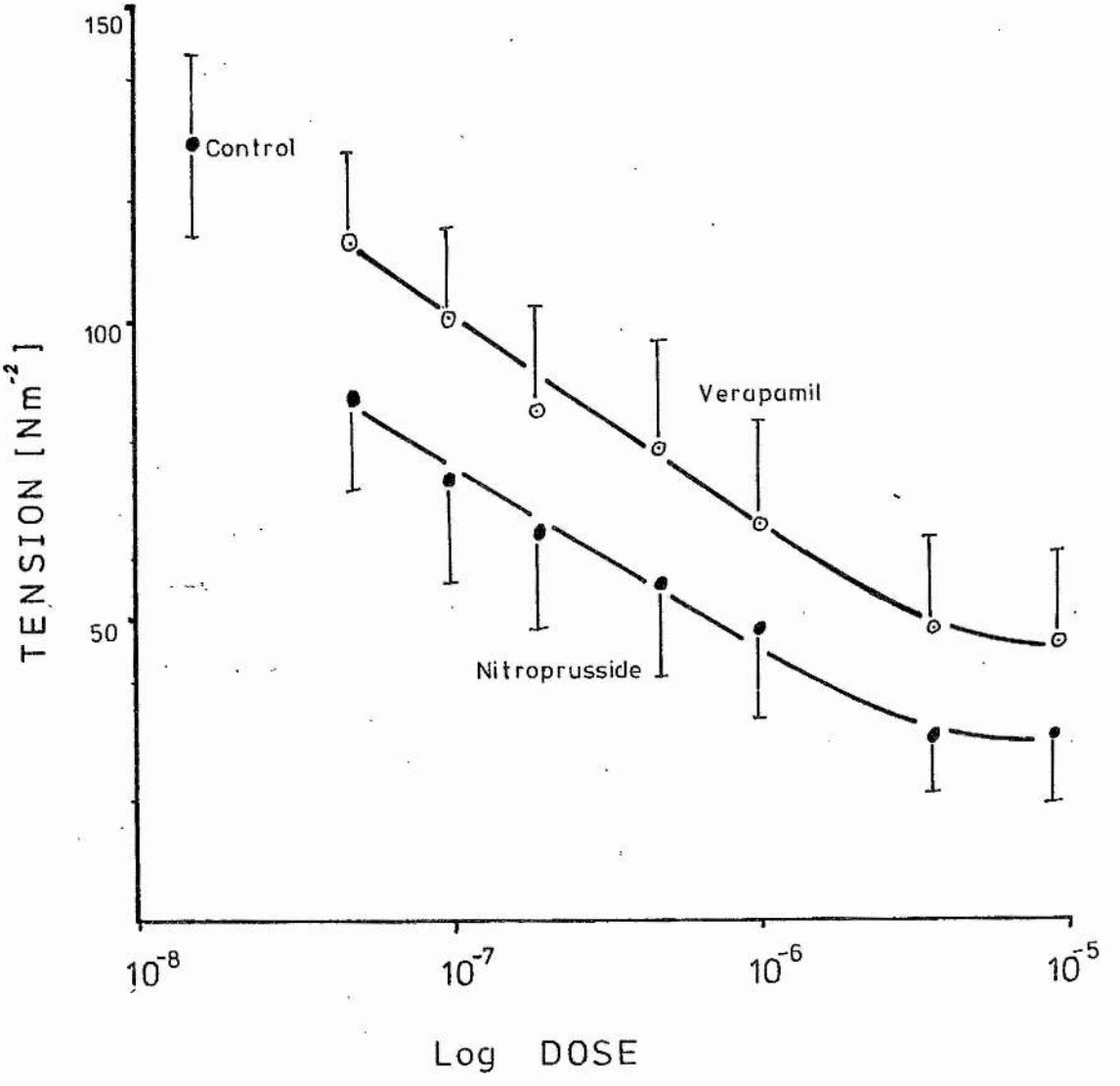

100
80
60
40
20
TENSION [Nm^{-2}]
Control
Nitroprusside
Verapamil
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Log DOSE

Fig R2-4 Antagonist Dose Response Curves; ACh (NP and V).

As for Fig R2-1, except that the response measured is the contraction induced by ACh (2×10^{-3}M).


150
100
50
TENSION [Nm^{-2}]
Control
Verapamil
Nitroprusside
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Log DOSE

1.2.2 Curves in normal Calcium; Stelazine and Theophylline. -

The inhibition produced by Stelazine and Theophylline is shown in Figs R2-5 to R2-8. The effect on the primary and secondary phases of the KCl contraction are so similar that the curves for both phase are identical. In both cases Stelazine has a greater inhibitory effect than Theophylline. This greater inhibition by Stelazine is also shown in the case of NA and ACh, although the difference in the antagonist potency is not so great in the case of ACh. This is illustrated by the potency ratio of the ED50 (concentration giving 50% inhibition) for Stelazine and Theophylline, as shown below.

Doses giving 50% inhibition; (Mol)

	Theophylline	Stelazine	Ratio Theo/STZ
NA	5.0×10^{-3}	1.8×10^{-4}	28
ACh	1.5×10^{-3}	2.2×10^{-4}	7
KCl	2.4×10^{-3}	6.0×10^{-5}	40

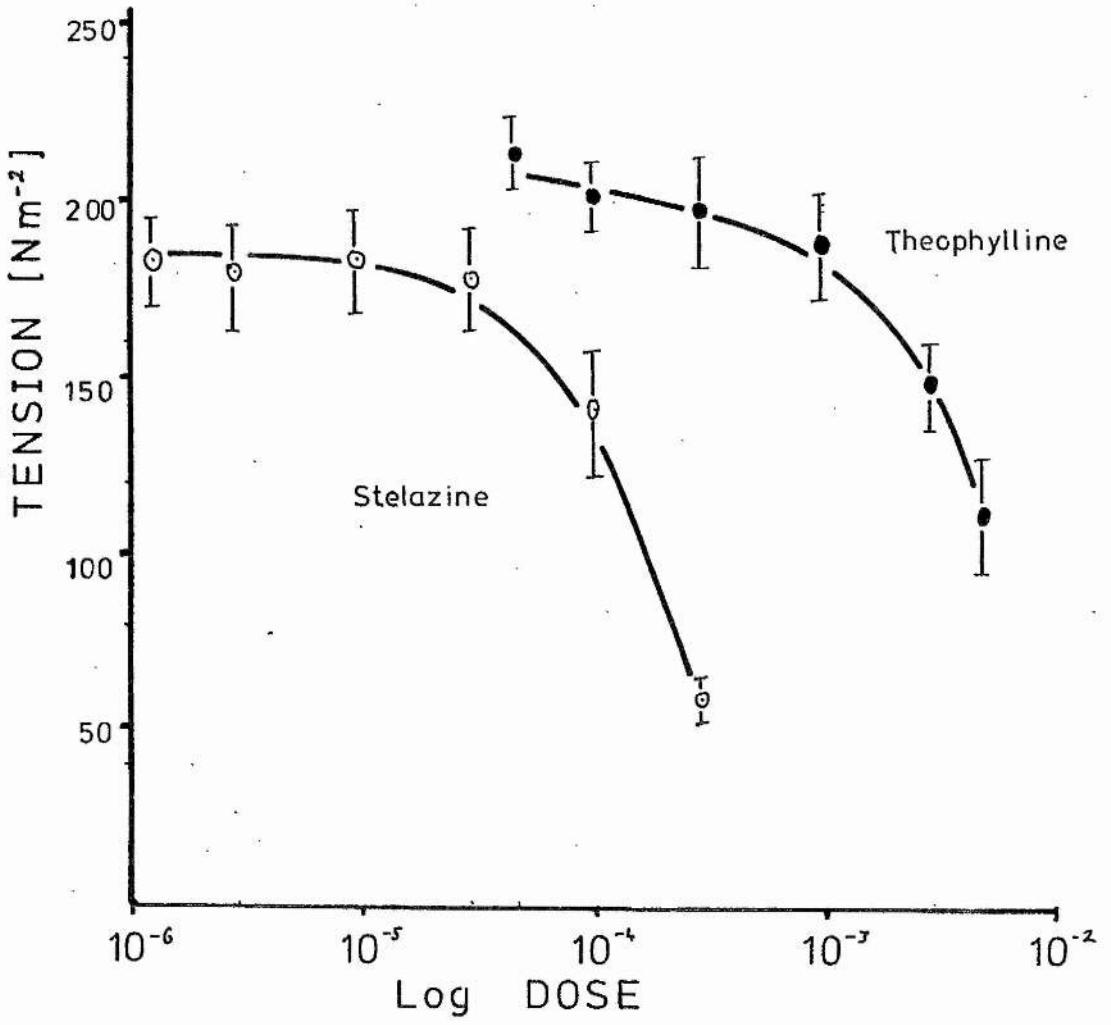
Note that it is not possible to use concentrations of Theophyline greater than 5×10^{-3}M, because of its low solubility in water.

1.3 Separation of Phases;

Antagonist dose response curves in low Calcium.

Fig R2-5 Antagonist Dose Response Curves; NA (STZ and Theo).

The effect of an increasing dose of STZ (open circles) and Theo (filled circles) on a contraction induced by a constant, maximal dose of NA (2.5×10^{-5}M). Error bars = 1SE. n = 8 for each point.


250
200
150
100
50
TENSION [Nm⁻²]
Theophylline
Stelazine
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
10⁻²
Log DOSE

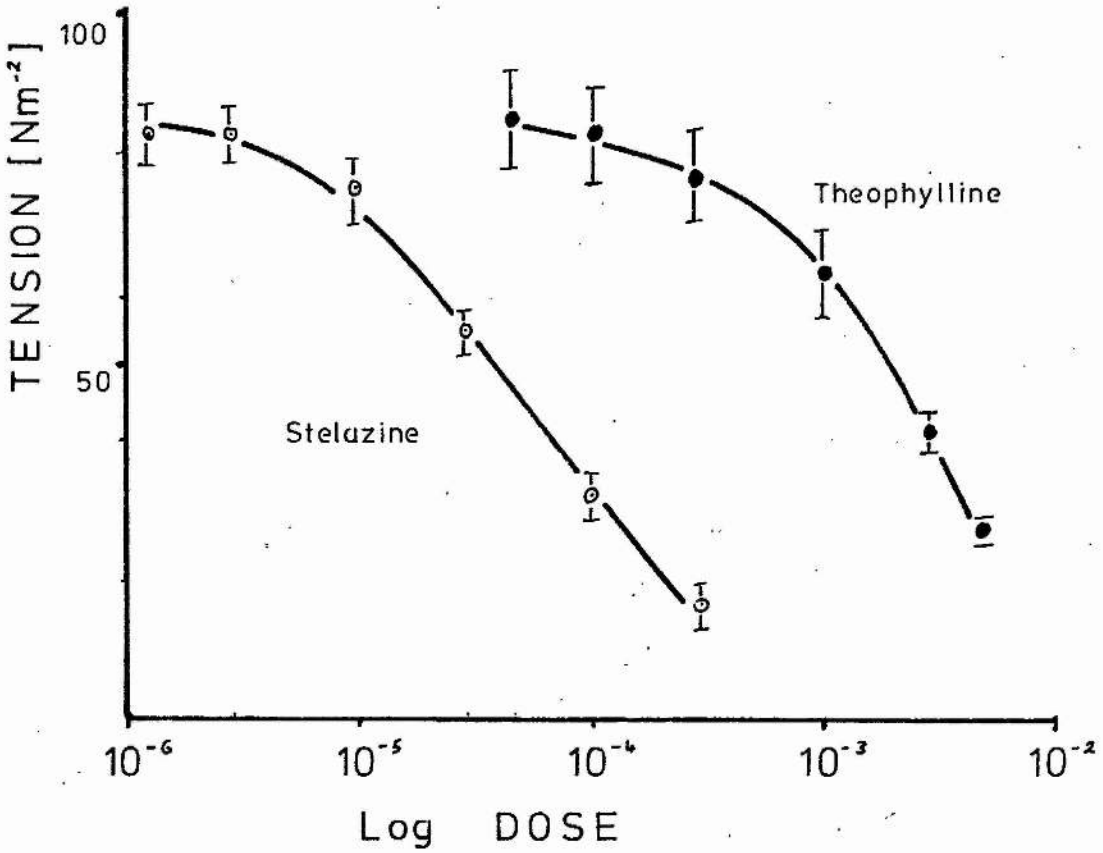

TENSION [Nm⁻²]
100
50
Stelazine
Theophylline
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
10⁻²
Log DOSE

Fig R2-6 Antagonist Dose Response Curves; KCl (primary), (STZ and Theo).

As for Fig R2-5, except that the response measured is the primary phase of a contraction induced by KCl (80mM).

Fig R2-7 Antagonist Dose Response Curves; KCl (secondary), (STZ and Theo).

As for Fig R2-5, except that the response measured is the secondary phase of a contraction induced by KCl (80mM).

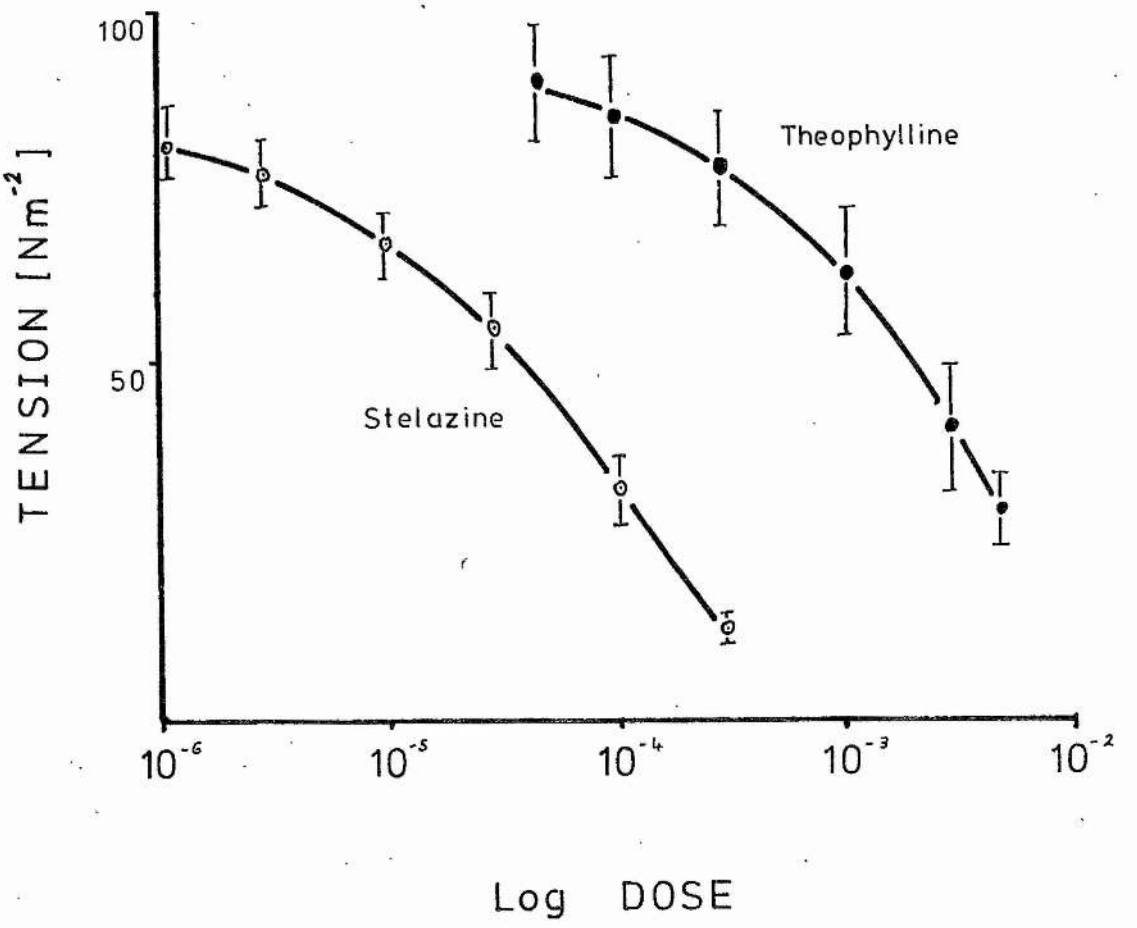

100
50
TENSION [Nm^{-2}]
Theophylline
Stelazine
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
Log DOSE

Fig R2-8 Antagonist Dose Response Curves; ACh (STZ and Theo).

As for Fig R2-5, except that the response measured is the contraction induced by ACh (2×10^{-5}M).

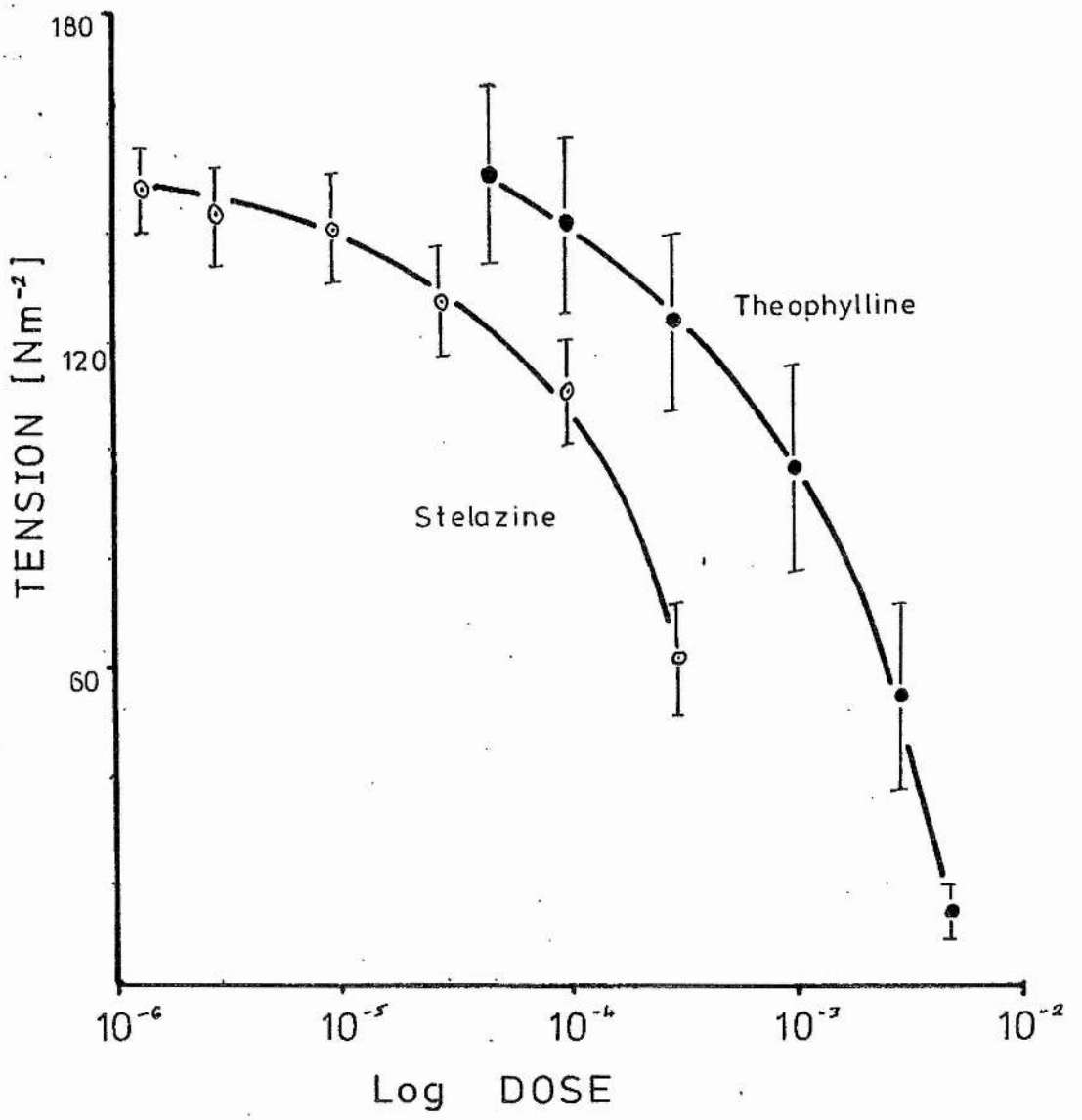

180
120
60
TENSION [Nm⁻²]
Theophylline
Stelazine
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
10⁻²
Log DOSE

Fig R2-9 Antagonist Dose Response Curves in Low Calcium; NA (primary)

The effect of an increasing dose of Verapamil (open circles) and Nitroprusside (filled circles) on the primary phase of a contraction induced by a constant, maximal dose of NA (2.5×10^{-5}M) in low calcium solution (0.12mM). Error bars = 1SE. n = 8 for each point. The point marked "Control" shows the response with no antagonist present.

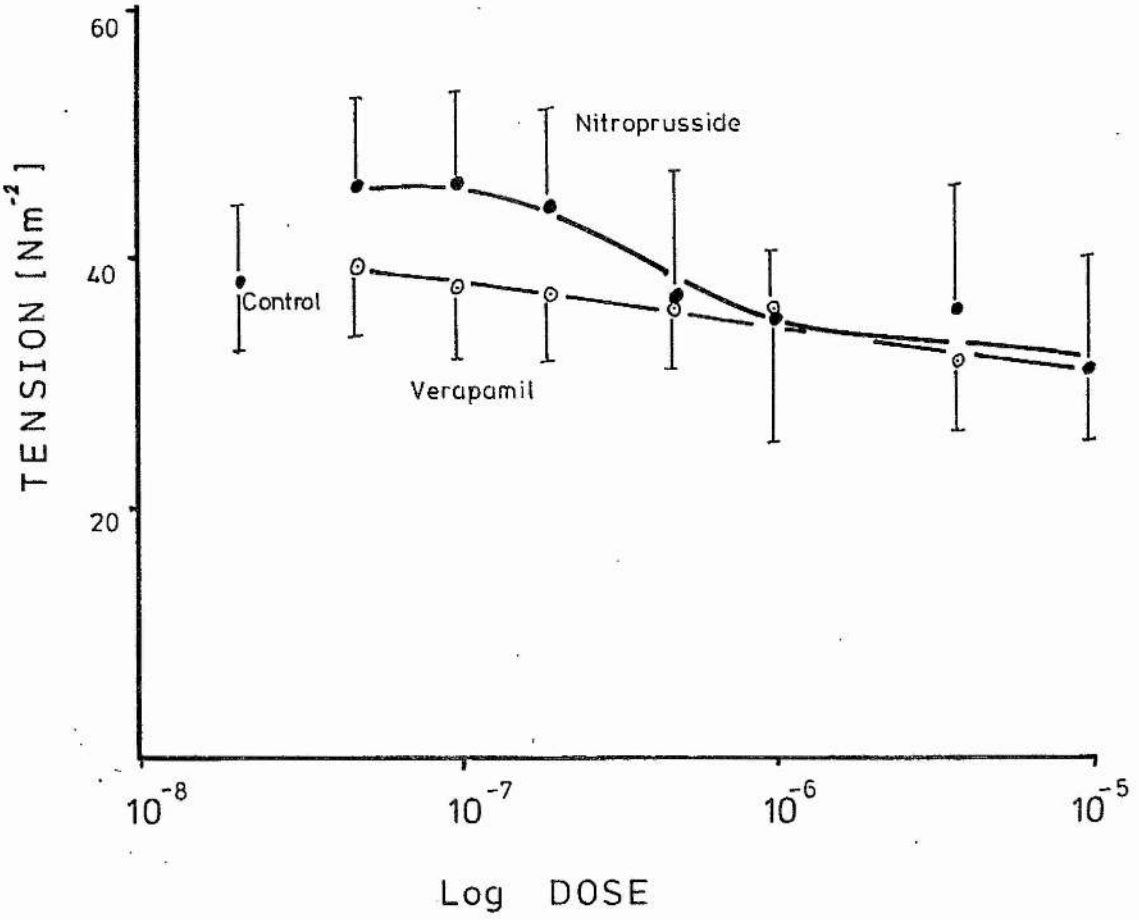

60
40
20
TENSION [Nm^{-2}]
Nitroprusside
Control
Verapamil
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Log DOSE

Fig R2-10 Antagonist Dose Response Curves in Low Calcium; NA (secondary).

As for Fig R2-9, except that the response measured is the secondary phase of the contraction induced by NA (2.5×10^{-5} M).

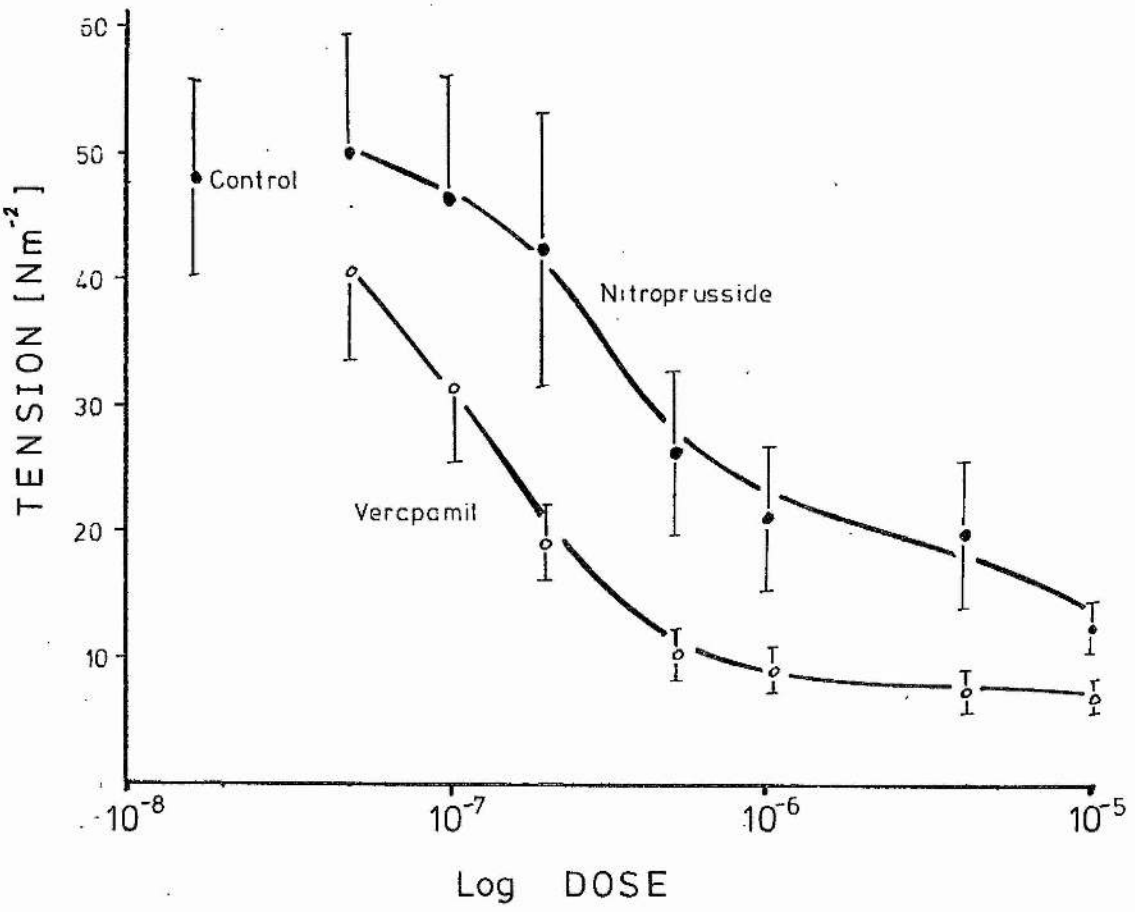

60
50
40
30
20
10
TENSION [Nm^{-2}]
Control
Nitroprusside
Verapamil
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Log DOSE

Fig R2-11 Antagonist Dose Response Curves in Low Calcium; ACh (primary).

As for Fig R2-9, except that the response measured is the primary phase of the contraction induced by ACh (2×10^{-5}M).

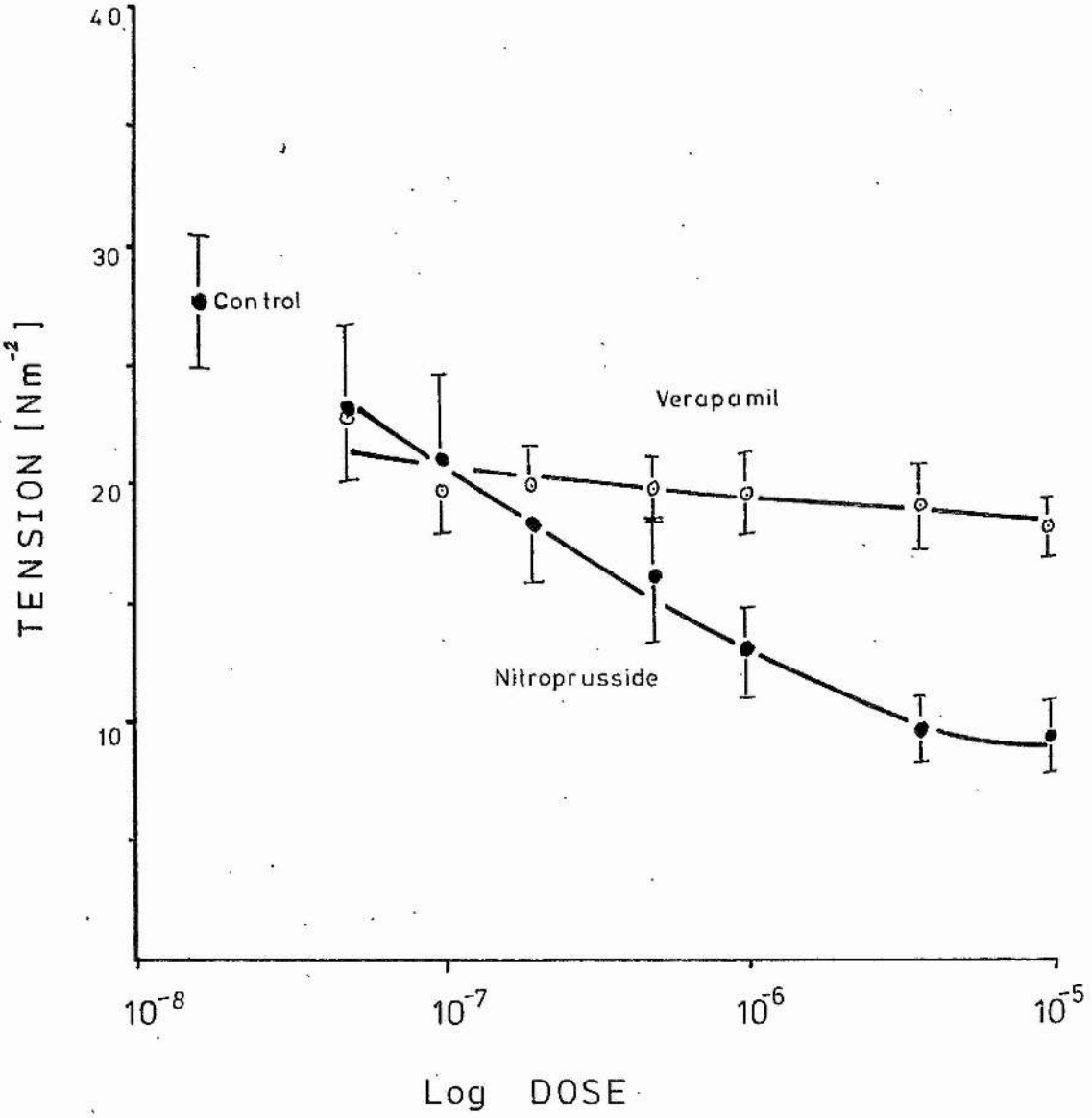
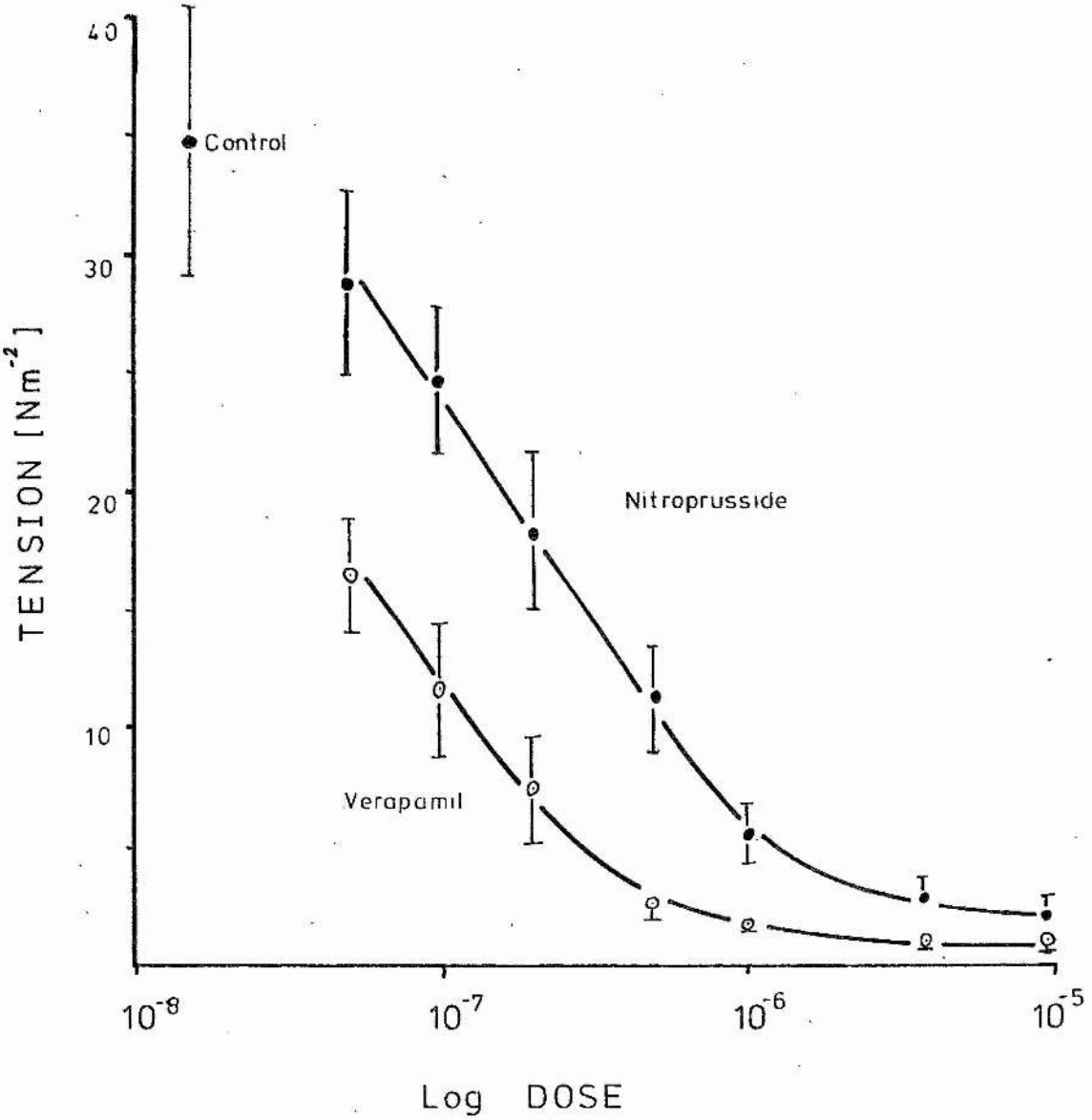

40
30
20
10
TENSION [Nm^{-2}]
Control
Verapamil
Nitroprusside
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Log DOSE

Fig R2-12 Antagonist Dose Response Curves in Low Calcium; ACh (secondary).

As for Fig R2-9, except that the response measured is the secondary phase of the contraction induced by ACh (2×10^{-5} M).


40
30
20
10
TENSION [Nm^{-2}]
Control
Nitroprusside
Verapamil
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Log DOSE

1.3.1 - Figs R2-9 to R2-12 show the dose response curves of the inhibitory effects of V and NP on the primary and secondary phases of contractions produced by NA and ACh in low calcium Krebs (0.12mM). In the case of NA a significant difference in the inhibition of the two phases is apparent. The secondary phase is inhibited to a large extent by both NP and V, V having the greatest effect. V produces a maximum inhibition of 80% at about 8 x 10^{-6}M, whereas NP produces a maximum inhibition of 55% at about 3 x 10^{-5}M. This is not shown in the case of the primary phase of the contraction. Rather, this is unaffected by V and indeed NP produces a slight potentiation of the contraction in concentrations of around 10^{-7}M. This effect is small, however, and the variation in these curves is quite large, since these contractions in low calcium solutions tend to be variable. The slight potentiation was seen in all the preparations, however.

The secondary contractions produced by ACh in low calcium are inhibited in a similar way to the contractions in normal Krebs, being markedly affected by both NP and V except that in normal Krebs NP is the more effective at producing inhibition, whereas in low Ca^{++} Krebs it is V which is the more effective (about 95% inhibition being produced by each). This data is consistent with the effects of V and NP on the Calcium dose response curve for the secondary phase of the ACh contraction (Fig R3-4). It can be seen from this that at low calcium concentrations V is more effective at producing inhibition, but the curves for V and NP cross at higher concentrations, so that NP is slightly more effective at the maximum calcium concentration used. The primary

phase of the contraction is relatively insensitive to V, but is inhibited by NP.

It can be seen that the primary and secondary phases of NA and ACh contractions in low calcium Krebs do indeed have dissimilar properties compared with each other, and compared to with the contractions in normal Krebs.

1.4 Calcium Dose Response Curves.

Calcium dose response curves are shown in Figs R3-1 to R3-6. Primary and secondary contractions are not shown for all three agonists. Since Ach and NA only produce bi-phasic contractions and a distinct, measurable primary phase at low calcium concentrations (below about 5×10^{-4}M), the variability of the contractions under these conditions makes it impossible to draw any firm conclusions. The doses of the agonists used were the same as those used for the antagonist dose response curves, ie KCl = 80mM, NA = 2.5×10^{-5}M, ACh = 2×10^{-3}M.

Secondary Phases of Contractions;

The Calcium dose response curves for the secondary phases of the contractions induced by the three agonists show marked differences with respect to the inhibitory effects of NP and V. The contractions produced by ACh are markedly inhibited by both NP and V, (as shown by the shift of the curve to the right and also the reduction in the maximum response obtained at higher calcium concentrations.) The extent of the inhibition produced by both is

Fig R3-1 Calcium Dose Response Curves; NA

The calcium dose response curve produced by increasing the calcium concentration of the Krebs solution while repeating a fixed dose of NA (2.5 x 10^{-5}M), (triangles, curve labelled "Control"). Also shown are the effects of a fixed concentration of Verapamil (5 x 10^{-7}M, open circles) and Nitroprusside (2 x 10^{-7}M, filled circles). Error bars = 1SE. n = 8 for each point.

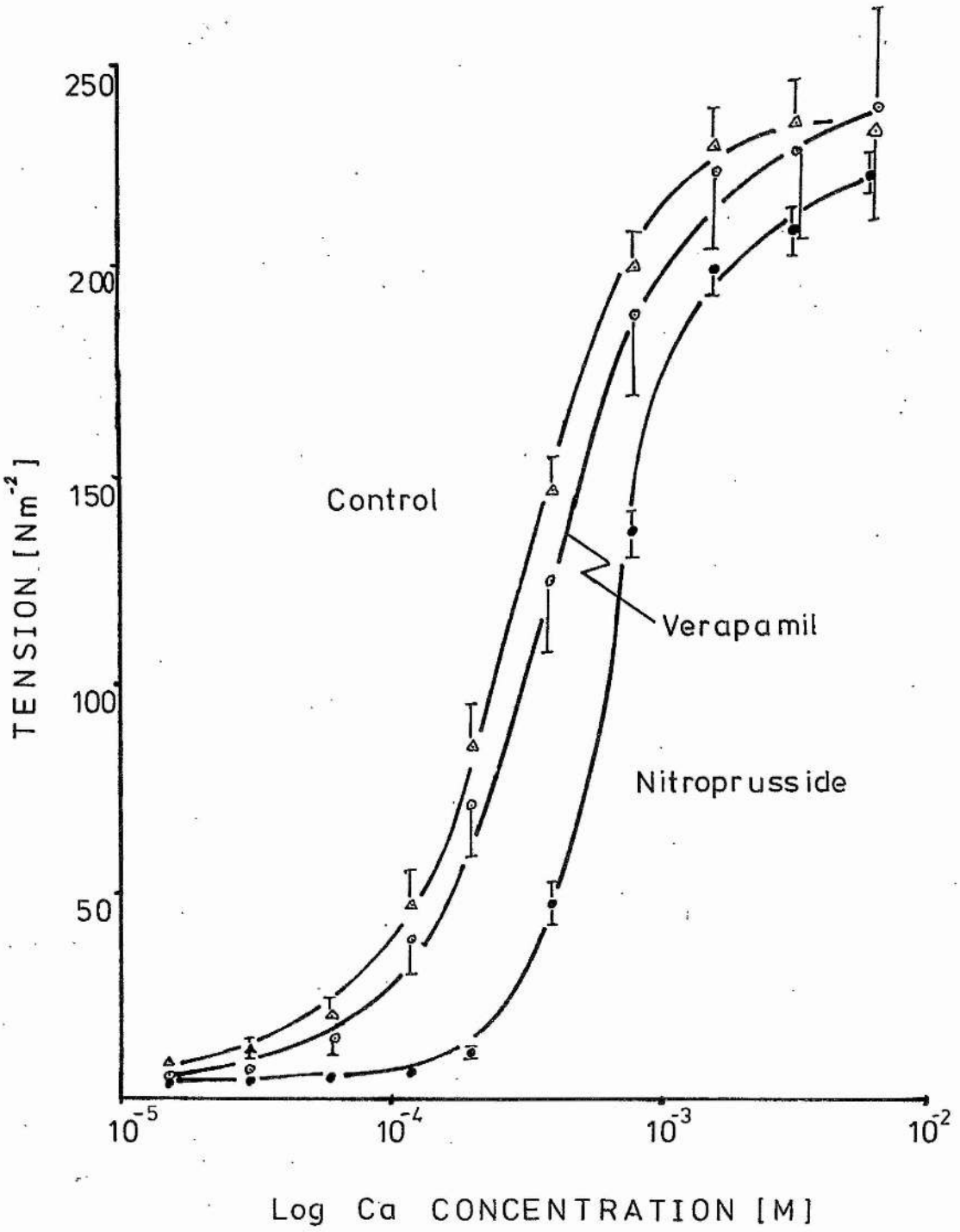

250
200
150
100
50
TENSION [Nm^{-2}]
Control
Verapamil
Nitroprusside
10^{-5}
10^{-4}
10^{-3}
10^{-2}
Log Ca CONCENTRATION [M]

Fig R3-2 Calcium Dose Response Curves; KCl (primary)

As for Fig R3-1, except that the response measured is the primary phase of the contraction induced by KCl (80mM).

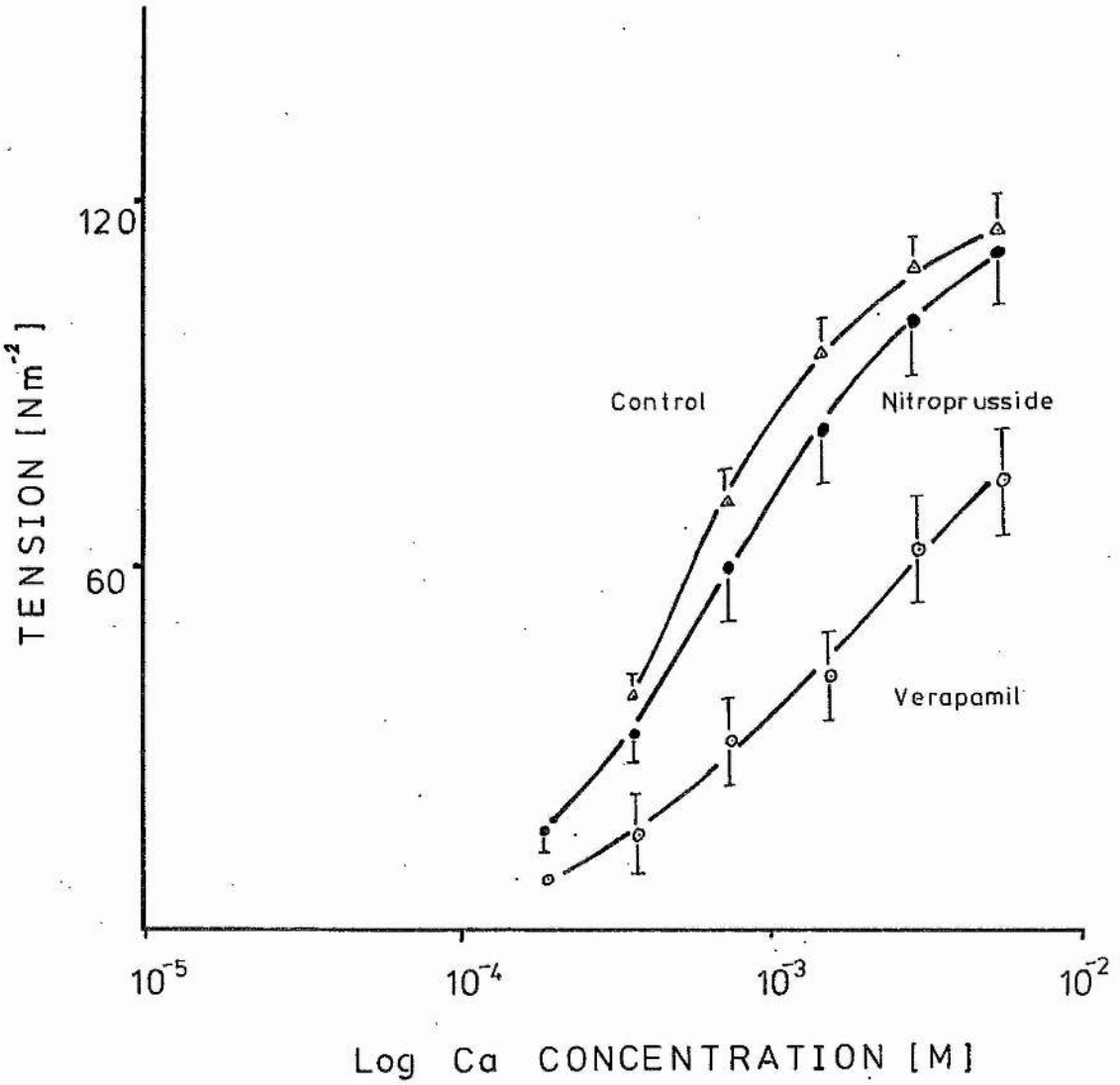

120
60
TENSION [Nm^{-2}]
Control
Nitroprusside
Verapamil
10^{-5}
10^{-4}
10^{-3}
10^{-2}
Log Ca CONCENTRATION [M]

Fig R3-3 Calcium Dose Response Curves; KCl (secondary)

As for Fig R3-1, except that the response measured is the secondary phase of the contraction induced by KCl (80mM).

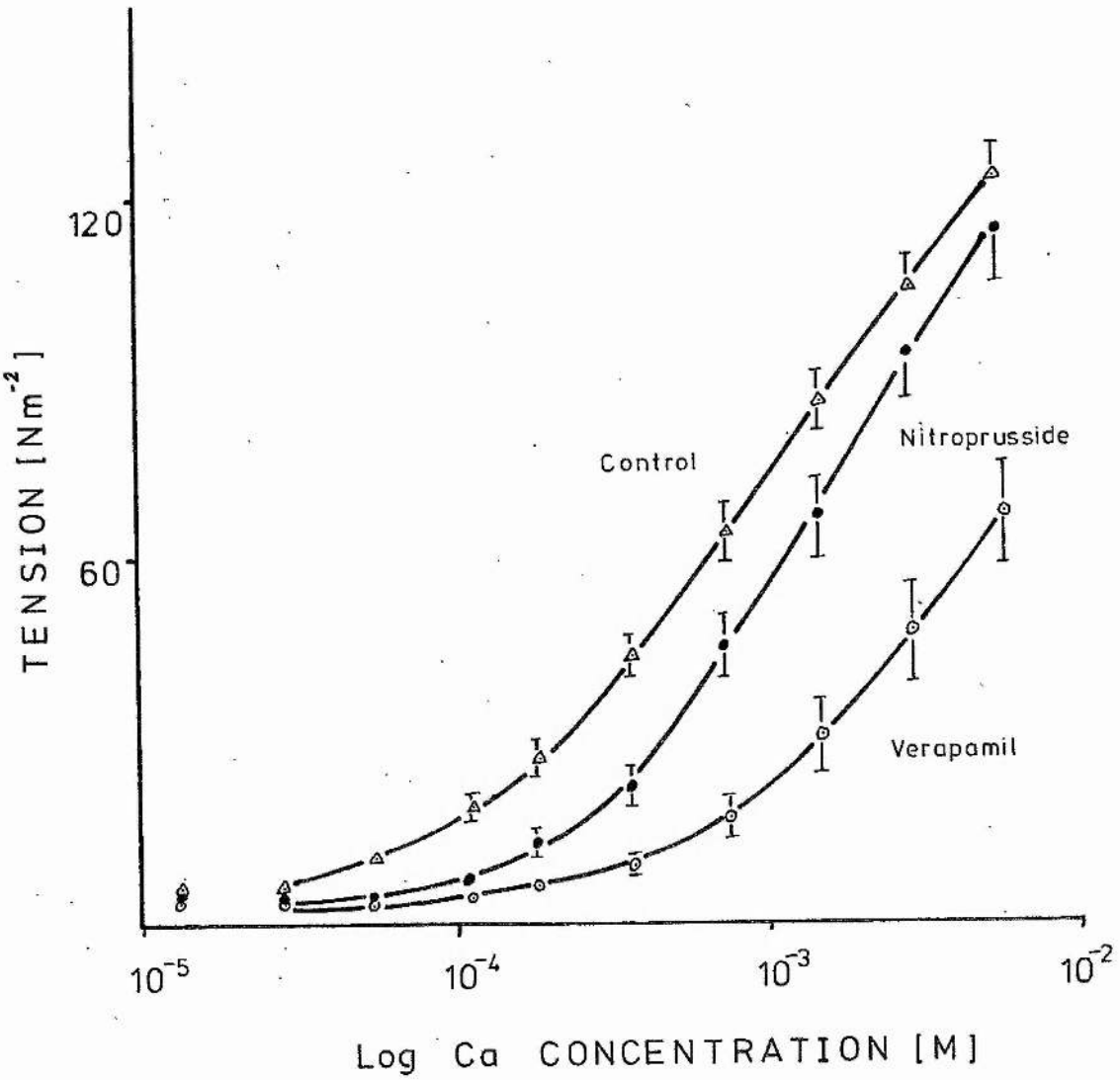
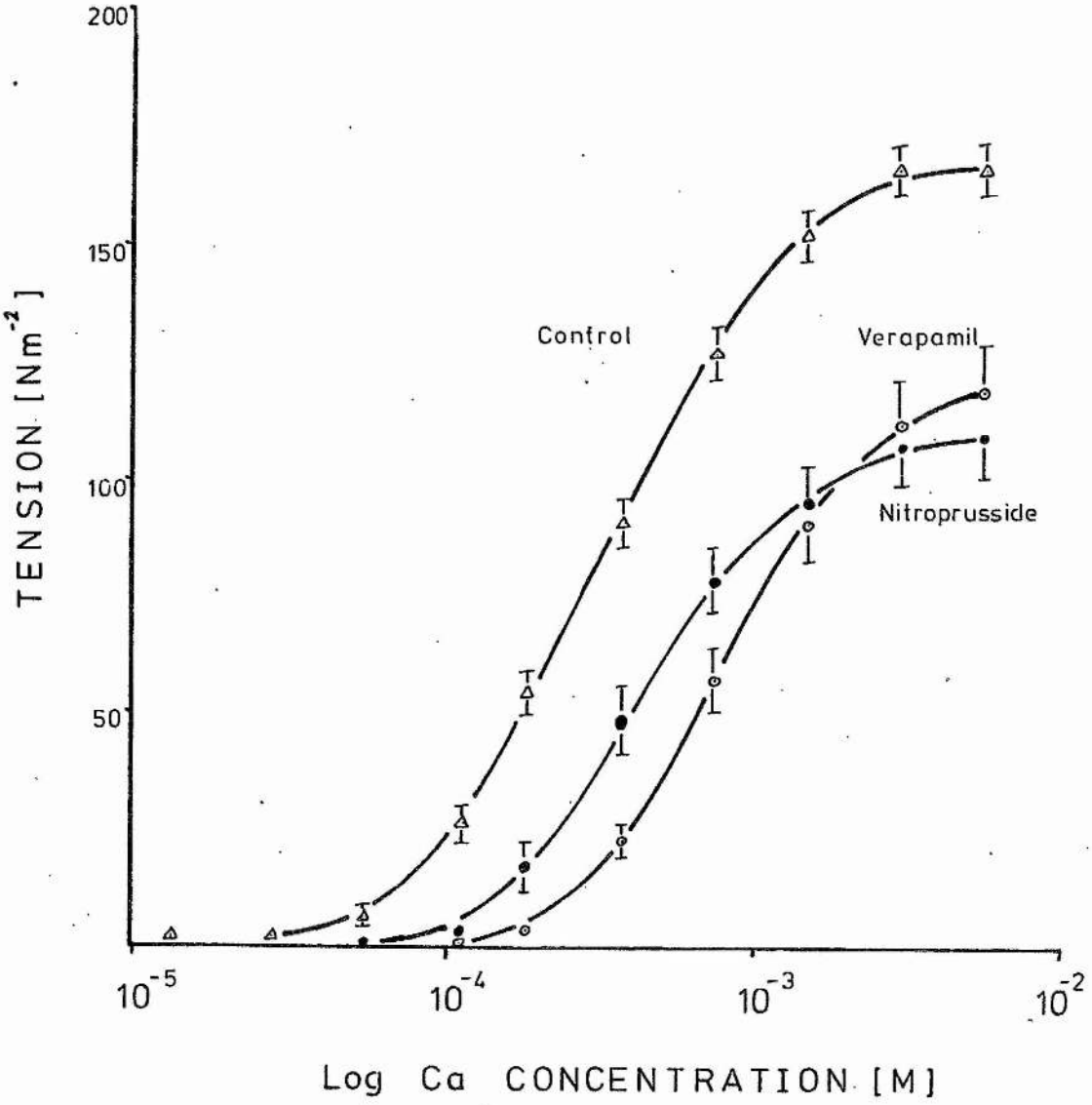
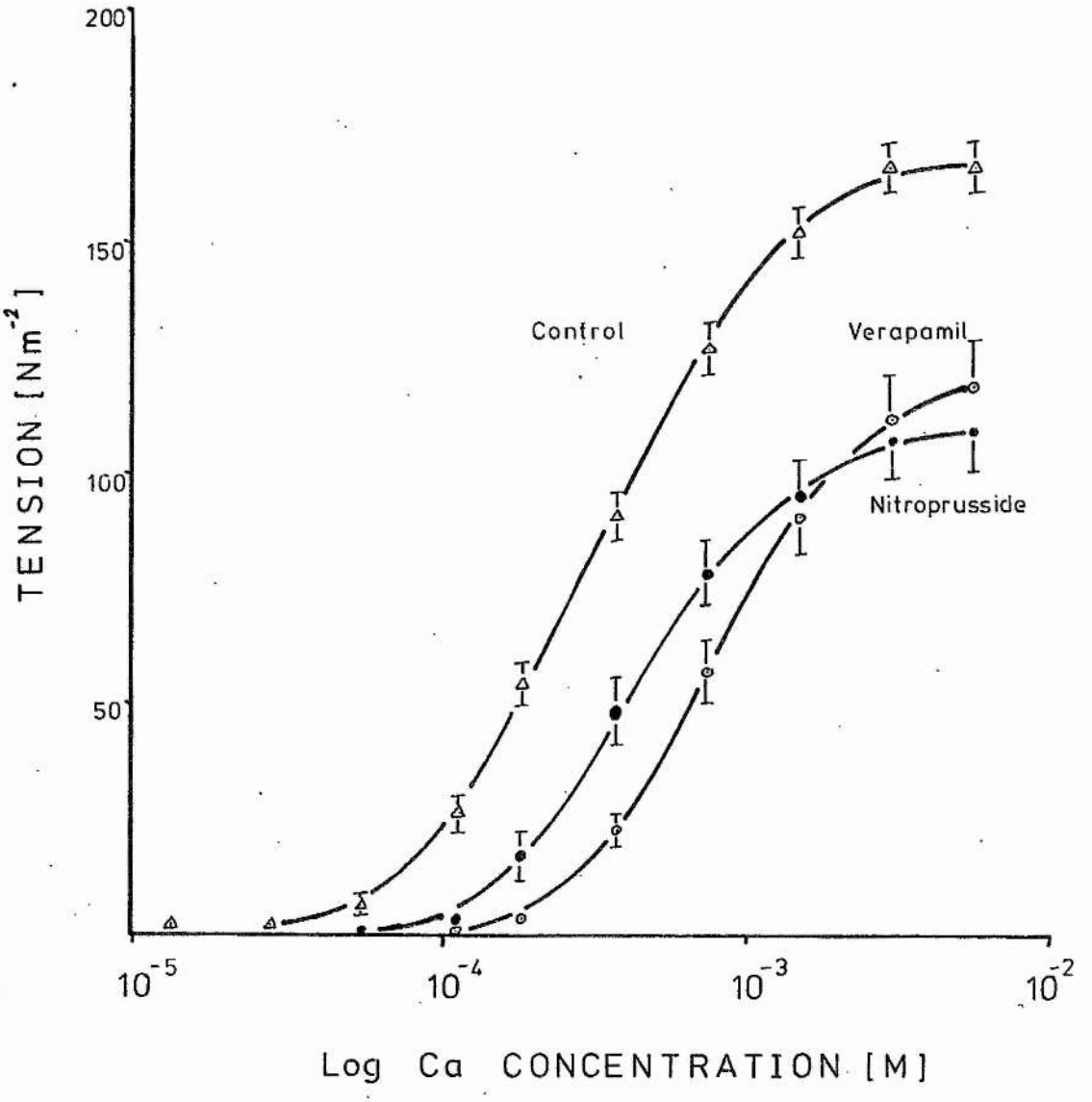

120
60
TENSION [Nm^{-2}]
Control
Nitroprusside
Verapamil
10^{-5}
10^{-4}
10^{-3}
10^{-2}
Log Ca CONCENTRATION [M]

Fig R3-4 Calcium Dose Response Curves; ACh

As for Fig R3-1, except that the response measured is the contraction induced by ACh (2×10^{-3}M).


200
150
100
50
TENSION [Nm^{-2}]
Control
Verapamil
Nitroprusside
10^{-5}
10^{-4}
10^{-3}
10^{-2}
Log Ca CONCENTRATION [M]


200
150
100
50
TENSION [Nm^{-2}]
Control
Verapamil
Nitroprusside
10^{-5}
10^{-4}
10^{-3}
10^{-2}
Log Ca CONCENTRATION [M]

similar (Fig R3-4). Both antagonists reduce the maximum response of the tissue from about 160 Nm^{-2} to about 110 Nm^{-2}. This reduction is highly significant in both cases, $P < 0.001$ for NP and $P = 0.001$ for V. This effect is not seen with NA, where it appears that V has no significant effect and NP only shows marked inhibitory effects at calcium concentrations below about $10^{-3}M$, almost 90% inhibition at a calcium concentration of $2 \times 10^{-4}M$ (Fig R3-1). This inhibition dissappears at higher calcium concentrations, so that the maximum response of the tissue is unaffected by NP or V.

The KCl secondary contractions do not reach a maximum in the control situation up to a calcium concentration of 6.4mM. Higher calcium concentrations are not possible because of the pecipitation of calcium phosphate. The KCl secondary curve (Fig R3-3) is slightly inhibited by NP at low calcium concentrations , at around $3x10^{-4}M$ NP ($2 \times 10^{-7}M$) produces an inhibition of 50%, but this inhibition is reduced at higher calcium concentrations (shown by the convergence of the curve with the control curve). Substantial inhibition is produced by V, however, and this effect is maintained at high calcium levels. The curve for V and the control curve are parallel above a calcium concentration of about $8x10^{-1}M$.

It should be noted that there is a fundamental difference in the shape of the control curves for the agonists. The curves for ACh and NA reach a maximum response below the maximum calcium concentration used, whereas the KCl curve continues to rise to this

point, with no sign of a maximum response being reached. The possible reasons for this are discussed later.

Primary Phases of Contraction;

ACh and NA only produce bi-phasic contractions below a calcium concentration of about $3x10^{-4}M$. KCl, however, only produces a bi-phasic contraction above $2x10^{-4}M$. The primary response of the KCl contraction is much less variable than those of NA and ACh.

Interpretation of the results for the primary responses is therefore made more difficult by the variability of the ACh and NA contractions. The only clear effect is that the primary response of KCl is inhibited by V but not by NP, V producing an inhibition of about 30% at the maximum calcium concentration used (6.4mM). (Fig R3-4). This is very similar to the effects of V and NP on the secondary response.

1.5 Drugs Added During the Plateau Phase of Contraction.

1.5.1 Effect of NP. (Fig R4-1A,B,C). - There are marked differences in the inhibitory effect produced by NP ($2 \times 10^{-7}M$) on the contractions produced by NA, ACh or KCl. It can be seen from Figs R4-1B and R4-1C that the inhibitory effect is extremely rapid in the case of ACh and NA, being greater in the case of ACh (about 60% inhibition) than for NA (about 40% inhibition). The inhibition of the KCl contraction is smaller (about 15%) and not

as rapid.

Note that the inhibition produced by NP in these cases is about the same as would be expected from the antagonist dose response curves for each of the agonists, except NA. The antagonist dose response curve for NA shows no significant inhibition at NP concentrations around 2×10^{-7}M, but this concentration of NP added during the plateau phase produces about 40% inhibition. The two sets of data agree quite well for KCl and ACh, however.

1.5.2 Effect of V. (Fig R4-2A,B,C). - The differences in the inhibitory effect of V on each of the three agonists are much less pronounced. In all cases the effect of V (5×10^{-7}M) is much slower than NP, (2×10^{-7}M), which makes interpretation of the results much more subjective. On the basis of typical traces it appears that the inhibitory effect of V on the three agonists is in the order NA > ACh >> KCl, with NA being inhibited the most. It should be noted that this is very different from the differential effects of verapamil when added prior to the agonists, in which case it is the KCl contractions which are inhibited most strongly. This difference is probably due to the fact that the effects of V on an established contraction are different to those on a developing contraction (see discussion).

1.6 Addition of Agonists.

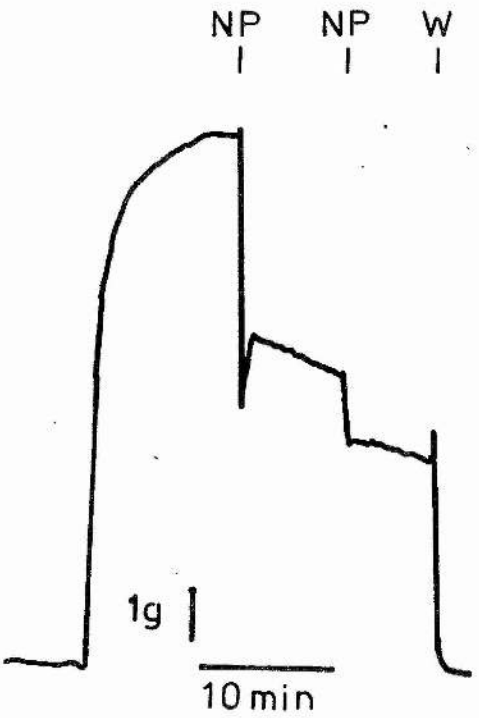
If KCl is added to the tissue during the plateau phase of a

Fig R4-1A NP added during the plateau of contraction; NA

The figure shows a typical contraction induced by NA (2.5×10^{-5} M). At "NP" a dose of Nitroprusside (2×10^{-7} M) was added to the bath. The dose was repeated at the second "NP" label (producing a bath concentration of 4×10^{-7} M). The bath was washed out at "W".

Fig R4-2A V added during the plateau of contraction; NA

The figure shows a typical contraction induced by NA (2.5×10^{-5} M). At "V" a dose of Verapamil (5×10^{-7} M) was added to the bath. At "NP" a dose of Nitroprusside (2×10^{-7} M) was added, in order to test the responsiveness of the tissue. The bath was washed out at "W".


NP
NP
W
1g
10 min

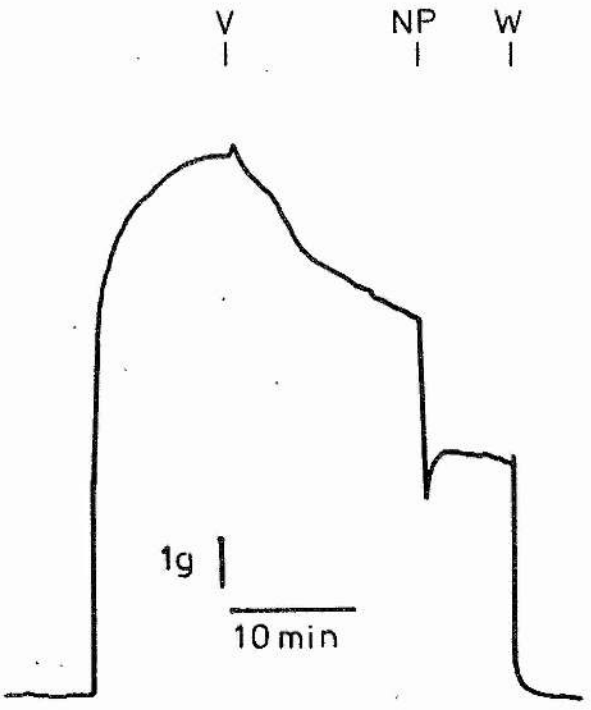
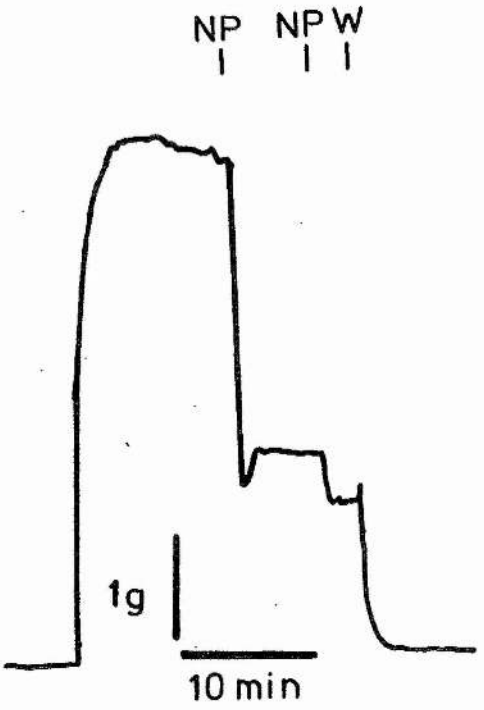

V
NP
W
1g
10 min

Fig R4-1B NP added during the plateau of contraction; ACh

As for Fig R4-1A, except that the agonist used was ACh (2×10^{-3} M). Note that the calibration is different from Fig R4-1A.

Fig R4-2B V added during the plateau of contraction; ACh

As for Fig R4-2A, except that the agonist used was ACh (2×10^{-3} M). Note that the calibration is different from Fig R4-2A.


NP
NP
W
1g
10 min

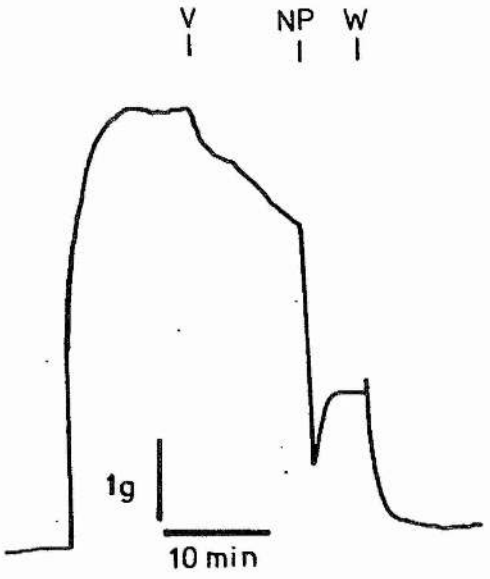
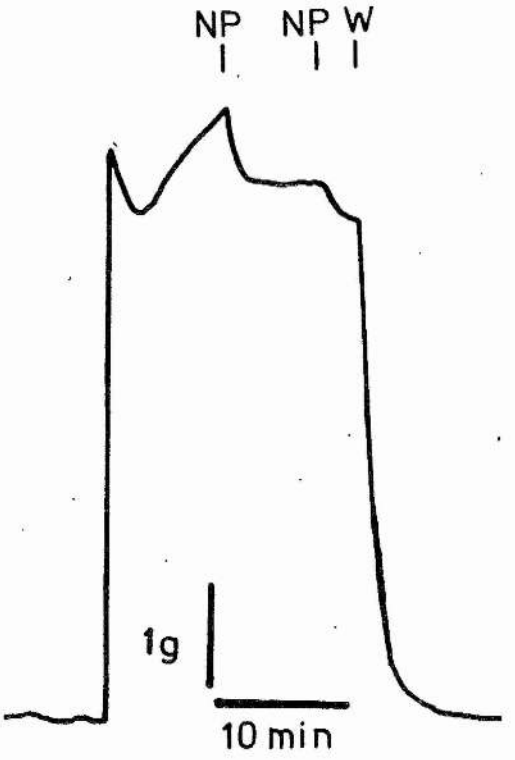

V
NP
W
1g
10 min

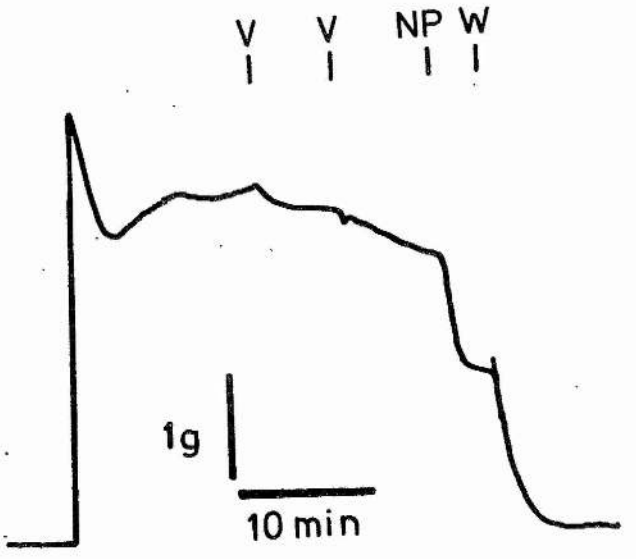
Fig R4-1C NP added during the plateau of contraction; KCl

As for Fig R4-1A, except that the agonist used was KCl (80mM). Note that the calibration is different from Fig R4-1A.

Fig R4-2C V added during the plateau of contraction; KCl

As for Fig R4-2A, except that the agonist used was KCl (80mM). Note that the dose of V was added twice in this case (so the bath concentration at the second "V" $=10^{-6}$M). This was done because of the apparent lack of effect of V. Note that the calibration is different from Fig R4-2A.


NP
NP
W
1g
10 min


V
V
NP
W
1g
10 min

pre-existing contraction to ACh or NA it produces inhibition. This fast inhibitory effect has been investigated by Gillespie (1972) and has been shown to be due to the release of inhibitory transmitter by depolarisation of nerve terminals. (there is also a slow inhibitory effect of KCl, which is not relevant in this case, -see discussion) However, if the agonists are added in the reverse order, KCl first then ACh or NA, then the second agonist produces an additional contraction. This effect is shown in Fig R5. This effect is interesting in that it implies a phrmaco-mechanical coupling of NA and ACh (ie. if the membrane is maximally depolarised by the presence of 80mM KCl, the subsequent addition of NA or ACh has produced an additional contraction without additional membrane depolarisation. This effect is investigated more fully in the section on electrophysiology.)

It was found that the combined tension produced by the addition of ACh to a KCl contraction approximates to the tension produced when an ACh contraction was inhibited by the addition of KCl (ie. the final tension is independent of the order of addition of the agonists). This applied also to a certain extent in the case of NA, although usually the tension produced by NA then KCl was greater than the that produced by KCl then NA (see Fig R5).

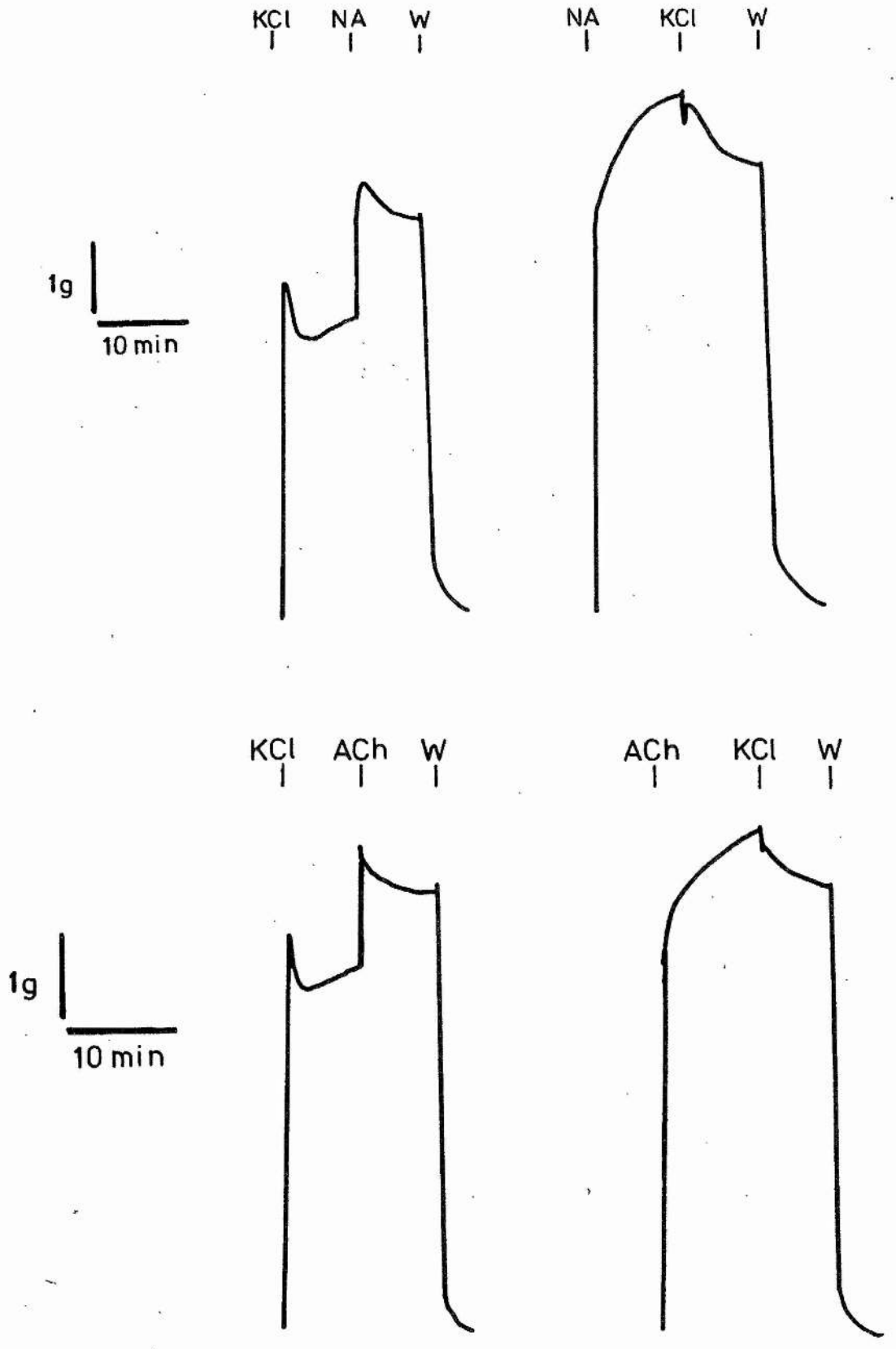
This relationship was only true in cases where the inhibitory effect of KCl was reasonably long lasting, however. In many cases the inhibition was transitory (lasting 1 to 2 minutes), which made interpretation difficult. It seems likely that these

Fig R5 A Addition of agonists; NA/KCl.

At "KCl" a contraction was induced by 80mM KCl. At "NA" a dose of NA (2.5×10^{-5} M) was added. The bath was washed at "W". The second trace shows the effects of the same doses of the agonists added in the reverse order.

Fig R5 B Addition of agonists; ACh/KCl

At "KCl" a contraction was induced by 80mM KCl. At "ACh" a dose of ACh (2×10^{-5} M) was added. The bath was washed at "W". The second trace shows the same doses of the agonists added in the reverse order.


KCl
NA
W
NA
KCl
W
1g
10 min
KCl
ACh
W
ACh
KCl
W
1g
10 min

differences in the inhibitory effect of KCl may be due to different degrees of inhibitory innervation in different muscles, leading to either more release of transmitter , or a greater removal of transmitter by uptake mechanisms, metabolism, etc.

The additional contractions produced by the agonists added in this way retain their distinct responses to the antagonists. This can be see in Fig R6-A. In this case ACh is added to a pre-existing KCl contraction. The effect of two different concentrations of V on this is shown. It can be seen that the initial KCl contraction is almost completely inhibited by V (5 x 10^{-6}M.), whereas the subsequent ACh contraction is unaffected, indeed it is actually slightly potentiated.

All three agonists can be added in sequence, each eliciting an additional contraction. Fig R6-B shows the effect of adding KCl then ACh then NA to the preparation. It can be seen that NA can produce an additional contraction even in the presence of a pre-existing contraction produced by KCl and ACh together.

Fig R6 A Addition of agonists; Effect of Verapamil

The trace shows the response to the two agonists KCl and ACh added as in Fig R5B$_7$(second trace). At the break Verapamil (5×10^{-7}M) was added to the bath and equilibrated for 15min. The combination of the agonists was then repeated. At the second break the dose of Verapamil was increased to 5×10^{-6}M. (again equilibrated for 15min.) The combination of the agonists was then repeated once more. "W" signifies bath washout in each case.

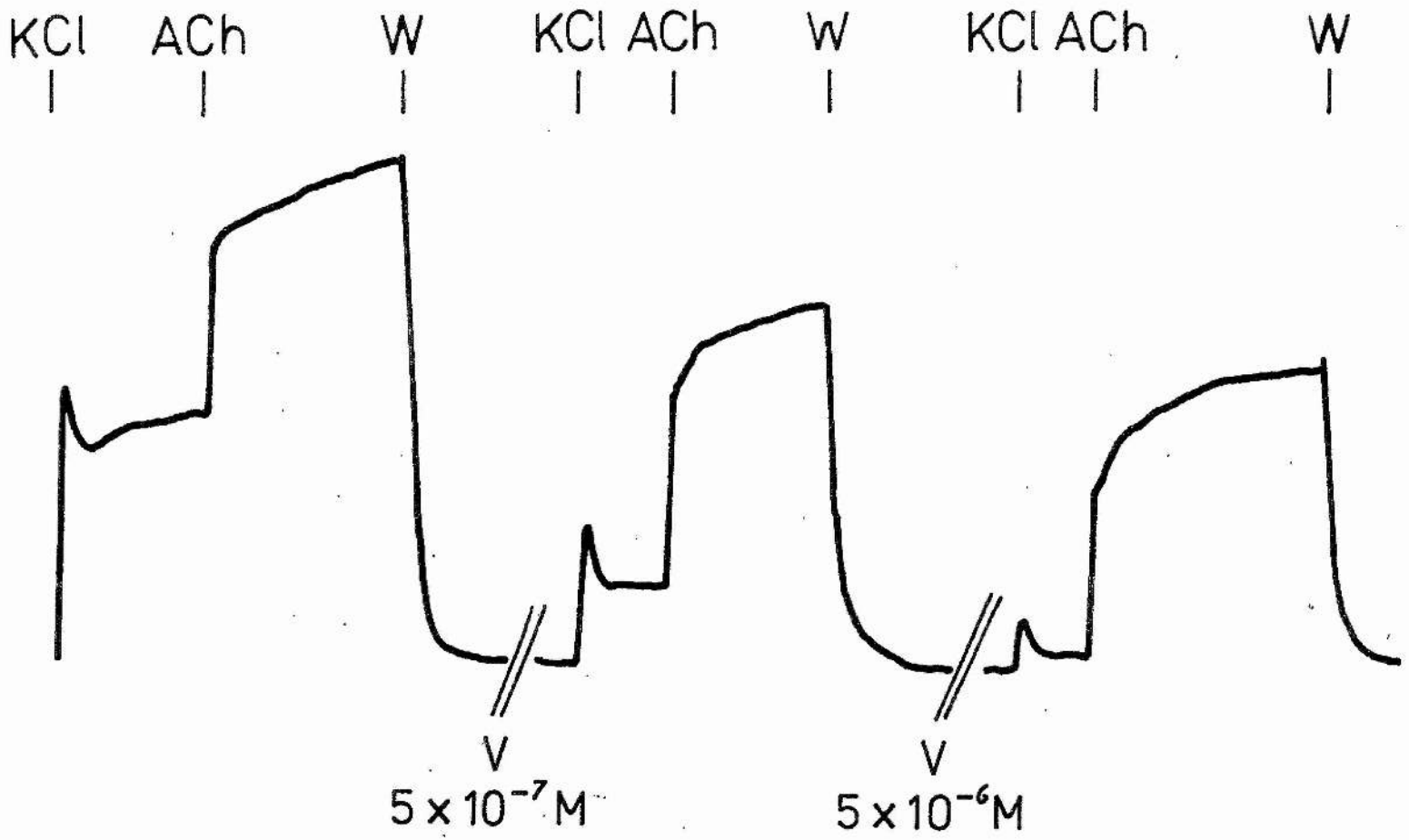
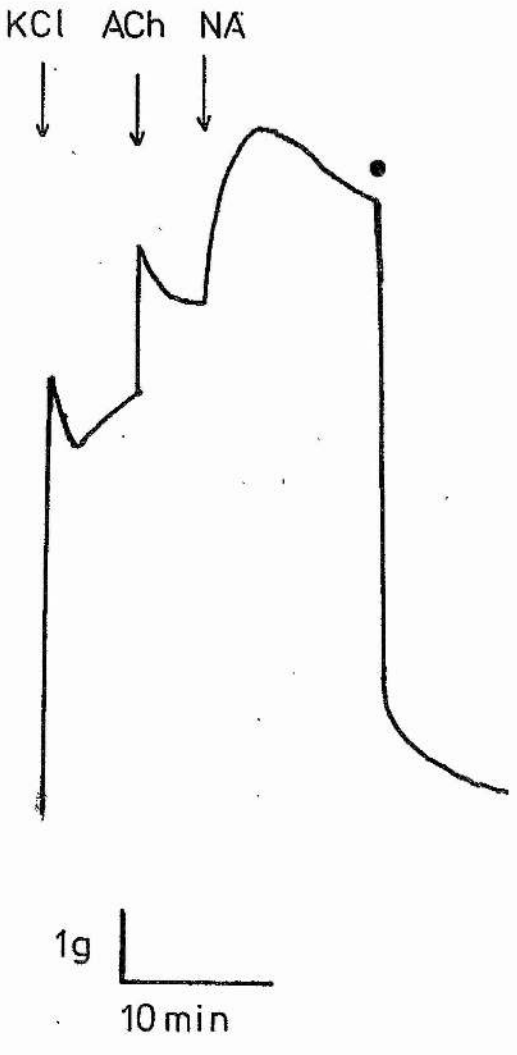

KCl
ACh
W
KCl
ACh
W
KCl
ACh
W
V
5 x 10⁻⁷ M
V
5 x 10⁻⁶ M

Fig R6 B Addition of agonists.

The trace shows that all three agonists can be added sequentially, each producing an additional rise in tension. The doses used were KCl =80mM, ACh =2 x 10^{-3}M and NA =2.5 x 10^{-5}M.


KCl
ACh
NA
1g
10 min

2.0 CYCLIC NUCLEOTIDE STUDIES

2.1 Justification of Methods.

Table R2 gives the results of a typical assay for cAMP. "Count" in this case is the number of counts in 10 minutes. Since no correction is made for variations in counting efficiency between the samples (ie by external standards ratio etc.), the data is used in the form of counts in 10 minutes directly, rather than being converted to DPM. It is unlikely that variations in counting efficiency between samples will be large enough to interfere with the accuracy of the assay.

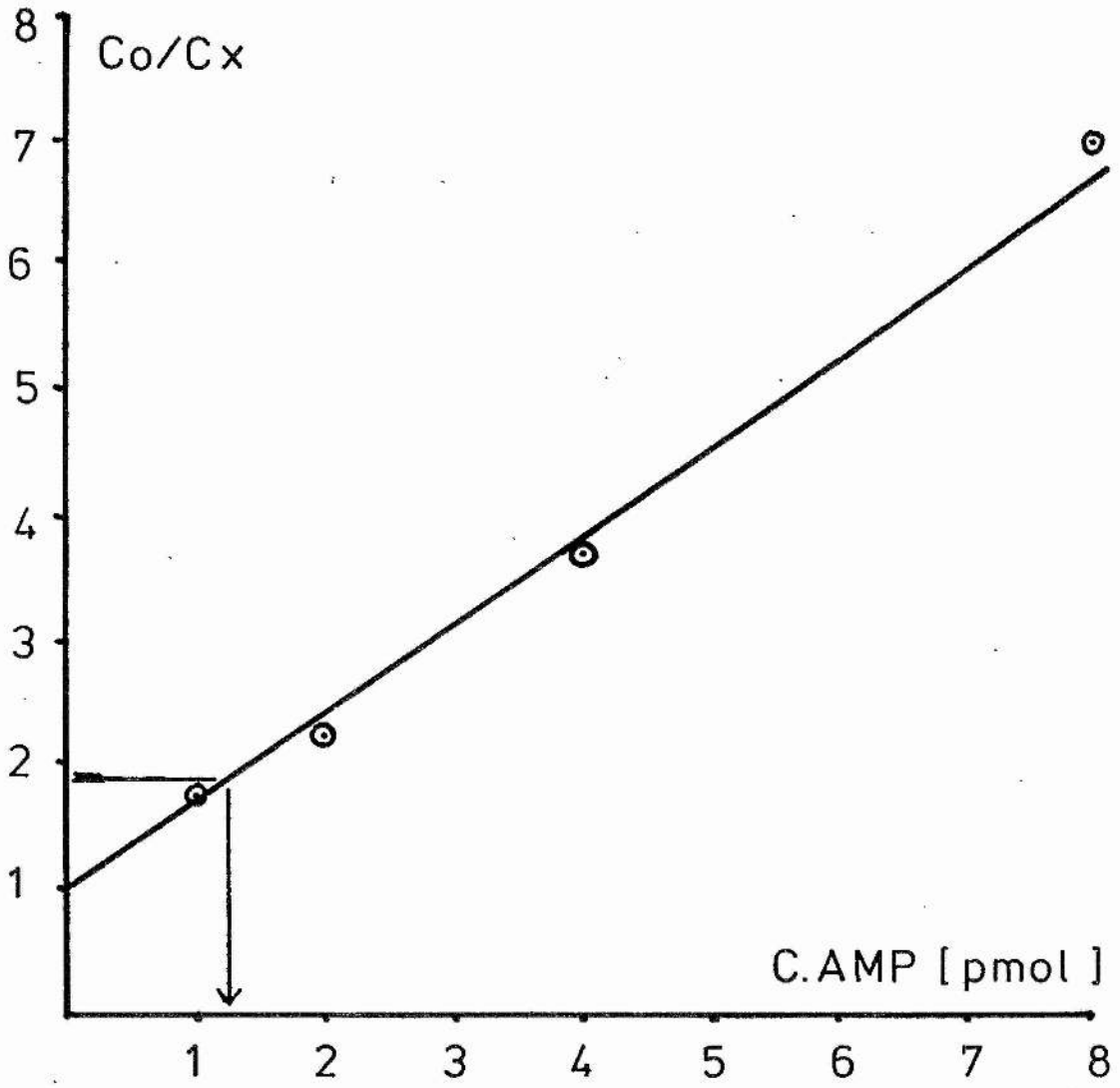
TABLE R2.

TUBE	C.P.M.	C.P.M-BLANK (Cx)	Co/Cx	CONTENTS
1,2	3122	-	—	Blank
3,4	7262	4140	1.0	Zero
5,5	5446	2324	1.78	1 pmol std.
7,8	4960	1838	2.25	2 pmol std.
9,10	4226	1104	3.75	4 pmol std.
11,12	3706	584	7.08	8 pmol std.
13,14	5326	2204	1.87	Unknown

This data gives the calibration curve shown in Fig R7. It was found generally that the calibration curves were linear up to 8 pmol, but at higher levels of cAMP or cGMP the calibration becomes less linear. However, most of the samples were below 4pmol, which is the most accurate part of the calibration. The assay has a co-efficient of variation of less than 11% over the range 0.2 - 16pmol and less than 7% over the range 0.5 - 4pmol. (data quoted by Amersham).

Fig R7 Calibration curve for cAMP assay.

The graph shows a typical calibration curve for the cAMP assay. Arrow shows an example of use (Co/Cx =1.9, cAMP =1.25pmol).


Co/Cx
8
7
6
5
4
3
2
1
C.AMP [pmol]
1
2
3
4
5
6
7
8

The calibration curve was checked for each assay, with a minimum of two standards being determined, unless a new kit was being used, some experimental procedure had been changed, or a kit had been stored for some time. In these cases the complete curve was re-determined.

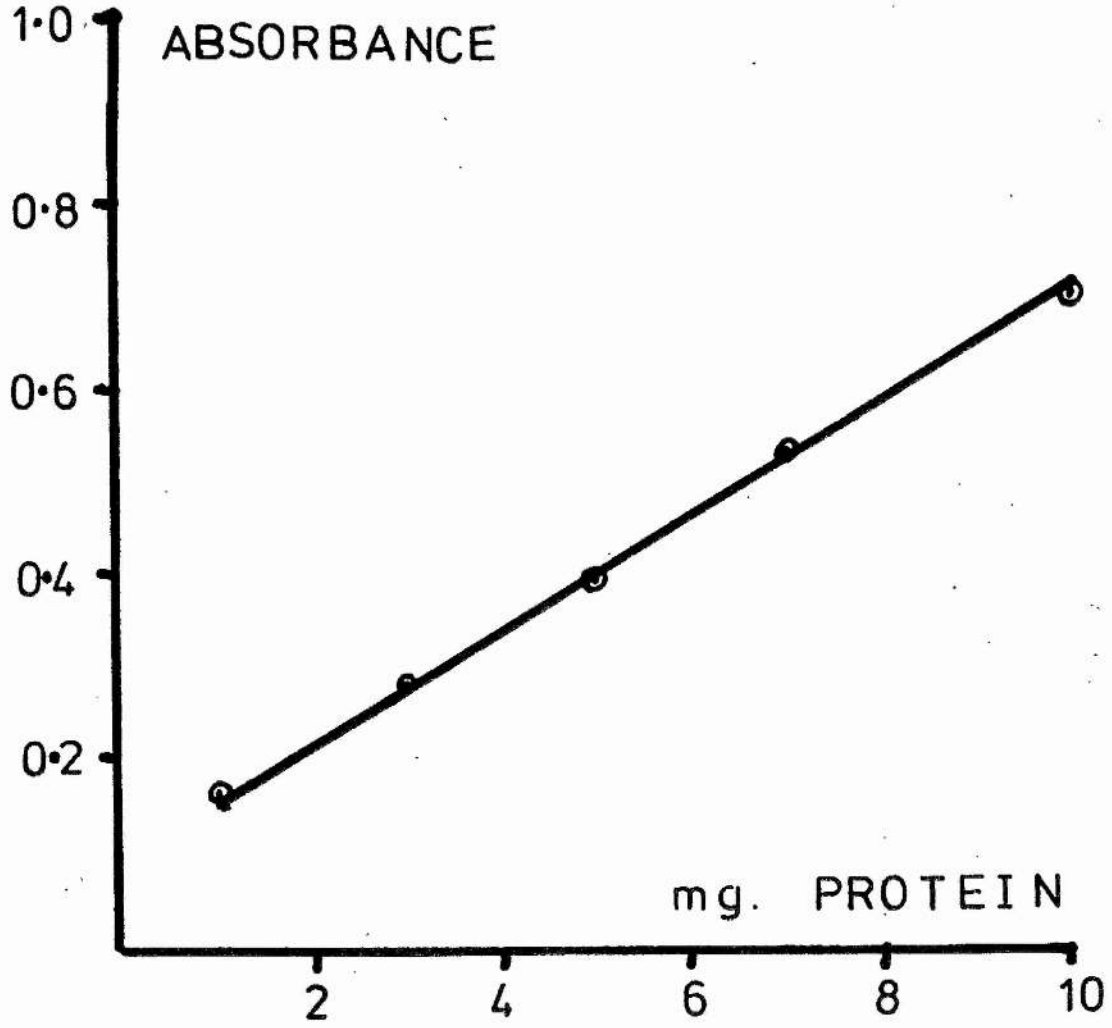
The main problem with storage of the kits is degredation of the binding specificity and affinity of the binding protein (in the case of cAMP) or of the antiserum (in the case of cGMP). Generally the binding protein, or antiserum, was split into aliquots for storage and only one thawed for use at a time. This minimises the number of freeze/thaw cycles that the binding protein or antiserum has to undergo. It was found that the binding protein or antiserum could be stored for 2 months, when not repeatedly thawed and re-frozen, without significant effects being produced on the calibration of the assay.

The unknown in this experiment, as read from the calibration curve shown, contains 1.25 pmol (in the 100ul sample). The total in the original 500ul of buffer is therefore 6.25 pmol.

The calibration curve for the protein assay for this particular experiment is shown in Fig R8. The unknown contains 9.85 mg protein.

The two results are then used to express the final result in terms of cAMP/mg protein, in this case 0.63 pmol/mg. The recovery in this particular experiment was 55% for protein and 75% for cAMP.

Fig R8 Calibration Curve for Protein Assay.


1·0
ABSORBANCE
0·8
0·6
0·4
0·2
mg. PROTEIN
2
4
6
8
10

The results for the cGMP assays were treated in the same way.

2.2 Results.

The data obtained from the experiments is shown in table R3. This shows the cAMP and cGMP levels (expressed in pmol/mg protein) for the control (untreated) muscles, and the changes produced by treatment with the three agonists. Also shown are the effects of pre-treatment of the muscle with NP (10^{-7}M). These experiments, (shown as NA/NP or KCl/NP), were performed in the same way as the NA and KCl experiments respectively, except that NP (2 x 10-7) was added to the bath 15min before the agonist.

The column headed "Protein (mg)" shows the amount of protein for each group of pooled muscles used in the experiments. Note that the result is for 6 to 8 muscles, so that the protein content of one muscle is about 1 to 1.5mg. The wet weight of one muscle is of the order 5 to 7mg. Note that the protein result obtained for experiment NA 1 is low compared to the rest. This is because the tube was dropped in this experiment, leading to some loss of the extract. The results for cAMP and cGMP are very similar to those for the other NA experiments, so the result is included here. It was assumed that the extract was homogeneous at this stage so the loss did not affect the levels of cyclic nucleotides expressed as pmol/mg protein.

The column headed "P value" shows the probability level associated with each result, compared to the control value. This was calculated using Student's paired T test. The P value for the NA/NP group compared to the NA group is < 0.001. The P value for

Table R3 Cyclic Nucleotide Assay Results.

TABLE R3

EXPERIMENT	PROTEIN (mg)	cAMP (pmol/mg protein)			cGMP (pmol/mg protein)		
		x	Mean	P value	x	Mean	P value
Control 1	8.25	0.94			0.39		
Control 2	9.0	1.27	0.90	-	0.58	0.37	-
Control 3	9.5	0.5			0.14		
NA 1	3.8	1.66			0.197		
NA 2	9.5	1.26	1.39	0.1 - 0.2	0.147	0.168	0.1 - 0.2
NA 3	7.8	1.25			0.16		
ACh 1	9.1	0.66			0.096		
ACh 2	10.1	0.87	0.76	> 0.5	0.123	0.109	0.2 - 0.4
KCl 1	9.85	0.63			0.137		
KCl 2	9.9	0.71	0.76	> 0.5	0.156	0.164	0.1 - 0.2
KCl 3	8.5	0.94			0.2		
NA/NP 1	6.6	0.68			0.71		
NA/NP 2	10.7	0.72	0.7	> 0.5	0.81	0.76	< 0.1
KCl/NP	7.25	0.98			0.63		

the KCl/NP experiment compared to the KCl group is < 0.01.

The results show that the level of cAMP in this tissue is low and variable in unstimulated muscles. (0.9 pmol/mg). It appears to be changed little by the action of the agonists, except possibly by NA, which causes a slight rise, (from 0.9 to 1.39 pmol/mg. $0.1 < P < 0.2$). All three agonists cause a pronounced fall in cGMP levels, however. The significance levels for these changes are low, but this is probably due to the small number of data in each group.

Pre-treatment with NP prevents the fall in cGMP levels caused by the agonists, rather it is replaced by a definite rise (from 0.37 to 0.76 pmol/mg) This effect is shown when either NA or KCl are used as the agonist. NP does not appear to affect the cAMP levels when the muscle is stimulated with NA or KCl, although the slight rise in cAMP levels caused by NA is not seen in this case. This may be a secondary effect due to the fact that cGMP does not fall- see discussion.

3.0 ELECTROPHYSIOLOGY

3.1 General

Only in exceptional circumstances could a cell be recorded from continuosly, using the intracellular microelectrodes obtained, for more than 20 seconds or so. Usually the potential was seen to drift after this time as the electrode came out of the cell. In most cases any attempt to stimulate the muscle while a cell was impaled resulted in the electrode being dislodged. Ths was in spite of the fact that the muscle was under fairly high resting tension and also that the cells were usually impaled near to an imobilised area of the muscle (ie. near to one of the pins). In some instances, however, it was possible to record from a cell while the muscle was stimulated in some way, possibly because the cells in these cases were larger than average. Examples of these traces are shown in Figs R9-1 to R9-5.

3.2 Results

The results of the "population" measurements of membrane potential are shown in tables R4 and R5.

Table R4 shows the membrane potential of a sample population of cells for control (unstimulated) muscles and the membrane potential of a sample population of cells after the muscle had been stimulated by each of the three agonists, NA, ACh and KCl. For each of these situations the number of cells penetrated (n), the

TABLE R4

Electrophysiology results; Effect of NP.

TABLE R5

Electrophysiology results; Effect of V.

	NO ANTAGONIST			NP (2×10^{-7} M)			
	n	Mean	SE	n	Mean	SE	P value
Control	26	-55.2	1.22	20	-58.0	1.16	0.1
NA	22	-30.8	1.26	24	-35.1	0.82	> 0.001
ACh	21	-27.3	0.87	22	-33.0	0.76	< 0.001
KCl	30	-19.8	0.26	18	-20.2	0.35	> 0.2

	NO ANTAGONIST			V (5×10^{-7} M)			
	n	Mean	SE	n	Mean	SE	P value
Control	26	-55.2	1.22	21	-51.8	1.26	> 0.05
NA	22	-30.8	1.26	19	-34.2	1.15	> 0.05
ACh	21	-27.3	0.87	22	-31.6	0.86	=0.001
KCl	30	-19.8	0.26	17	-19.1	0.42	> 0.01

mean of the membrane potentials measured (Mean), and the standard error of this mean (S.E.), are shown. The second column of results, headed NP (2×10^{-5}M), gives the results of the same measurements in the presence of NP. The final column shows the significance level of the result in the presence of NP as compared to that in the "no antagonist" situation.

Table R5 shows the same results as table R4 for the "no antagonist" situation in the first column, but these are compared to results obtained in the presence of V (5×10^{-7}M), shown in the second column. Again, the P value is that compared to the "no antagonist" situation.

It can be seen that the resting membrane potential measured in the control situation was -55.2mV. This is not significantly changed by the presence of NP or V. A maximal dose of NA produces a depolarisation of the membrane to a potential of -30.8 mV. The depolarisation produced in the presence of NP is slighly less, to a potential of -35.1mV. The difference between these results is significant, ($0.01 > P > 0.001$). The depolarisation produced by NA in the presence of V is again slightly less than that produced in the "no antagonist" situation, although in this case the difference is less significant, ($P > 0.05$).

A maximal dose of ACh produces a depolarisation of the membrane to -27.3mV in the "no antagonist" situation. Both NP and V reduce this deplolarisation by a significant amount. In the presence of NP the depolarisation is to -33.0 mV, ($p < 0.001$).

In the presence of V the depolarisation is to -31.6 mV, ($P < 0.001$).

KCl (80mM) produced a membrane depolarisation to a potential of -19.8 mV. This was not significantly affected by NP or V.

To Summarise;

NA produces a depolarisation to about -31mV. This depolarisation is slightly reduced in the presence of NP (slightly significant) and also slightly reduced in the presence of V (not significant).

ACh produces a depolarisation to about -27mV. This depolarisation is reduced in the presence of NP and V (both highly significant).

KCl produces a depolarisation to about -20 mV. This depolarisation is not affected by NP or V.

Note that although KCl produces the greatest depolarisation, it produces the smallest increase in tension in the mechanical experiments.

Fig R9-1

Trace showing a penetration of a cell which meets the criteria stated in the text. Upper trace shows potential recording, lower trace shows tension (not operative in this case). At "A" a cell was penetrated, recording a membrane potential of approximately -52mV. At "B" the electrode potential drifts, indicating that the electrode is leaving the cell.

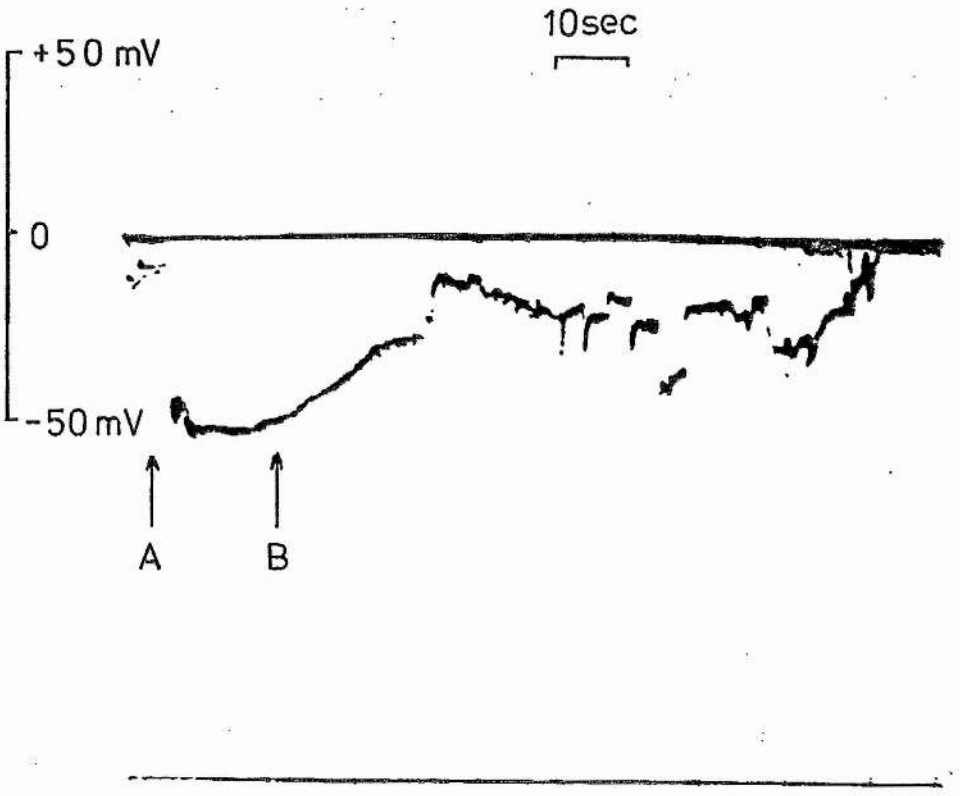
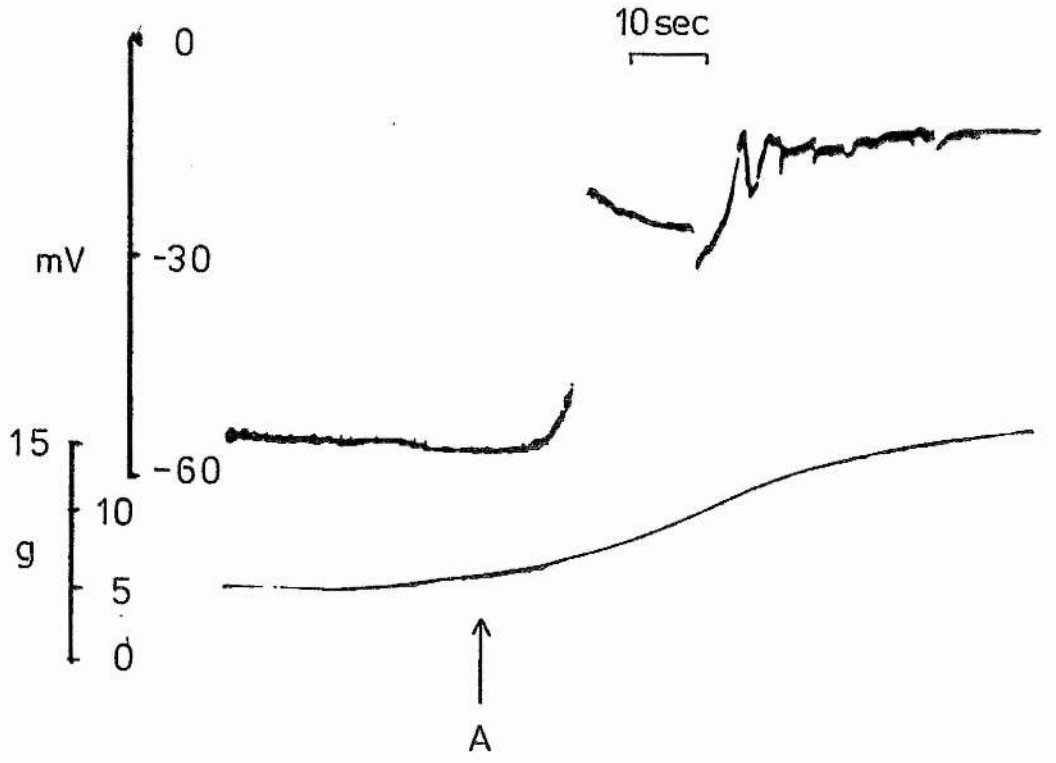

10sec
+50 mV
0
-50 mV
A
B

Fig R9-2 Effect of NA.

Upper trace shows a resting membrane potential of approximately -55mV. At "A" a maximal dose of NA was added to the bath. This causes a rise in tension from 5g to about 15g and a depolarisation to about -25mV. The temporal difference in the traces is due to the fact that the agonist is added to one end of the bath and distributed by the perfusion and by diffusion.

Fig R9-3 Effect of ACh.

Upper trace shows a resting membrane potential of approximately -60mV. At "A" a maximal dose of ACh was added to the bath. This causes a rise in tension from 5g to about 10g and a depolarisation to approximately -20mV.


10 sec
0
mV
-30
-60
15
10
g
5
0
A

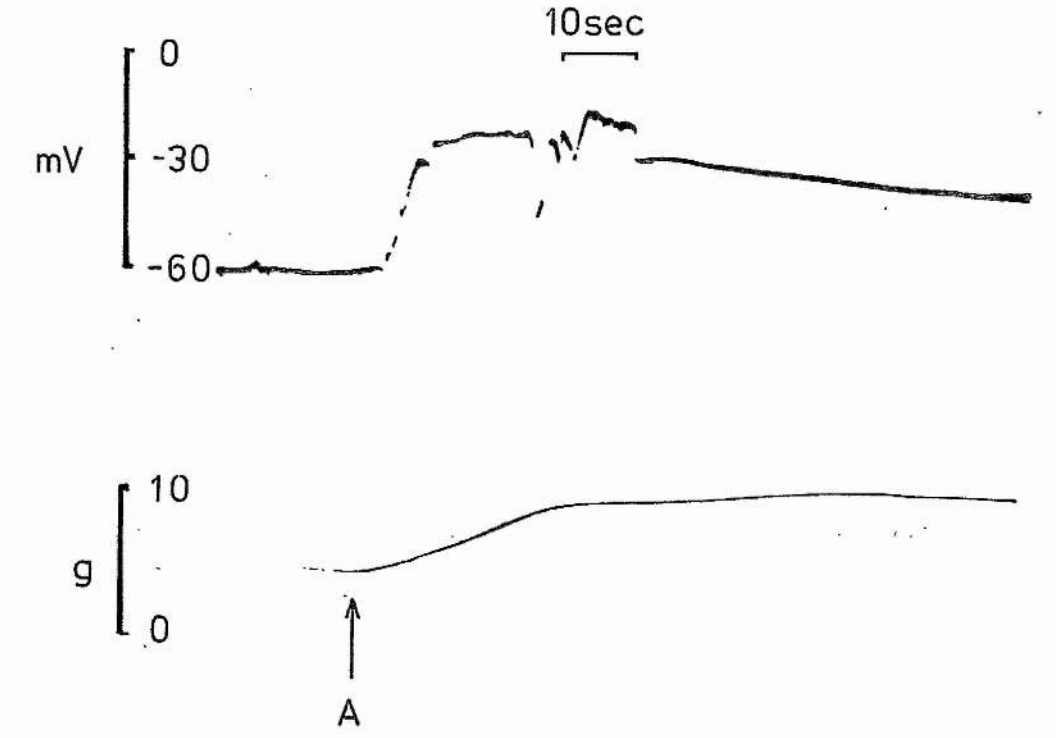
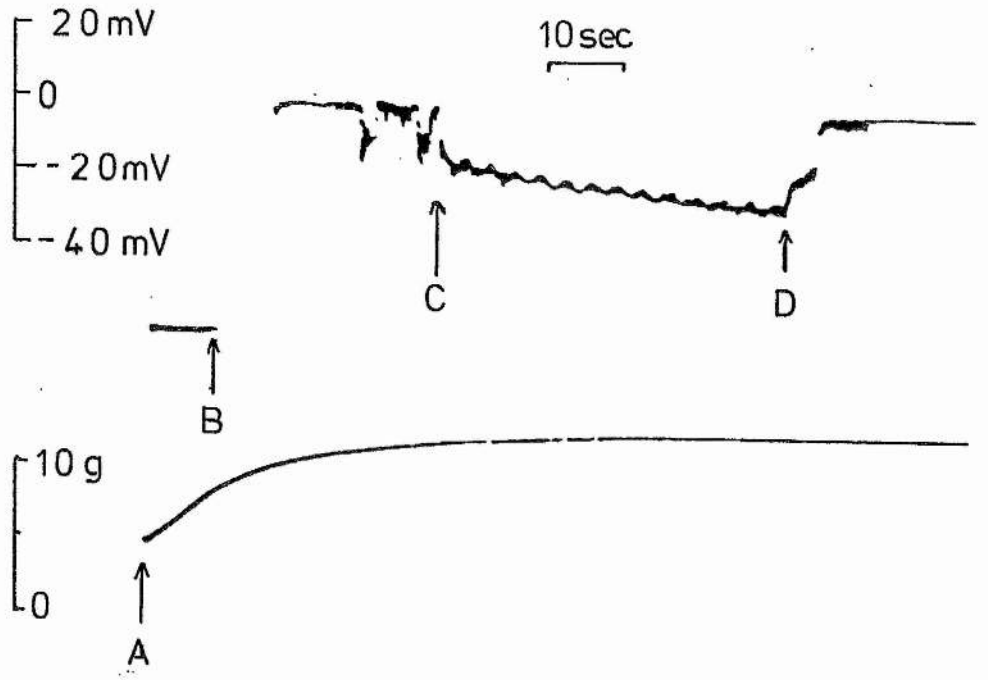

10sec
0
mV
-30
-60
10
g
0
A

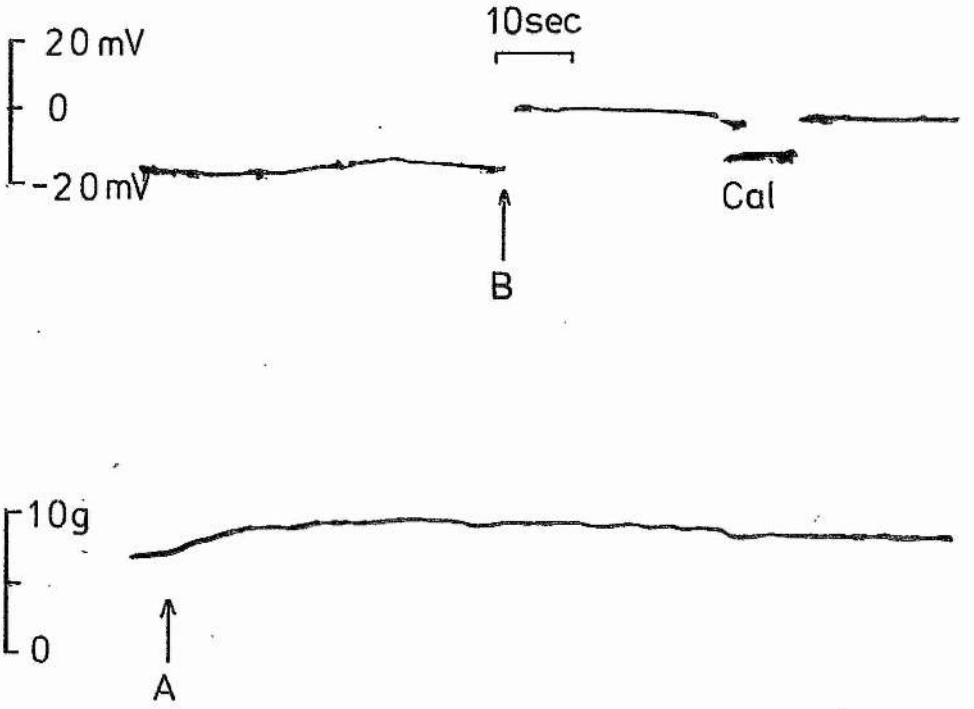
Fig R9-4 Membrane Oscillations.

At "A" a maximal dose of ACh was added to the bath. This causes the rise in tension seen on the lower trace, but dislodges the electrode from the cell at "B" (previously recording a resting potential of approximately -60mV). At "C" another cell is penetrated, giving a membrane potential of approximately 20mV, which slowly increases to approximately -30mV. The membrane potential shows oscillations of about 2mV amplitude at a rate of 0.3Hz. The electrode leaves the cell at "D".

Fig R9-5 Addition of Agonists.

Upper trace shows the membrane potential recorded from a cell in 80mM KCl solution. Lower trace shows the tension recorded. At "A" a maximal dose of NA was added to the bath. This produces a rise in tension but only a small additional depolarisation. The electrode was dislodged at "B". "Cal" indicates a 10mV signal from the pre-amplifier.


20 mV
0
-20 mV
-40 mV
10 sec
C
D
B
10 g
0
A


20 mV
0
-20 mV
10sec
Cal
B
10g
0
A

DISCUSSION

SECTION 1; ACTIVATION BY THE THREE AGONISTS.

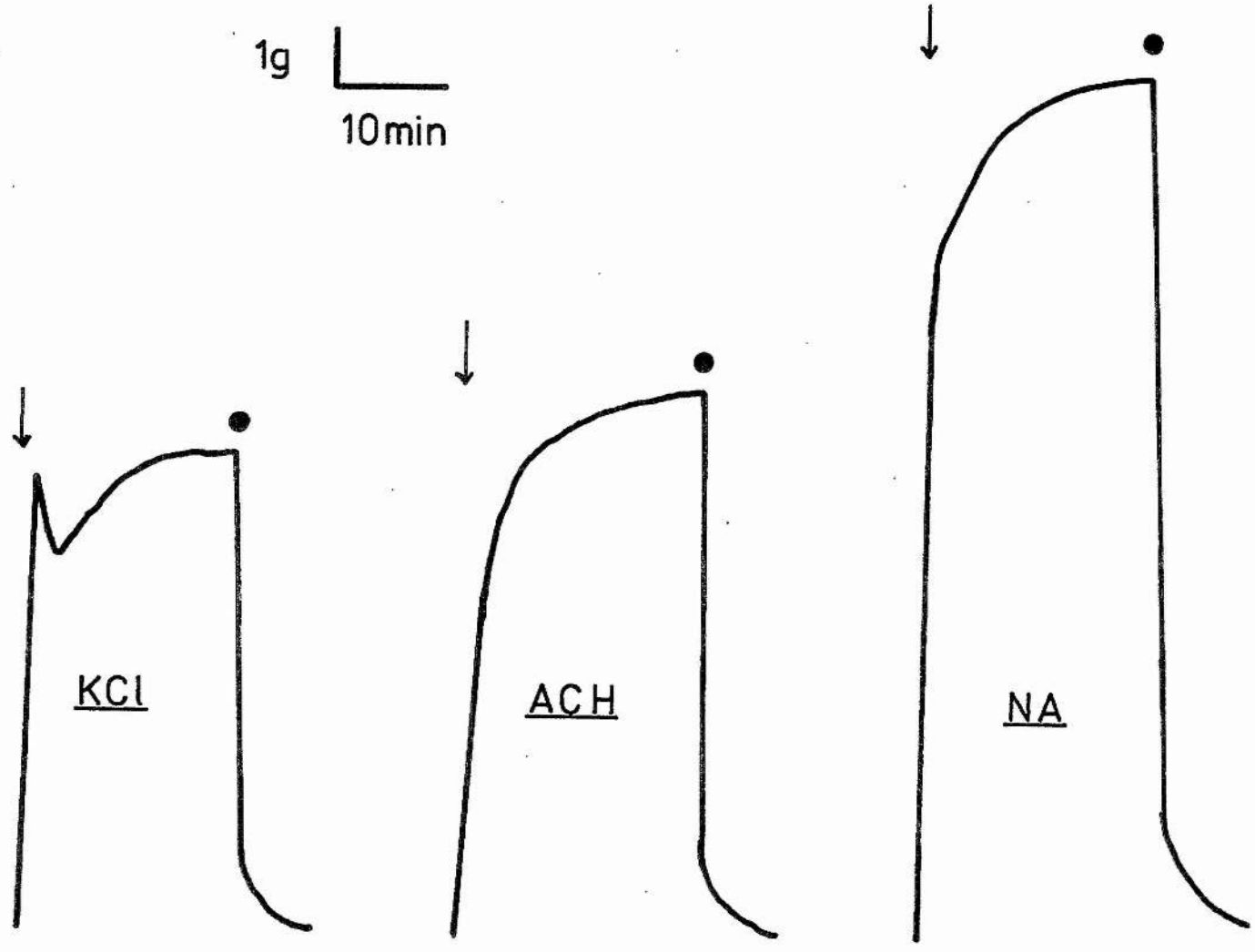
1.0 Introduction;

Fig D1 shows the typical contraction produced by a maximal dose of each of the three agonists. It can be seen that ACh and NA both produce a very similar type of contraction, with a rapid rise in tension to about 75% of the final tension, then a slow further increase in tension to the maximum finally attained, over a period of some 15 to 20 minutes. A maximal dose of KCl, however, produces a contraction showing a bi- phasic form.

Evidence presented later (section 2) indicates that KCl contractions depend to a large extent upon an influx of extracellular calcium. The properties of the two phases were found to be very similar with respect to the effects of antagonists (especially Verapmil). This indicates that the two phases of KCl contractions are probably not due to different activation pathways, rather, the bi- phasic form of the contraction appears to be due to calcium sequestration or pumping mechanisms. If the initial influx of calcium causes a rise in intracellular levels of calcium which is then taken up into intracellular stores, this could cause the transient primary phase seen. If these stores subsequently become saturated, the level of calcium would once again rise. In this way the bi- phasic form of the contraction can be accounted for by calcium eqilibration within the cell. It is possible that the calcium induced release of calcium described by Cheng (1976) plays a part in this process. If this release of calcium is

Fig D1 Typical Contractions induced by each Agonist.

Contractions induced by 80mM KCl, 2×10^{-3}M ACh and 2.5×10^{-5}M NA.


1g
10min
KCl
ACH
NA

somewhat delayed, this could also contibute to the production of a bi- phasic type of contraction.

It appears that NA and ACh do not produce this type of bi-phasic contraction because they use different activation pathways. NA and ACh induced contractions do not depend to any great extent upon the influx of extracellular calcium (section 2), but rather they produce an amplification of a small, initial calcium signal. This would mean that the calcium equilibration processes during contraction are different.

The other main difference in the contractions is that the maxima are very different. This is illustrated better by the agonist dose response curves shown in Fig R1. These different maxima may be due to several factors.

1) It is accepted that the contraction of smooth muscle is due to a rise in the intracellular concentration of calcium, in the same way as skeletal muscle (Sandow 1965 , Gordon 1978).
NA and ACh may produce a greater permeability change in the membrane, so allowing a greater influx of calcium, hence a greater rise in cytoplasmic calcium levels (as will be seen, this is unlikely).

2) NA (and to a lesser extent, ACh) may cause the release of intracellular stores of calcium, while KCl does not. If NA is more effective at producing this effect, it would produce a greater

rise in cytoplasmic calcium levels, without a greater influx. Such a release of intracellular calcium by NA activation has been proposed in many smooth muscles. (Collins, Sutter and Teiser 1972, Deth and Van Breemen 1974, Godfraind 1976, Keating 1972).

3) It is possible that NA and ACh are capable of indirectly increasing the response of the contractile apparatus to a given calcium concentration. In this way it would be possible for them to produce a greater response although the rise in intracellular calcium levels would not be as great as with KCl activation.

These possibilities are disscussed in more detail in the following sections.

Another point of interest in the agonist dose response curves is that while NA and ACh produce a graded response from very low doses, KCl shows a distinct mechanical threshold, at around 20mM. Below this concentration no consistent response is produced, while above it the developed tension rises rapidly with increasing KCl concentration.

This type of effect has been investigated by Syson and Huddart (1973) in the rat vas deferens and ileum, where they found that KCl produced no appreciable mechanical threshold. This difference may be due to the fact that rat vas deferens and ileum are spontaneously active and produce action potentials (APs). Since KCl, at low concentrations, was found to increase spike frequency,

(although at higher concentrations it will suppress spike generation and produce a depolarisation of the membrane, Syson and Huddart 1976), this may be the way in which it increases tension in this preparation. A gradual increase in spike frequency would be expected to produce a graded increase in tension, since one of the main current carriers during the AP is calcium (Kuriyama 1970).

The rat anococcygeus does not produce APs (this study, also Creed, Gillespie and Muir 1975), so such a mechanism is not possible. The mechanical threshold seen in this muscle is a reflection of the fact that the depolarisation caused by KCl opens voltage dependent channels at some threshold value, this threshold being at a KCl concentration of about 20mM. This gives the effect of a sudden "switch on" of the activation process with increasing KCl concentrations, at the point at which membrane depolarisation is sufficient to open voltage dependent channels (VDCs), so allowing the influx of extracellular calcium.

The fact that NA and ACh do not produce such a mechanical threshold indicates that the activation processes used by them are different. This would support the idea that membrane depolarisation leading to the opening of voltage dependent channels is not the most important event in the activation by NA and ACh. It may be that they produce opening of receptor operated channels (ROCs) in the membrane, and this could produce the finely graded responses seen. However, the electrophysiology section of this study shows that a dose of NA which produces a depolarisation of

the membrane to -30.8mV produces a greater response than a dose of ACh which depolarises the membrane to -27.4mV (a greater depolarisation). 80mM KCl produced the greatest depolarisation (to -19.8mV), but the least rise in tension. This indicates that NA especially, but also ACh, must produce some "amplification stage" in the activation process. If this process can respond in a graded manner, this would also give the graded responses seen.

The following sections consider the ways in which the agonists could produce different maxima, and the evidence that membrane depolarisation per se is not the fundamental factor in determining the response of the muscle. The evidence indicates that important "second messenger" changes ocurr, especially in the level of cyclic nucleotides, and these may control the contractility of the muscle.

2.0 Electrophysiology

The resting membrane potential of a population of cells in the rat anococcygeus muscle was -55.2mV (S.E. 1.22), measured in control conditions (see table R4). Creed (1974) quotes a value for this of -62.1mV (S.D 7.8) . However, Creed, Gillespie and Muir (1975) reported a slight change in the resting membrane potential when the resting tension was changed. At a resting tension of < 0.75g they recorded a potential of -58.1mV (S.D. 5mV). When the muscle was stretched to the point where stimulation no longer produced any movement, the potential was -61.5mV (S.D. 8.1) Although this difference was not statistically significant, the results do indicate that a slightly different resting membrane potential can be recorded depending upon the experimental

conditions. On this basis the value of -55.2mV (S.E. 1.22) recorded in this study indicates that the recording system used gave results which were comparable to those obtained by other workers.

NA (2×10^{-5}M) produced a membrane depolarisation to a value of -30.8mV. This concentration of NA produced a rise in tension of about 250Nm^{-2}. This value agrees well with other published results (Creed et al 1975). The effects of NA (and other agonists) are different in the rat anococcygeus muscle to those in many smooth muscles, since the anococcygeus muscle does not produce action potentials under normal circumstances (Creed 1975). The rat anococcygeus is also reported to give no resonse to B adrenergic stimulation , so the response seen to NA is an response.

Alpha adrenergic stimulation is generally accompanied by depolarisation, and an increase in the frequency of APs (in those muscles producing APs) (Bolton 1979), although a number of papers have been published which report that activation can produce a contraction with little or no change in the membrane potential in a number of smooth muscles (Casteels et al 1977, Droogmans and Casteels 1977, Su, Bevan and Ursillo 1964, Droogmans et al 1977). The effect of receptor stimulation generally appears to be an increase in membrane conductance, which implies that the receptors are capable of opening ion channels in the membrane (Receptor Operated Channels, ROCs). The basis of the permeability change which accompanies α receptor activation is thought to be an

increase in the permeability to sodium, chloride and possibly potassium (Casteels et al 1977, Droogmans et al 1977). The role of calcium permeability changes is uncertain. Some authors have suggested that there is an increase in calcium influx (or efflux) during activation, (Godfraind 1976, Wahlstrom 1973, Turlapaty, Hester and Carrier 1976). although others have found no change (Keating 1972) or a decrease (Hudgins and Weiss 1968, Seidel and Bohr 1971). It should be noted, however, that in many instances calcium influx (or even membrane depolarisation) appears to be unecessary for the action of NA. The release of calcium from intracellular stores is often sufficient to produce a contraction. In this connection, α receptor activation has been reported to reduce calcium binding to aortic microsomes (Baudouin-Legros and Meyer 1973).

ACh (2×10^{-3}M) produced a depolarisation of the membrane in this study to a value of -27.4mV (S.E. 0.87). This concentration of ACh produced a rise in tension of about 165Nm^{-2}. Like NA, ACh produces a variety of effects on smooth muscles. In all smooth muscles the effect of ACh is mediated via muscarinic receptors. In those smooth muscles in which it produces a contraction, ACh also produces depolarisation. It is interesting to note that pronounced inter- species differences occur. In the rat anococcygeus, ACh produces contraction and depolarisation, (this study), whereas in the rabbit and the cat, the anococcygeus muscle is relaxed by ACh (Gillespie and McGrath 1974, Creed, Gillespie and McCaffery 1977).

Muscarinic receptor activation generally is thought to increase membrane permeability to sodium and potassium, (and possibly chloride), by opening ROCs (Bolton 1972). Some workers have found an increase in calcium influx upon muscarinic receptor stimulation (Banergee and Lewis 1963, Lammel 1977) whereas others have found no increase (Hodgson and Daniel 1973, Van Breemen and Daniel 1966). Calcium permeability changes induced by various agonists in many smooth muscles have been measured using calcium isotope efflux techniques (Saad and Huddart 1981). The results of this type of experiment indicate that smooth muscles vary widely in their dependence upon calcium influx for activation, and this appears to correllate well with the developement of intracellular stores. It would appear that, as for α receptors, calcium permeability changes are not the most important ones induced by muscarinic receptor stimulation and thus calcium influx is not of primary importance in the production of a response.

KCl (80mM) produced a depolarisation of the membrane in this study to a value of -19.8mV (S.E. 0.26). This concentration of KCl produced a rise in tension of about $90Nm^{-2}$. The typical bi-phasic contraction induced by KCl in the rat anococcygeus is similar to that reported in other smooth muscles (Gabella 1978, Syson and Huddart 1973), especially in those types which produce APs. In these the initial fast rise in tension appears to be associated with an initial burst of APs. In muscles which do not produce APs, KCl usually produces a slow rise in tension which gradually approaches a maximum. The rat anococcygeus is unusual in this respect, in that although it does not generate action

potentials the initial rise in tension is very rapid. This is not due to the action of NA released from nerve terminals, since in all experiments with KCl phentolamine is included in the Krebs solution.

High K^+ solutions exert their effect on membrane potential primarily by altering the potassium gradient across the membrane. Resting membrane potential is largely determined in smooth muscles by the potassium equilibrium potential (Casteels et al 1977, Holman 1958, Kuriyama 1963), but sodium and chloride also have an appreciable effect, so that changing the ionic gradient for potassium will change the membrane potential. It is thought that the change in membrane potential opens voltage dependent ion channels (VDCs) which permit the entry of calcium down its electrochemical gradient and so initiate contraction. Internal K^+ concentrations have been shown to be of the order of 150mM in most smooth muscles (Scheid and Fay 1980). If this is true of the rat anococcygeus muscle, then the potassium equilibrium potential in normal Krebs will be approximately -87mV. The resting membrane potential was measured as -55.2 mV, indicating that other ions besides K^+ are involved in the maintainence of the resting membrane potential. In 80mM KCl the potassium equilibrium potential will be about -15.8mV. However, it has been shown that smooth muscles deviate from the expected membrane potential in high K^+ solutions to various degrees. Teania coli cells produce a relationship between external K^+ and membrane potential which agrees fairly well with the predicted relationship, whereas vascular smooth muscle exibits little change in membrane potential

with variations in external K^+ concentration. The deviation from the predicted relationship is no doubt due to rectification by the membrane due to the opening of various voltage dependent channels. Also, electrogenic pumping is increased by high external K^+, which leads to the intracellular accumulation of K^+.

Although this deviation from the predicted relationship means that it is impossible to calculate in advance what the membrane potential will be for a given K^+ concentration, the value found in this study, (-19.8mV for 80 mM KCl), would indicate that the deviation from the theoretical value in the case of the rat anococcygeus is not as great as found in many other smooth muscles.

The results obtained for the three agonists indicate that there is no simple relationship between the membrane potential produced by an agonist, and the ability of that agonist to induce a contraction. This is due to the different activation processes used by each of the agonists to couple membrane permeability changes to contraction of the muscle (and also because the inital permeability changes are different).

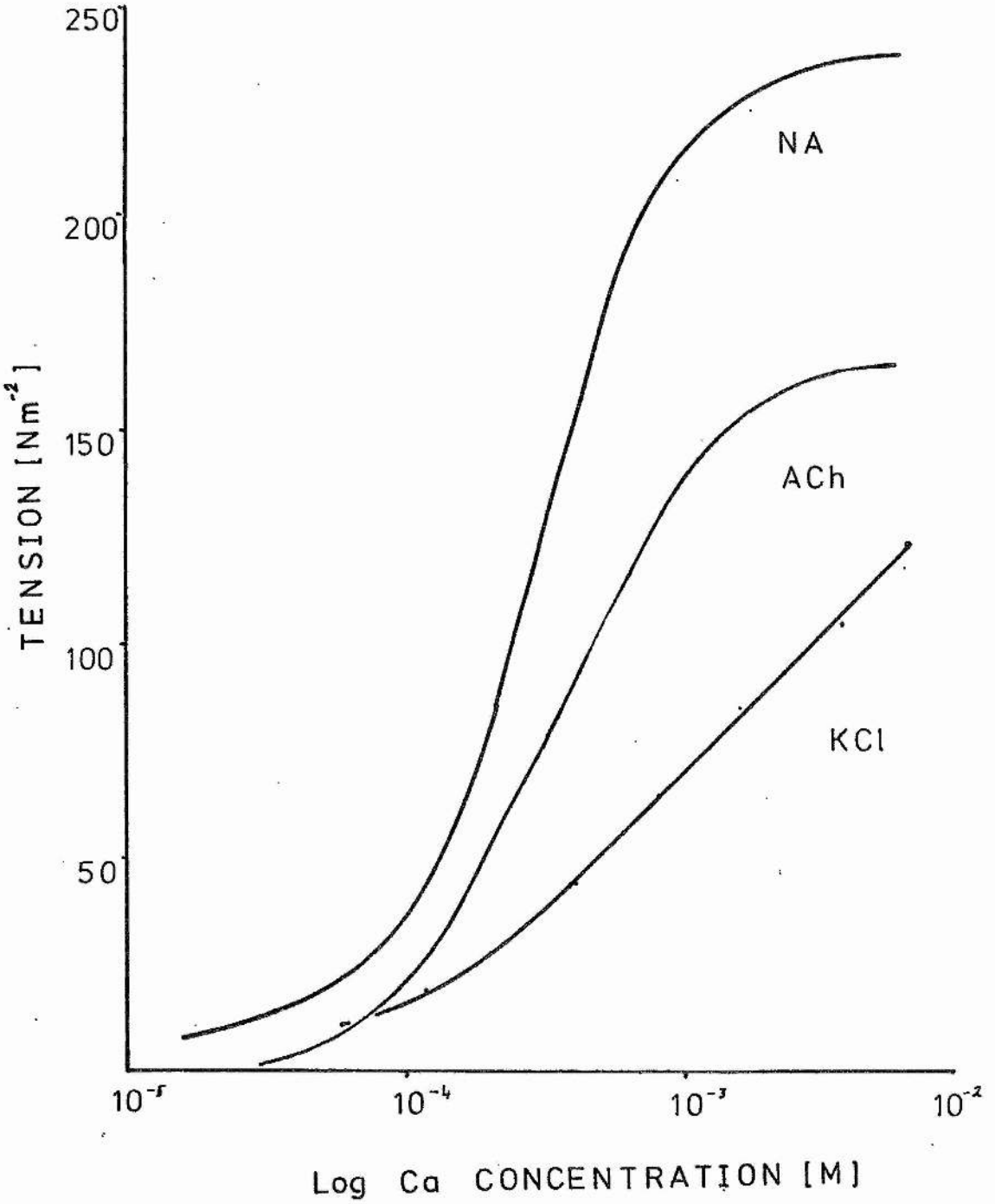
More evidence for the existence of these activation processes used by the agonists to regulate contractility can be obtained from the calcium dose response curves.

3.0 Calcium Dose Response Curves; Control Curves.

Fig D2 shows the control curves for each agonist plotted on the same scale. The curves provide useful imformation about the

Fig D2 Control Calcium Dose Response Curves

Control calcium dose response curves for each agonist taken from the Calcium dose response section, plotted on the same scale.


250
200
150
100
50
TENSION [Nm^{-2}]
NA
ACh
KCl
10^{-5}
10^{-4}
10^{-3}
10^{-2}
Log Ca CONCENTRATION [M]

activation processes used by the agonists. Although the depolarisation produced by increasing concentrations of KCl is often not simply related to the KCl concentration, (see above), this problem is not too crucial in these experiments, since the concentration of KCl used was constant at 80mM. It can be assumed that the permeability change produced is therefore constant. If this is so, then the linear relationship seen when tension is plotted against log $[Ca^{++}]$ for KCl induced contractions merely reflects the dependence of the contraction on calcium influx, since increasing the extracellular calcium concentration will increase the electrochemical gradient which drives calcium influx. This linear relationship is produced since the contractile proteins themselves respond to the log of the intracellular calcium concentration in this way (Gordon 1978, Endo, Kitazawa, Yagi, Iino and Yakuta 1977). This line of reasoning can be represented thus;

For the contractile proteins themselves;

$$\text{Response} \propto \text{Log}\,[Ca^{++}]i \quad \text{(intracellular calcium conc.)}$$

If we assume that the internal calcium concentration is related directly to the calcium influx, then;

$$\text{Response} \propto \text{Log}\,(\text{Influx})$$

If we add a proportionality constant, K;

$$\text{Response} = K\,\text{Log}\,(\text{Influx})$$

Now

Influx = Calcium Gradient x Membrane permeability

So;

Response = K Log (Gradient x Permeability)

Since the permeability of the membrane is assumed to be constant, (due to the constant concentration of KCl used), this equation describes the relationship between response and Log $[Ca^{++}]$ shown in fig D2 very well.

It can be seen that ACh and NA also produce this linear relationship between calcium concentrations of 10^{-4} to 10^{-3}M. The slope of the lines is much greater, however. It is apparent from the equation derived above that a change in the permeability of the membrane will result in a shift in the line parallel to the Log $[Ca^{++}]$ axis, but that the slope will remain constant. A change in the proportionality constant, K, however, will result in a change in the slope of the line. This proportionality constant can be thought of as an index of the amplification processes which are used by NA and ACh to increase the response to a given calcium signal (ie. an index of the pharmaco-mechanical coupling). The data indicate, therefore, that NA and ACh produce a greater contraction by an amplification of the initial calcium signal, rather than by an increased permeability.

As stated earlier, in the rat anococcygeus muscle NA induced contractions are less affected by removal of the extracellular calcium (or by Verapamil) than KCl induced contractions. This indicates that NA causes contraction by release of intracellular stores of calcium, although it may be that the amplification of the calcium signal produced by NA is so powerful that a small contraction can be produced as a result of the very small calcium influx under these conditions. Results quoted by van Breemen (Van Breemen, Farinas, Casteels, Gerba, Wuytack and Deth 1973) indicate that, at least in the rabbit aorta, NA stimulation produces less calcium influx than does KCl depolarisation. The evidence supports the view, therefore, that NA and ACh do not produce a greater contraction by a greater change in membrane permeability, hence a greater calcium influx. rather, they have a greater secondary pharmaco-mechanical coupling effect, which can act to amplify the response to what may be a smaller initial calcium signal.

3.1 Maximum response of NA and ACh;

The calcium dose response curve for KCl induced contractions is still rising at the highest calcium concentration used, (6.4mM), whereas the curves for NA and ACh induced contractions reach a maximum at about 2mM. Initially it was thought that this 'leveling off' might be due to calcium phosphate precipitation, leading to a maximum attainable ionised calcium concentration. This is unlikely to be the cause, however, since the KCl induced contractions are still rising. The possible explanations of this effect on NA and ACh induced contractions could be;

1) That high concentrations of calcium are producing a membrane stabilising effect, influencing the permeability changes produced by NA and ACh. This type of membrane stabilising effect of calcium has been shown in other preparations (Collins, Sutter and Teiser 1972 and Frankenhauser and Hodgkin 1957) However, a membrane stabilising effect of high calcium solutions would be expected to affect KCl induced contractions also, if this effect was on membrane permeability. The other possibility is that calcium bound to the membrane can regulate the release of the stores of calcium associated with the inner surface of the membrane or the receptors themselves. The existence of such regulatory calcium binding has been suggested by Weiss (1977).

2) High calcium solutions may interfere with the binding of the agonist to the receptor. Calcium has been shown to be necessary for the binding of NA to alpha receptors (Bulbring and Tomita 1977), so it may be possible that high calcium solutions can interfere with this process in some way. No similar information exists for muscarinic receptors.

It is possible that the amplification stage in the activation processes used by NA and ACh becomes saturated in its ability to amplify the small initial calcium signal. This would lead to a maximum contraction being reached, with these maximum contractions, however, still greater than the KCl induced contractions. If the amplification process is different (and/or more powerful) for NA, this concept would also explain the greater response possible to NA before the process becomes saturated. This more powerful

amplification stage produced by NA activation (and to a lesser extent ACh) as compared to KCl activation is also illustrated by the experiments in which NA and/or ACh were added to a pre-existing KCl contraction.

4.0 Addition of agonists.

The fact that agonists can produce additional contractions in KCl depolarised muscle has been reported in various preparations (Peiper, Griebel and Wende 1971). NA and ACh can induce additional contractions in the rat anococcygeus muscle depolarised by 80mM KCl. It was shown earlier that this concentration of KCl depolarises the membrane to about -20mV. It is reasonable to suppose that this is not changed by the subsequent addition of NA or ACh. (see electrophysiology). The fact that this addition of NA or ACh can produce an additional contraction must therefore be taken as an effect of these agonists on events subsequent to membrane depolarisation. It would appear, therefore, that the data supports the hypothesis that NA and ACh produce some additional amplification stage, which acts on events subsequent to membrane depolarisation.

The observation that the three agonists can be added in the order KCl/ACh/NA, each producing an additional contraction, and the observation that the combination of KCl/ACh produces a lower total response than the combination of KCl/NA, indicates that ACh produces a less powerfull "amplification" than NA. This fits the idea that NA and ACh use subtly different second messenger "amplification" systems.

That agonists added together in this way retain their characteristic sensitivities to antagonists has been reported by Peiper, Greible and Wende (1971). They found similar results to those quoted in this study for the rat anococygeus in the rat aorta, ie that V inhibited the inital KCl contraction, but did not substantially affect the subsequent NA contraction.

The data in the previous sections supports the view that NA and ACh do not produce a greater contractile response by membrane effects (eg by producing greater calcium influx), but rather by some amplification step in the activation process which is involved in the control of contractility. The main candidates proposed for this role in the control of contraction are the cyclic nucleotides, cyclic AMP and cyclic GMP.

5.0 Cylic Nucleotide Studies;

Cyclic nucleotide changes have been implicated in the changes in contractility of various smooth muscle preparations. The relaxing effect of β adrenergic stimulation has been linked to a rise in intracellular cAMP levels (Andersson 1972,1973, Murad and Kimura 1974, Ohkubo, Takayanagi and Tagagi 1976). However, the changes in cyclic nucleotide levels which accompany the excitatory effects of drugs are much more variable (Bar 1974). In some muscle preparations an increase in cGMP has been associated with contraction. Thus, it was found by Lee et al (1972) that muscarinic stimulation in various tissues resulted in a rise in cGMP and a slight fall, or no change, in cAMP. Similar results have been found by Schultz Hardman, Hurwitz and Sutherland

(1973), in the ductus deferens and small intestine. Subsequently this work was confirmed (Schultz, Schultz and Hardman 1975, Schultz et al, 1973), but some doubt was cast upon the role of cGMP by the finding that changes in cGMP did not correllate well with the time course of the contraction. Diamond and Holmes (1975) showed also that KCl depolarisation caused a fall, rather than a rise in cGMP in the rat myometrium associated with contraction, and that the relaxant effects of papaverine and nitroglycerine did not correllate well with their ability to produce changes in cGMP levels.

Although it seems, therefore, that the role of either cGMP or cAMP alone is not well defined, the possibility exists that the two act in an antagonistic way to regulate cell function. Thus an increase in cGMP may be linked to contraction, while an increase in cAMP is linked to relaxation. This so called Ying/Yang hypothesis was first proposed by Goldberg (Goldberg et al 1975). This idea has subsequently been refined so that the proposed controling mechanism is now thought to be the ratio of cAMP to cGMP. (Dunham, Haddox and Goldberg 1974, Lee, Kuo and Greengard, 1972)

The Rat Anococcygeus;

It can be seen from table R3 that NA stimulation of the muscles caused a slight rise in cAMP levels, whereas ACh and KCl did not. This may be due to the existence of a small number of B receptors in this tissue, although experiments have shown that B agonists have little or no pharmacological effect in the rat

anococcygeus (this study, unpublished results). It is widely accepted that in general B receptor stimulation causes a rise in cAMP levels in many tissues (Scheid, Honeyman and Fay 1979) and it is possible that the slight rise in cAMP observed could be due to a small number of β receptors, which do not contribute to the contractile responses of the tissue under normal circumstances.

More pronounced changes in the levels of cGMP are produced by the agonists. All three cause a fall in cGMP from the control value of 0.337 pmol/mg protein. The greatest fall is caused by ACh, to 0.109 pmol/mg. Both NA and KCl produce a fall to around 0.16 pmol/mg. This result is the opposite to that most often quoted, ie. that agents which cause contraction generally raise cGMP. However, one or two cases have been quoted where contraction is accompanied by a fall in cGMP levels (Diamond and Holmes 1975). At first it seems difficult to reconcile these results with any proposed role of cAMP or cGMP in the contraction of the rat anococcygeus muscle, since the levels of the nucleotides do not appear to correllate with the tension produced by each agonist.

However, a good correllation is obtained between the ratio of the levels of cAMP/cGMP produced by activation by each of the agonists, and the maximum tension produced by those agonists. It should be noted that the maximum tension values used here are not those produced by the muscles which were actually assayed for cAMP and cGMP. Although desirable, this was not possible since the muscles were freeze clamped at the time of maximum tension. This precludes the weighing step used in the mechanical experiments to

convert the tension to Nm^{-2}. Instead the maximum tension produced by each agonist in the mechanical experiments was used. Since the muscles used for the assay were freeze clamped at the point of maximum tension (induced by the same concentration of the agonists used in the mechanical experiments), it was felt that any errors would not be great enough to affect the conclusion.

Table R6 shows the max. tension and the cyclic nucleotide ratio produced by each agonist, compared to the control situation.

Table R6:

AGONIST	Max TENSION [Nm^{-2} SE]	RATIO cAMP/cGMP	MEAN RATIO
NA	247 13	8.4,8.5,7.8	8.35
ACh	170 9	6.8,7.0	6.9
KCl	99 5	4.5,4.6,4.7	4.6
CONT	15	2.4,2.1,3.5	2.6

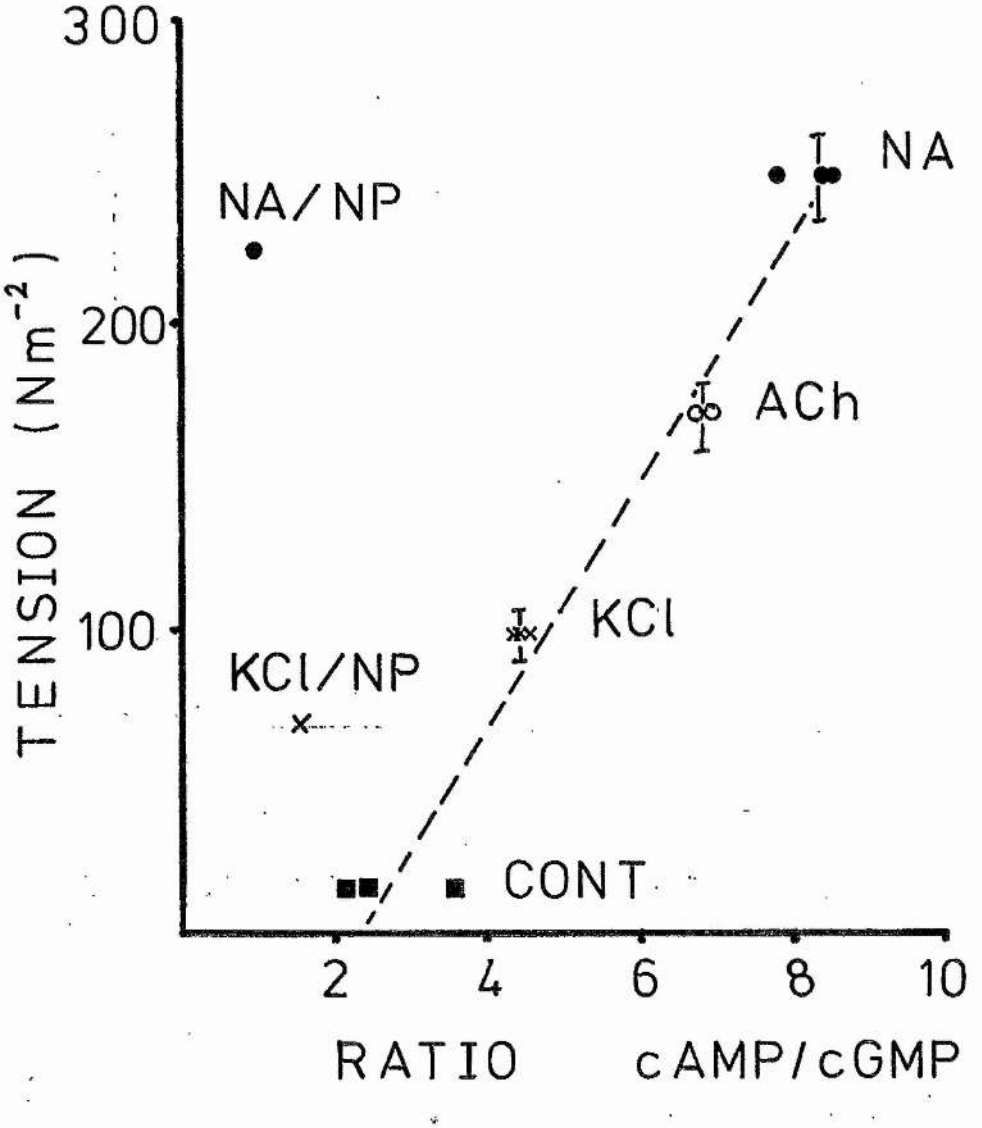
Fig D3 shows the result of plotting the tension induced by each agonist against the ratio of the levels of cAMP/cGMP produced by that agonist. It can be seen that a good straight line can be drawn through the points for the three agonists and the points for the control muscles, (set up under 1g resting tension, approx 15 Nm^{-2}). Regression analysis of the data points gives an equation for this line of $y=-86 + 39.3x$, with a correllation coefficient of 0.9825.

This good correllation between the tension produced and the

Fig D3 Ratio cAMP/cGMP versus Tension.

The graph shows a plot of the ratio cAMP/cGMP produced by a maximal dose of each agonist against the tension produced by that dose. Doses are; KCl 80mM, ACh 2×10^{-3}M and NA 2.5×10^{-5}M. Ratios are plotted as points, each point being a result from Table R2. Tension is taken from the mechanical studies section and is plotted as mean tension, error bar is 1SE. n=8 for each agonist. The dotted line is that generated by regression analysis of the points. The equation for this line is $y = -86 + 39.3x$. The correllation coefficient is 0.9825.

The point marked NA/NP is that produced by pre-treatment with NP (2×10^{-7}M) prior to NA addition. The point marked KCl/NP is that produced by pre-treatment with the same concentration of NP prior to the addition of KCl.


300
200
100
TENSION (Nm^{-2})
NA
NA/NP
ACh
KCl
KCl/NP
CONT
2
4
6
8
10
RATIO cAMP/cGMP

ratio of cAMP/cGMP produced by each agonist would seem to be good evidence in favour of the hypothesis that the ratio of the two cyclic nucleotides acts in some way to regulate the contractility of the muscle.

Exogenous cAMP and cGMP analogs;

If this is indeed the case, then raising the level of one or other of the nucleotides by some means (or using analogous compounds) should cause differential changes in the contractility of the muscles, in accordance with the scheme above. Di- butryl cAMP, an analogue of cAMP, has been shown to relax vascular smooth muscle (Webb and Bohr 1981), guinea pig trachea (Moore, Iorio and McManus, 1968) and rat ileum and uterus (Overweg and Schiff 1978). However, some authors have reported that di- butyryl cAMP causes activation of rat vas deferens and ileum (Saad and Huddart 1980), and they conclude that cAMP is not important in the relaxation mechanism in these two muscles.

8- bromo cGMP is an analogue of cGMP, which can be used in the same way. 8- bromo cGMP and di- butyryl cAMP have been used in the study of the control of cardiac contractility and have been shown to exert antagonistic effects in this tissue (Sing and Flitney 1981). Thus if the ratio of cAMP/cGMP is important in regulating the contractility of the rat anococcygeus muscle, then exogenous di- butryl cAMP should cause a contraction, (or enhance a contraction caused in some other way) and exogenous 8- bromo cGMP should cause a relaxation. (or inhibit a contraction caused in

some other way). Fig D4 shows the effect of exogenous 8- bromo cGMP (5 x 10^{-5}M) on the muscles when the muscle is prevously contracted by either NA or KCl. (maximal doses). 8- bromo cGMP caused a relaxation in both cases. This relaxation is greater in the case of NA, which may be an indication of the greater reliance of NA on changes in cyclic nucleotides in the production of a contraction. The effects of exogenous di- butyryl cAMP are shown in Fig D5. It would be expected that this would cause a contraction. However, it can be seen that, on a preparation contracted with KCl, di- butyryl cAMP (5 x 10^{-5}M) causes a slight, transient relaxation. This may be due to the other efects of cAMP and its analogues in the cell (eg. on phosphorylase kinase, etc.). On muscles contracted with NA, however, di- butyryl cAMP caused a pronounced, sustained relaxation. This may be because the muscle is already maximally contracted by NA, so futher contraction is not possible. The relaxant effect may be due to the effefcts of cAMP analogues on cGMP metabolism (eg. it has ben shown that cAMP can inhibit the hydrolysis of cGMP by phosphodiesterase (Brostrom and Wolff, 1976). Also, in tracheal smooth muscle, a protein has been demonstrated which will bind cAMP, and which shows the same type of cross reactivity to cAMP and cGMP (Meich, Niedzwichi and Smith, 1979). Thus it is possible for exogenous cAMP analogues to increase the levels of cGMP. This would be expected to be important in NA induced contractions, where further stimulation is not possible, but where the contraction is dependent to a large degree on the ratio of the two nucleotides. However, it was not possible to measure the changes in cyclic nucleotide levels actually produced in these experiments, so the

Fig D4 Exogenous 8-bromo cGMP

The effect of adding 8-bromo cGMP (5×10^{-5}M) to a contraction induced by KCl (80mM), or NA (2.5×10^{-5}M). The agonist was added at "X", 8-bromo cGMP at "G". The dose was repeated at the second "G", giving a bath concentration of 10^{-4}M. The bath was washed out at the dot.

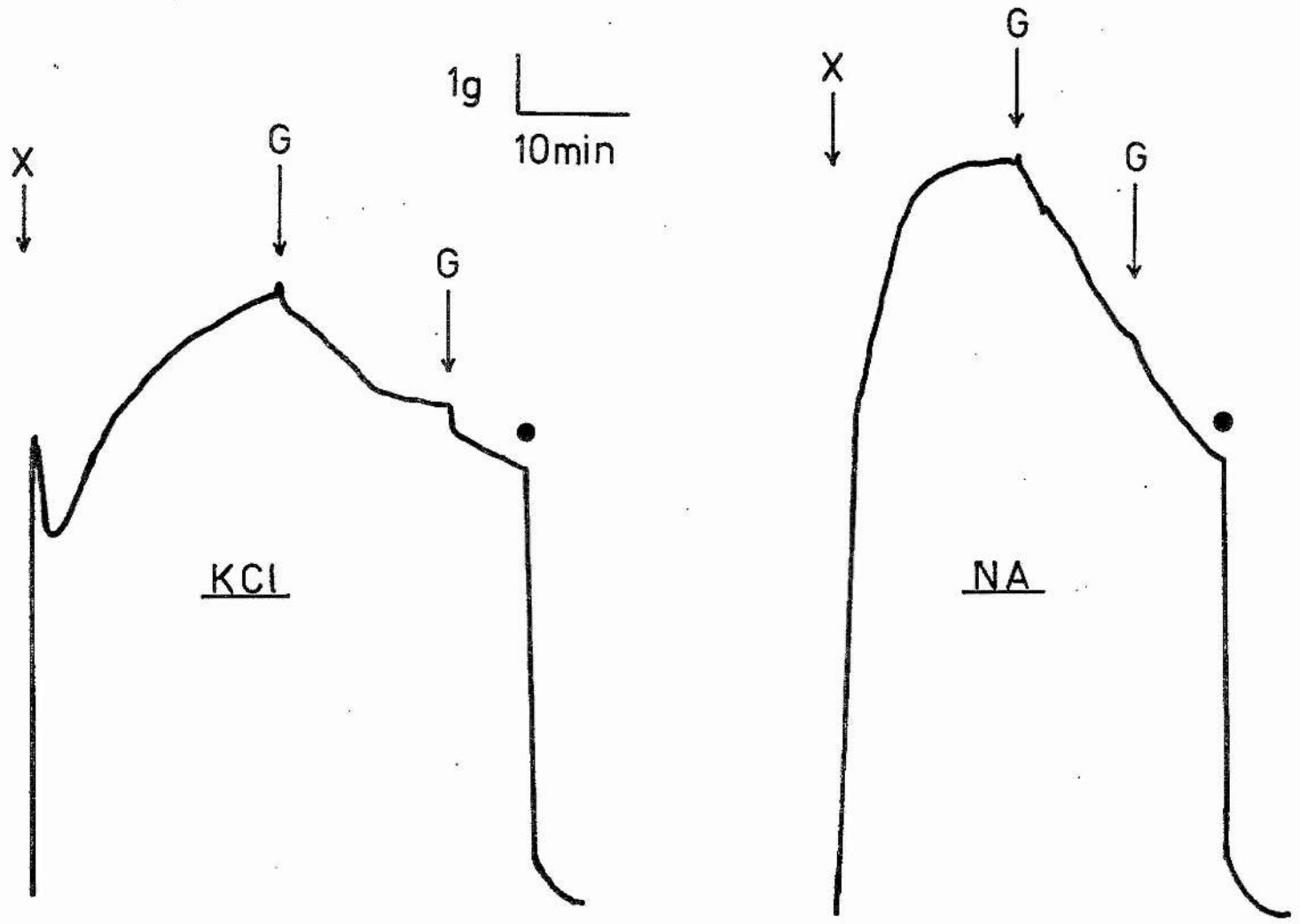
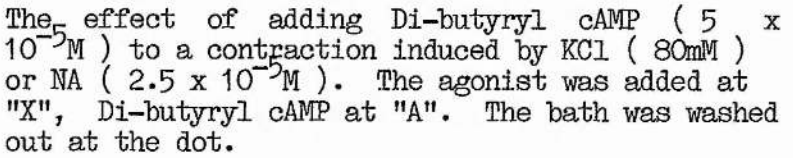
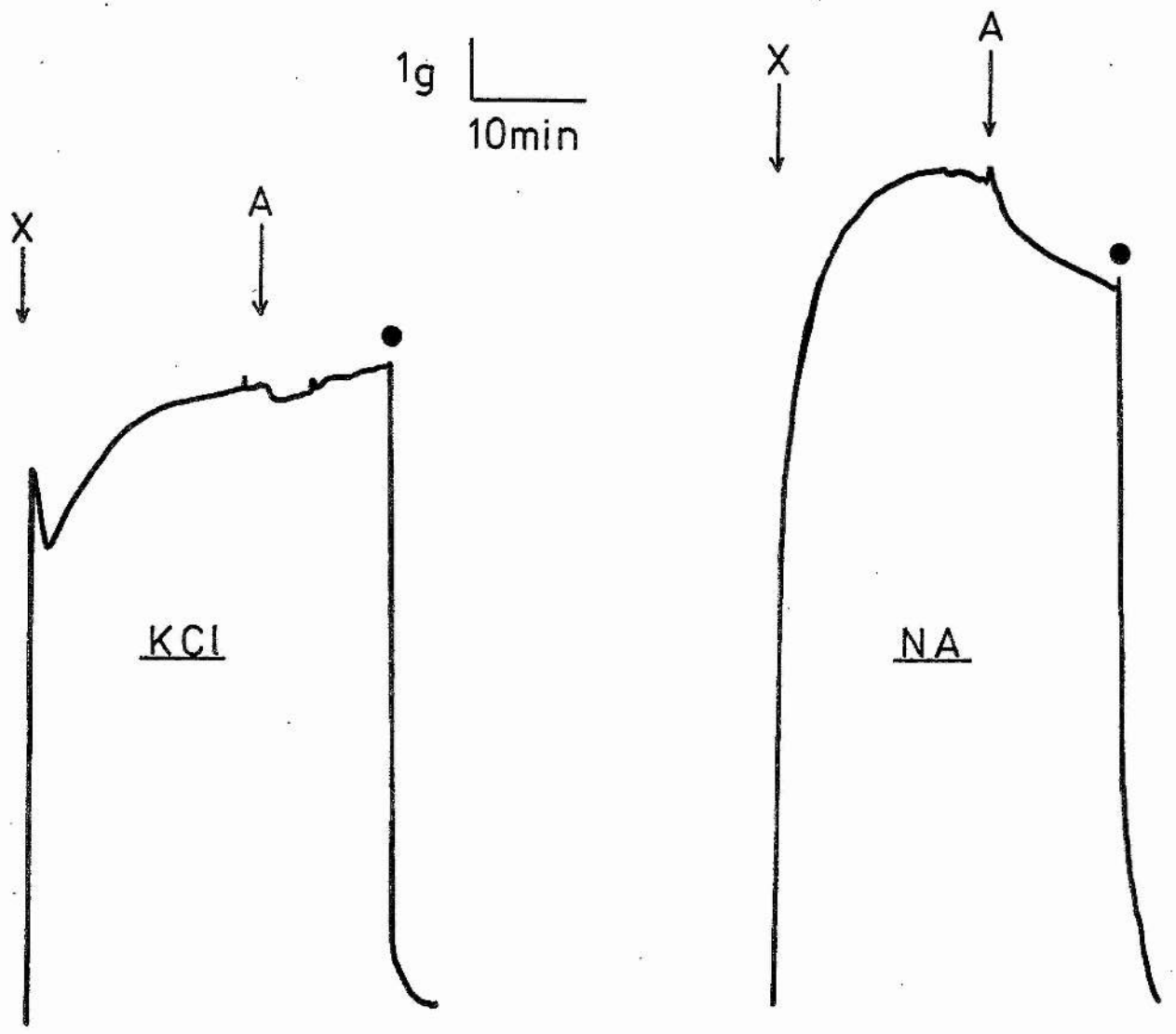

1g
10min
X
G
G
KCl
X
G
G
NA

Fig D5 Exogenous Di-butyryl cAMP



The effect of adding Di-butyryl cAMP (5×10^{-5}M) to a contraction induced by KCl (80mM) or NA (2.5×10^{-5}M). The agonist was added at "X", Di-butyryl cAMP at "A". The bath was washed out at the dot.


1g
10min
X
A
KCl
X
A
NA

results may be open to other interpretations.

6.0 Conclusion.

The data presented in this section supports the veiw that the three agonists use different methods of activation, in that KCl contractions depend to a large extent upon calcium influx, while NA and ACh contractions depend upon this much less. The electrophysiological results support the idea that membrane potential changes per se are not important in the regulation of contraction.

In order to explain the different effects of the agonists one must postulate the existence of some second messenger process which can regulate the contractility of the muscle in a way which is largely independent of the membrane potential changes. The changes in the cyclic nucleotides produced by the agonists suggest that this regulation of contraction may be related to the ratio of cAMP/cGMP in the cells, since a good correllation is obtained between this and tension.

SECTION 2; ANTAGONISTS; V and NP.

1.0 Effect of Verapamil; Introduction.

Verapamil was first investigated by Fleckenstein and co-workers (Fleckenstein, Kammermeier, Doring and Freund 1967) who showed that, in cardiac muscle, it specifically inhibited calcium influx, without affecting the resting membrane potential. Subsequently a similar effect, (inhibition of calcium induced contraction in K^+ depolarised muscle), was demonstrated in rabbit main pulmonary artery (Haeusler 1972). Similar calcium antagonistic effects have been reported in many other smooth muscle preparations, (Gruen, Fleckenstein and Tritthart 1971, Fleckenstein, Gruen, Tritthart and Byron 1971). In the light of these results it has been postulated that verapamil acts by preventing trans-membrane fluxes of calcium. Differential effects of verapamil on the contractions induced by different agonists have been observed in various smooth muscle preparations.

The inhibition of KCl induced contractions and NA induced contractions has been investigated in a number of preparations. In all instances the KCl induced contractions are inhibited to a greater degree, although the effect on the NA induced contractions tends to be more variable (Peiper et al 1971, Golenhofen and Hermstein 1975, Golenhofen, Hermstein and Lammel 1973, Hausler 1972, Bilek, Laven, Peiper and Regnat 1974). These results are to some extent dependent on the concentration used, but are nevertheless consistent with the idea that verapamil blocks

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transmembrane flux of calcium, and so will inhibit those contractions which are dependent upon an influx of extracellular calcium. Evidence that Verapamil acts in the rat anococcygeus as a calcium antagonist can be seen in the calcium dose response curves.

1.1 Calcium Dose Response Curves;(Figs R3-1 to R3-4)

It can be seen from Fig R3-3 that Verapamil (V) strongly inhibits KCl induced contractions. Since the curve for KCl does not reach a maximum, it is impossible to determine the effect of V on the maximum. However, it is apparent that V causes a shift of the curve to the right, while the slope remains unchanged. This is indicative, as explained earlier, of a change in the membrane permeability. This result is consistent with the generally accepted mode of action of V, which is to block trans- membrane fluxes of calcium. NA induced contractions are not significantly affected by V (Fig R3-1) since they do not depend primarily upon calcium influx. The slight effect of V, to produce a small shift of the curve to the right, may indicate a small, verapamil-sensitive component of the contraction (possibly due to a small initial influx of calcium through verapamil sensitive channels, rather than ROCs). This effect is not important in normal conditions, however.

ACh induced contractions are inhibited by V. Fig R3-4 shows that up to calcium concentrations of 2×10^{-3}M, V causes a shift in the calcium dose response curve to the right. This indicates, as for KCl contractions, that V has caused a reduction in the

membrane permeability to calcium, and that this is important for the ACh induced contractions. Above 2 x 10^{-3}M, however, V causes a reduction in the maximum response produced. This would appear to indicate that V exerts some sort of non-competitive inhibition of the contraction that cannot be overcome by increasing calcium concentrations. This additional effect may be linked to the effect of high calcium itself, as discussed earlier (ie effects on the receptor binding or membrane stabilising effects). The alternative view is that V can interfere in some way with the amplification process postulated in the action of ACh. These results indicate that V is acting in the rat anococcygeus primarily as a calcium antagonist.

The data from the experiments in which V is added to a pre-existing contraction induced by each of the agonists are also consistent with this action of V. The relative lack of effect on pre- existing KCl induced contractions (Fig R4-2) can be explained since the initial calcium influx (and therefore rise in cytoplasmic calcium concentration) has already ocurred when V is added. The slight inhibition of NA or ACh induced contractions may ocurr if there is a slight verapamil sensitive component involved in these. When V is added the calcium influx will be slightly reduced. Calcium sequestration (or extrusion) will then tend to lower cytoplasmic sequestration, which will lead to a slow relaxation.

These results and the differential inhibition of contractions induced by the three agonists is consistent with the veiw that V

can block influx of calcium and that KCl induced contractions depend to a large extent upon this, while NA and ACh induced contractions depend upon it only to a small extent.

In most smooth muscles the blockage of the influx of calcium by V takes the form of a suppression of action potentials, or a reduction in their frequency (Golenhofen and Lammel 1972, Reiner and Marshall 1975, Riemer, Dorfler Mayer and Ulbrecht 1974). The anococcygeus muscle, however, does not generate action potentials in normal circumstances (Creed, Gillespie and Muir, 1975), rather it produces a slow, graded depolarisation in response to stimulation. Although permeability changes to more than one ion are probably involved in this response, (Creed 1972), it seems likely that this depolarisation is at least partially due to an increase in calcium permeability, resulting in an influx of calcium and subsequent muscle activation, this calcium influx being particularly important in the case of KCl activation. However, more than one calcium channel may be responsible for this calcium influx, with different properties (ie voltage dependent channels, which are verapamil sensitive, and receptor operated channels, which are not.)

The effect of verapamil on the membrane potential changes induced by the agonists may clarify the mechanism of the differential inhibition produced by V on the contractions induced by them.

1.2 Electrophysiology;

Verapamil (5 x 10^{-7}M) was found to produce a slight depolarisation in control cells, from -55.2mV to -51.8mV. (P > 0.05). Although this effect is small, and not highly significant, it is the opposite of the result expected, if calcium permeability contributes to the resting membrane potential. The expected effect, if V produces a reduction in calcium permeability, would be a slight hyperpolarisation. However, there is evidence that in other smooth muscles calcium plays little part in the determination of the resting membrane potential, this being largely due to permeability to the other ions (Siegel et al 1976, Kuriyama 1963). It may be that, in this instance, V is competing for the calcium- binding sites which are thought to be responsible for membrane stabilisation, hence causing a small, generalised increase in membrane permeability to other ions (eg. sodium). This generalised effect of V on the membrane may account for the slightly reduced depolarisation produced by NA. The lack of a specific effect on the depolarisation produced by NA correllates well with the fact that this concentration of V does not significantly inhibit NA induced contractions.

However, a more specific action is seen in the case of ACh. Here the depolarisation produced by ACh is significantly reduced. (see Table R5). This would appear to be an effect specific to ACh activation, rather than a generalised effect on the membrane. This effect correllates well with the mechanical effects of V, since this concentration of V produces about 40% inhibition of ACh

induced contractions. It may be that the ROCs opened by ACh have a verapamil- sensitive component, so that V can slightly reduce the depolarisation produced by them. The alternative is that V may interfere in some way with the mechanism which couples receptor activation with opening of the ROCs. It has been shown that there is a measurable delay, or latency, in the response to activation of muscarinic (Hill-Smith and Purves 1978) and adrenergic (Purves 1978) receptors, which may be due to this coupling process. If this coupling process is different for NA and ACh, (as seems likely), then it may be possible that V affects one preferentially. This may be due to V interfering in some way with calcium bound to the membrane in the region of the receptors. This finding may explain why V can reduce the maximum contraction produced by ACh in the calcium dose response curves, and may be the effect postulated earlier in addition to the calcium antagonistic effect.

The effect of V on KCl depolarisation (ie. no significant effect), was unexpected. It was expected that V would reduce the depolarisation produced, by preventing the calcium permeability change induced by KCl. However, as stated previously, KCl depolarises the membrane by changing the electro- chemical gradient for K^+, (and hence the equilibrium potential.) Depolarisation by this means would thus still be possible in the face of little or no change in the permeability to other ions. Also, the fact that this depolarisation itself produces changes in the permeability to other ions, by opening voltage dependent channels, complicates the situation. If the contribution of calcium permeability changes is

small compared to these other effects, it seems poosible that a reduction in calcium permeability would have little effect. In fact, when the further complications of ionic pumps in the membrane are considered, the result is not surprising.

It is notable that the primary and secondary phases of the KCl contractions are almost identical in their sensitvity to V, (and very similar with regard to NP). This is strong evidence that these two phases are not fundamentally diferent in this muscle, (ie. they are not due to different activation processes, like the P and T systems described in other preparations.) This supports the idea proposed in section 1, that the bi- phasic contraction produced by KCl is due to the re- equilibration of calcium within the cell.

The results of these experiments indicate that in the rat anococcygeus muscle KCl contractions are primarily due to an influx of calcium, whereas NA and ACh induced contractions do not rely to such a great extent upon calcium influx, (since V inhibits KCl contractions well, but only poorly inhibits NA and ACh contractions.) It must be born in mind in the interpretation of these results, however, that it is possible that NA and ACh produce an influx of calcium through channels that are not sensitive to V. Such receptor operated channels (ROC's) have been proposed to exist in many preparations and to have different properties to the channels opened by KCl depolarisation, (voltage dependent channels, VDC). If the number of ROCs is much greater than the number of VDCs, this could go some way to explaining the greater

maximum contraction produced by NA and ACh, as compared to KCl.

It should be noted that V causes the greatest inhibition of KCl induced contractions, (compared with the other agonists), but that it produces no appriecable change in the membrane potential. This is further evidence that the membrane potential per se is not fundamental in the regulation of contraction.

1.3 NA and ACh; Effects of low calcium;

The efects of NP and V on the contractions induced by NA and ACh in low calcium solution were investigated. Although the concept of P and T system activation does not seem applicable to explain the differences in activation produced by the three agonists in this muscle, (see earlier), it was felt that perhaps the different phases of the bi-phasic contractions produced in low calcium for NA and ACh would exibit some of the properties of P and T systems, (ie. selective inhibition by NP and V). As can be seen from the results (Figs R2-9 to R2-12), the different phases did display different properties.

The primary phase of the contraction induced by NA in low calcium is largely unaffected by V, as is the contraction in normal calcium. Since V has little effect it is reasonable to suppose that this primary contraction does not, therefore, depend upon influx of extracellular calcium. It seems likely that this contraction is produced by the release of intracellular stores of calcium by NA. A possible candidate for such a store is the

calcium associated with the cell membrane itself, or possibly calcium bound to the receptors themselves (Bolton 1979). If the small amount of calcium so released into the cytoplasm is then subsequently sequestered by calcium binding within the cell, possibly by mitochondria, sarcoplasmic reticulum or parallel structures, etc., then the rise in free calcium levels will be transient, producing the observed transient contraction. A general model of this type of internal calcium cycling has been proposed by Bianchi (1969). Presumably this primary contraction is not sensitve to NP for the same reasons that the contraction in normal calcium is not. (see earlier). If the small primary contraction produced by ACh in low calcium is due to the same sort of mechanism as that produced by NA, it should be relatively insensitive to V. This is what is found (Fig R2-11).

The later, secondary contraction induced by NA in low calcium is inhibited to a large extent by high concentrations of V (above 7×10^{-7}M). This inhibitory effect of V indicates that the contraction under these conditions is dependent upon influx of extracellular calcium, whereas the contraction in normal calcium is not. However, it is reasonable to suppose that the inhibitory effect of V will be greater in low calcium since it has been shown that V and low calcium produce similar results (Golenhofen and Lammel 1972), and also that the effect of V can be overcome by high calcium (Ferrari 1970). It may be that the contraction induced by NA in normal calcium does have a Verapamil- sensitive component, but that inhibition of this only becomes important at low calcium concentrations.

The secondary phase of contraction induced by ACh in low calcium shows very similar properties to the contractions in normal calcium. One of the effects of reducing the calcium concentration is to increase the effectiveness of V, which is consistent with the mechanism of action of V as a calcium antagonist.

1.4 Summary

The data presented in this section supports the view that V acts as a calcium antagonist in the rat anococcygeus. The inhibitory effect of V on the contractions induced by the three agonists support lead to the following conclusions.

1) KCl induced contractions depend largely upon an influx of extracellular calcium. V causes inhibition by preventing this influx.

2) The two phases of the contractions induced by KCl are not due to different activation mechanisms, but are due to calcium re- equilibration within the cell.

3) NA induced contractions are not dependent upon calcium influx in normal circumstances.

4) ACh induced contractions appear to have a Verapamil-sensitive component. Electrophysiological evidence suggests that this may be an effect of V on receptor coupling or on the ACh operated channels.

5) The initial event in NA and ACh induced contractions is either a small calcium influx which is not verapamil sensitive, or (more likely) a small release of intracellular stores of

calcium, possibly from sites associated with the receptors themselves.

2.0 Effect of NP; Introduction;

Nitroprusside is generally accepted as producing a pronounced rise in cGMP levels in a variety of smooth muscle preparations, while leaving cAMP levels substantially unchanged (Schultz et al 1977, Katsuki and Murad 1977). This rise in cGMP levels usually occurs along with a relaxant effect, for example in muscles depolarised by high K^+ solutions. The suggestion that the relaxant effects of NP are mediated via a rise in cGMP has recently been challenged by other workers however (Diamond and Janis 1978). They have found little correllation between the levels of cGMP and the relaxant effects produced by NP in the rat vas deferens.

It has also been suggested that NP exerts its relaxant effects via some effect on the cell membrane, so that the changes in cGMP levels which occur later in the activation process are incidental to contraction or relaxation and not causally related to it. In support of this view, NP has been reported to hyperpolarise arterial smooth muscle (Haeusler 1975) and rabbit portal vein (Ito, Suzuki and Kuriyama, 1978), although it is disputed whether NP reduces the calcium influx produced by high K^+ solutions (no effect or increase- Zoster, Henein and Wolchinski 1977, reduction- Kreye, Baron, Luth and Schmidt- Gayk 1975). NP was also found to produce slight hyperpolarisation of the rat anococcygeus in this study. Although the mechanism of action of NP remains a matter of some dispute, it is undoubtably true that its mode of action is very different from that of Verapamil. If there are differences in the activation pathways of the different agonists, it is reasonable

to suppose that V and NP will affect them in different ways. The differential effects of V and NP have been investgated in a number of preparations. NP has been shown to inhibit a component of contraction in some preparations that is resistant to nifedipine (an analogue of Verapamil) (Golenhofen, Wagner and Weston 1977).

NP and V were used by Golenhofen (1973,1976) to support the hypothesis that there are two different activation mechanisms in smooth muscle that differ in their responses to these antagonists. These two systems are designated P (Phasic), which is inhibited selectivly by V, and T (Tonic), which is inhibited by NP. It should be noted, however, that NP is not a T system antagonist in all tissues, and also that Golenhofen does not acknowledge the possible involvement of cGMP in the action of NP.

On the basis of this finding it would be supposed that KCl contractions in the rat anococygeus would be insensitive to NP but that NA contractions would be inhibited (ie. KCl acts primarily via a P type system, whereas NA acts via a T type system). ACh contractions would be expected to be intermediate in their sensitvity to NP, as for V. As can be seen from figs R2-2 and R2-3, KCl contractions are indeed largely insensitive to NP (both primary and secondary phases). However, NA contractions are also unaffected by NP (up to 10^{-5}M). The inhibition of ACh contractions is as predicted, being about 40%.

The results indicate that the concept of two activation

systems which are stimulated to different degrees by the agonists, (such as the P and T systems described by Golenhofen in other muscles), is not applicable to the action of the three agonists on the rat anococcygeus muscle. The fact that ACh contractions are inhibited by NP, whereas NA contractions are not, must indicate that ACh and NA use different activation pathways. It appears, therefore, that a slightly different mode of activation must be proposed for each of the three agonist in the rat anococcygeus muscle. It must be emphasised here that the concept of P and T systems was evolved largely from results with spontaneously active smooth muscles, which usually produce bursts of action potentials (eg stomach fundus). It may be that the response to different agonists in the anococcygeus muscle, which does not produce action potentials, is more subtle and the different agonists use slightly different activation pathways. These activation pathways may involve changes in second messenger substances within the cell, (eg cyclic nucleotides).

The evidence presented in the previous section, dealing with the effects of V, supports the idea that membrane potential per se is not important in the regulation of contraction, or in the inhibition produced by V. The membrane effects of NP were investigated in order to asses if these were important in the inhibition produced by it.

2.1 Electrophysiology

NP has been reported as producing hyperpolarisation of smooth

muscle cells (Ito et al 1978). In this study it was found that NP (2×10^{-7}M) also produced a small hyperpolarisation of the cells of the rat anococcygeus (of about 2.8mV). This effect was not significant when compared to the control value, however. NP had pronounced effects on the depolarisation produced by NA and ACh. In the presence of NP (2×10^{-7}M) both NA and ACh produced less depolarisation than in the control group. This effect was significant in both cases. It is difficult to reconcile these results with the most generally accepted mode of action of NP, namely that it increases cGMP levels in the cells which then acts in some way to produce relaxation, although it has been suggested that cGMP could exert a negative feedback effect which could reduce calcium influx (Schultz et al 1977). As stated previously, calcium permeability changes are unlikely to be of importance in the action of adrenergic or muscarinic receptors. The primary effect of these is thought to be an increase in sodium permeability. However, it is possible that cGMP could affect all of the permeability changes produced by ROCs (by interfering with the ROCs themselves). In this way NP could indirectly (via cGMP) reduce the depolarisation produced by NA or ACh.

KCl is thought to act primarily by opening voltage dependent channels which increase the membrane permeability to calcium and other ions such as sodium. Since NP was found not to reduce the depolarisation produced by KCl, one is forced to conclude that, if NP is acting via cGMP to reduce the permeability changes, the effect is confined to channels opened by NA or ACh receptors, (ROCs), ie. that only ROCs are sensitive to intracellular

changes in cGMP. The other possibility is that NP is acting directly on the coupling of receptor activation with the opening of these ROCs. This may be possible since organic nitrate vasodilators have been reported to interact with sulphydryl groups, either on the cell membrane (Smith and Krusyma, 1976, Winbury, Howe and Hefner 1969), or associated with specific receptor sites (Needleman and Johnson, 1973). However, later papers (Ito, Kityamura and Kuriyama, 1980) have suggested that nitroglycerine (and presumably related compounds) act on the final, calcium releasing stage of activation, rather than at the membrane. This was suggested on the basis of its non- selective action. The evidence of this study would suggest that it is not likely that NP acts in this way in the rat anococcygeus, since NP has different effects on the activation produced by the three agonists.

It would seem that NP acts in the rat anococcygeus either by affecting the coupling of receptor activation to the opening of ROCs, by interfering in some way with the ROCs, to reduce the permeability change produced, or by acting on some later stage in the different activation processes used by each agonist. The differential effect of NP on the contractions produced by ACh and NA suggests that, at least part of its effect is to interfere with the different activation processes used by NA and ACh, subsequent to the initial membrane events. However, since it is able to reduce the depolarisation produced by both NA and ACh, some of its effects must be on the coupling of receptor activation to membrane depolarisation. This effect is not due to a generalised effect on the membrane, since KCl depolarisation is not affected.

It appears, therefore, that NP acts in more than one way to produce inhibition in this preparation. One of these is by interfering with the depolarisation of the membrane produced by receptor activation. The other is by interfering with the later stages in the activation process, either directly or indirectly (via cGMP changes). However, these results are those obtained at normal calcium concentrations. The pattern of inhibition produced by NP is influenced by the calcium concentration, as can be seen in the calcium dose response curves.

2.2 Calcium Dose Response Curves (Figs 3-1 to 3-4)

Although NP produces no significant inhibition of NA or KCl induced contraction at normal calcium concentrations, a significant inhibition is produced in both cases at lower calcium concentrations, (around 5×10^{-4}M), ie. NP is a more effective antagonist at low calcium concentrations. This point is important in the consideration of the mode of action of NP. This effect (significant inhibition at low calcium concentrations) is also shown for ACh induced contractions, although in this case NP shows an additional effect, in that the inhibition is maintained at higher calcium concentrations leading to the 40% inhibition seen in normal solutions.

This increased efectiveness of NP at low calcium concentrations supports the idea that NP acts by increasing cGMP levels in the tissue which may then act to reduce calcium influx, or may produce other inhibitory effects.

Interesting results in this context were obtained from two experiments designed to investigate the calcium dependency of the changes induced in cyclic nucleotide levels by the agonists. The conditions of the experiments were the same as for those which investigated the changes in cyclic nucleotide levels due to the action of NA or KCl, except that calcium was omitted from the Krebs solution. The results of these experiments were;

	PROTEIN	cAMP	cGMP	RATIO
	mg.	pmol/mg.		
NA/Low Ca	10.5	1.35	0.54	2.3
KCl/Low Ca	9.75	0.81	0.30	2.7
CONTROL	8.25	0.90	0.37	2.6

It can be seen that the fall in cGMP normally produced by NA or KCl is not produced in low calcium solution. Note that NA still produces a slight rise in cAMP, thought to be due to a small β effect, so this effect is not calcium dependent. The slight rise in cGMP produced by NA in low calcium conditions may be linked in some way to this rise in cAMP. (since the two can affect each other, as stated previously, eg. cAMP may compete as a substrate for phosphodiesterase.). Note that the ratio produced by NA and KCl in low calcium solutions were very similar to the values for control muscles.

Similar results showing the calcium dependency of changes in cGMP levels in a number of preparations have been reported (Diamond and Janis 1980, Nilsson and Andersson 1977, Schultz et al 1973). Note that the changes produced by NP were not calcium dependent in many cases. Unfortunatly, it was not possible to investigate this in this study.

The results of these experiments would explain the mechanical effects of NP very well. Since the cyclic nucleotide changes induced by the agonists are calcium dependent, the amplification stage of the activation process due to them will be less effective in low calcium conditions. Since the changes in cyclic nucleotide levels produced by NP, however, are not dependent upon extracellular calcium, the effect of NP on cyclic nucleotide levels, (and therefore presumably on tension produced also), will become more important at low calcium concentrations.

Similar theories can be advanced to explain the data for ACh induced contractions. The main difference in this case is that the activation process used by ACh in normal calcium is more sensitive to NP (and to some extent V), so that one is forced to conclude that NP has some additional effect on ACh induced contractions which is important at normal calcium concentrations (as well as the effect at low calcium concentrations seen also with NA and KCl induced contractions). Just what this effect could be is difficult to see. Since the inhibition of the membrane depolarisation produced by NP is very similar for NA and ACh, it would seem that the effect is not due to some specific efect of NP on the ACh ROCs. It may be that NP can exert some specific effect

on ACh induced contractions by interaction with some later stage in the activation process. A similar type of effect has been proposed by Kreye et al (1975). This effect may be unrelated to the effects of NP on cGMP levels, although it is possible that the cyclic nucleotide changes induced by NA and ACh take place in different cellular compartments, with different sensitivities to NP. This concept of compartmentalisation may also be necessary to expain the lack of correllation found between the cyclic nucleotide changes and the relaxant effects induced by NP in the next section.

3.0 Cyclic Nucleotides; Effect of NP.

The data presented in the previous sections supports the hypothesis that cyclic nucleotide levels are important in the regulation of contractility in the rat anococcygeus. However, it has been reported in many instances that it is possible to change the level of one or other of the cyclic nucleotides without concommitant changes in contractility, and vice-versa (Diamond and Holmes, 1975, Schultz, Schultz and Hardman, 1975, Andersson 1973). One of the agents which can produce this dissociation of the changes in cyclic nucleotide levels and contractility is sodium nitroprusside (NP) (Diamond and Janis, 1978).

The experiments in this study showed that NP can produce a large rise in cGMP levels in the rat anococcygeus, as has been found in many other preparations. If this change in CGMP is responsible for the relaxant effect of NP, then when the change in the ratio of cAMP/cGMP produced by NP is plotted against the

tension change also produced, this point should lie on or near to the straight line on Fig D3. This is assuming that the cyclic nucleotide ratio is important in determining the tension produced by the agonists. NP (2×10^{-7}M) actually produces a rise in cGMP to about 0.76 pmol/mg., with little change in cAMP levels. The ratio cAMP/cGMP so produced is 0.9 (see cyclic nucleotide results, table R3). This ratio is produced when the muscles are treated with NA after NP. NA alone produces a ratio of 8.2 This is a dramatic change in cyclic nucleotide levels. However, the same concentratio of NP produces only about 10% relaxation of NA induced contractions, ie. the tension falls from about 250Nm^{-2} to around 230 Nm^{-2}. This point (ie a tension of 230Nm^{-2} and a ratio of 0.9) is plotted on Fig D3. It can be seen that it lies well off the line through the points produced by the agonists alone. A similar experiment was performed using muscles pre- treated with NP and then contracted with KCl. Unfortunatly it was only possible to assay one pool of 8 muscles. The ratio cAMP/cGMP was 1.56 in this case. The tension in the mechanical experiments was reduced to around 70 Nm^{-2} by this same concentration of NP. This point is also plotted on Fig D3 and it can be seen that it also lies well off the line produced by the agonists.

The results of these experiments with NP support the view of Diamond and Janis (1978), that increases in the levels of cGMP may not mediate the relaxant effects of NP. However, one is reluctant to abandon altogether the idea that the cyclic nucleotide ratio is important in the regulation of contraction. The evidence presented in section 1, (the good correllation between the ratio

and the tension produced by the three agonists), appears to be very strong evidence in favour of this theory. The theory also explains the data from the calcium dose response curves very well (that NP is more effective at low calcium concentrations), since the changes in the cyclic nucleotides induced by the agonists were found to be calcium dependent.

The alternative to abandoning the theory altogether is to suppose that some compartmentalisation occurs within the cell. One can suppose that cyclic nucleotide changes occur in many different cellular compartments, only one of which is important in the regulation of contraction. This important compartment is presumed to be very small in comparison to the others. It may be that under most circumstances the changes in the cyclic nucleotide ratio in all of the compartments are similar, so that a good correllation exists between the gross changes in cyclic nucleotide ratio and tension. NP may act differently in some compartments compared to others, however. In this case the gross changes in cyclic nucleotide ratio recorded may not be a true representation of that occurring in the small compartment which is important in the regulation of contraction. This would lead to the result obtained with NP, that the ratio of the nucleotides produced does not correllate well with the changes in tension. Such a compartmentalisation has been proposed in other preparations by Andersson, Nilsson, Wikberg, Johansson, Mohme-Lundholme and Lundholm (1975) and Polson, Krzanowski, Fitzpatrick and Sztenivanyi (1978).

3.1 Summary

The data presented in this section make it possible to produce theories regarding the mode of action of NP, although it is difficult to reach definite conclusions.

NP raises cGMP levels. This effect is probably not dependent upon extracellular calcium.
The effects of the agonists on cyclic nucleotide levels are calcium dependent.
This means that NP is more effective at low calcium concentrations.

This data supports the veiw that the primary action of NP is to raise cGMP levels intracellularly, which then produces some inhibitory effect, either a reduction in calcium influx, or some other effects. (eg effects on MLCK). The relative importance of these effects will depend upon just how much calcium influx is necessary for contraction in each case.

In order to explain satisfactorily the lack of correllation between cGMP levels and tension, however, a model of cellular compartmentalisation must be proposed in which the levels of cyclic nucleotides measured do not necessarily reflect those which are important in the regulation of contraction.

In addition to these effects it appears that NP can exert an effect specific to ACh activation, which leads to significant inhibition of ACh induced contractions even at normal calcium concentrations. This effect may be related to the concept of compartmentalisation also.

ANTAGONIST DOSE RESPONSE CURVES; STZ and Theo.

1.0 Introduction

The effects of Stellazine (STZ), (Trifluroperazine) and Theophylline (Theo) on the contractions induced by each of the three agonists were investigated. STZ is thought to exert its effects at the level of MLCK activation by calcium. Smooth muscle is thought to produce tension when the 20,000 Dalton subunit of myosin light chains is phosphorylated. This allows the myosin-actin interaction which generates tension (Chacko, Conti and Adelstein 1977, Gorecka, Aksoy and Hartshorne 1976). Myosin light chains are phosphorylated by a kinase, MLCK, which has as part of its stucture a calcium sensitive subunit (Drabowski, Aromatoril, Sherry and Hartshorne 1977, Drabowski, Sherry, Armatorio and Hartshorne 1978). This subunit confers calcium sensitivity upon MLCK,and hence upon the contractile system as a whole. This calcium sensitive protein has also been shown to be involved in the activation of numerous other enzymes and is generally refered to as calmodulin, (CALcium sensitive MODULator proteIN) (Walsh, Vallet, Cavadore and Demaille 1980).

STZ has been shown to bind to calmodulin in various enzyme systems (Levin and Weiss 1976,1977). It is thought that this prevents the activation of these enzymes by calcium. It has also been shown that STZ can inhibit the calcium activation of smooth muscle MLCK and this is thought to be responsible for the inhibition of tension (Cassidy et al 1980). The basic hypothesis

for the mode of action of STZ, therefore, is that it prevents the calcium sensitive activation of MLCK by calmodulin, thus preventing myosin light chain phosphorylation and subsequent tension development (see Fig G3). However, it must be born in mind that many diverse enzyme systems have been shown to depend on activation by calmodulin, eg. phosphodiesterase (Cheung 1979), adenyl cyclase (Brostrom, Huang, Breckenridge and Wolff 1975), phosphorylase kinase (Cohen 1979) and so STZ may have an influence on many diverse processes within the cell.

Theophylline (Theo) has been shown to inhibit phosphodiesterase in a number of tissues (Newman, Colella, Spainhow, Bramn, Zobko-Potapovich and Wardell 1978, Polson, Krzanowski, Adersson, Fitzpatrick, Huang and Szentivanyi 1979). There is generally a good correllation between the inhibition of phosphodiesterase catalysed cAMP hydolysis and the inhibition of tension in many tissues and this has been proposed as the mode of action of Theo. However, in many instances the concentration of Theo necessary for phosphodiesterase inhibition was many times greater than that necessary for inhibition of tension. Also, phosphodiesterase occurs in many forms, (ie. a high and a low Km form), and many of these enzyme studies have been done on the high Km (low affinity) form, using high substrate concentrations. It has been shown that, at low substrate concentrations, phosphodiesterase from brain hydrolyses cGMP faster than cAMP. (Brostrom and Wolff 1976). Also, the Km is lower for cGMP. Phosphodiesterase from glial tumour cells has been shown to be more sensitive to calcium when cGMP is the substrate, rather than cAMP.

(Brostrom and Wolff 1974).

These results cast doubt upon the hypothesis that Theo produces its relaxant effects by inhibition of phosphodiesterase. It seems possible that Theo could alter levels of cAMP and cGMP in ways which could not be predicted from the in vitro data. Very few experiments have been done to investigate the changes in cyclic nucleotide levels actually produced in vivo by Theo.

An alternative mode of action for Theo has been proposed by Meich, Niedzwicki and Smith (1979). They have shown that there is a cAMP binding protein in tracheal smooth muscle. The binding of cAMP to this protein is increased by Theo (leading to a reduction in cAMP levels) at concentrations much lower than those needed for phosphodiesterase inhibition. cGMP can reduce the binding of cAMP, by substrate competition, so accounting for the interrelationship often seen between the two nucleotides.

2.0 The Rat Anococcygeus.

The results obtained in this study showed that the contractions produced by all three agonists were inhibited by STZ and Theo (Figs R2-5 to R2-8). In all cases STZ was the most potent antagonist. This difference in potency is markedly less for ACh induced contractions, however. This can be seen more clearly when the results are replotted as % of control tension against concentration of STZ or Theo (Figs D6A/B). When the data is plotted in this way it is noticeable that NA induced contractions

Fig D6 A Effect of STZ

The results of the antagonist dose response curve for STZ for each agonist plotted as % control response for each agonist.

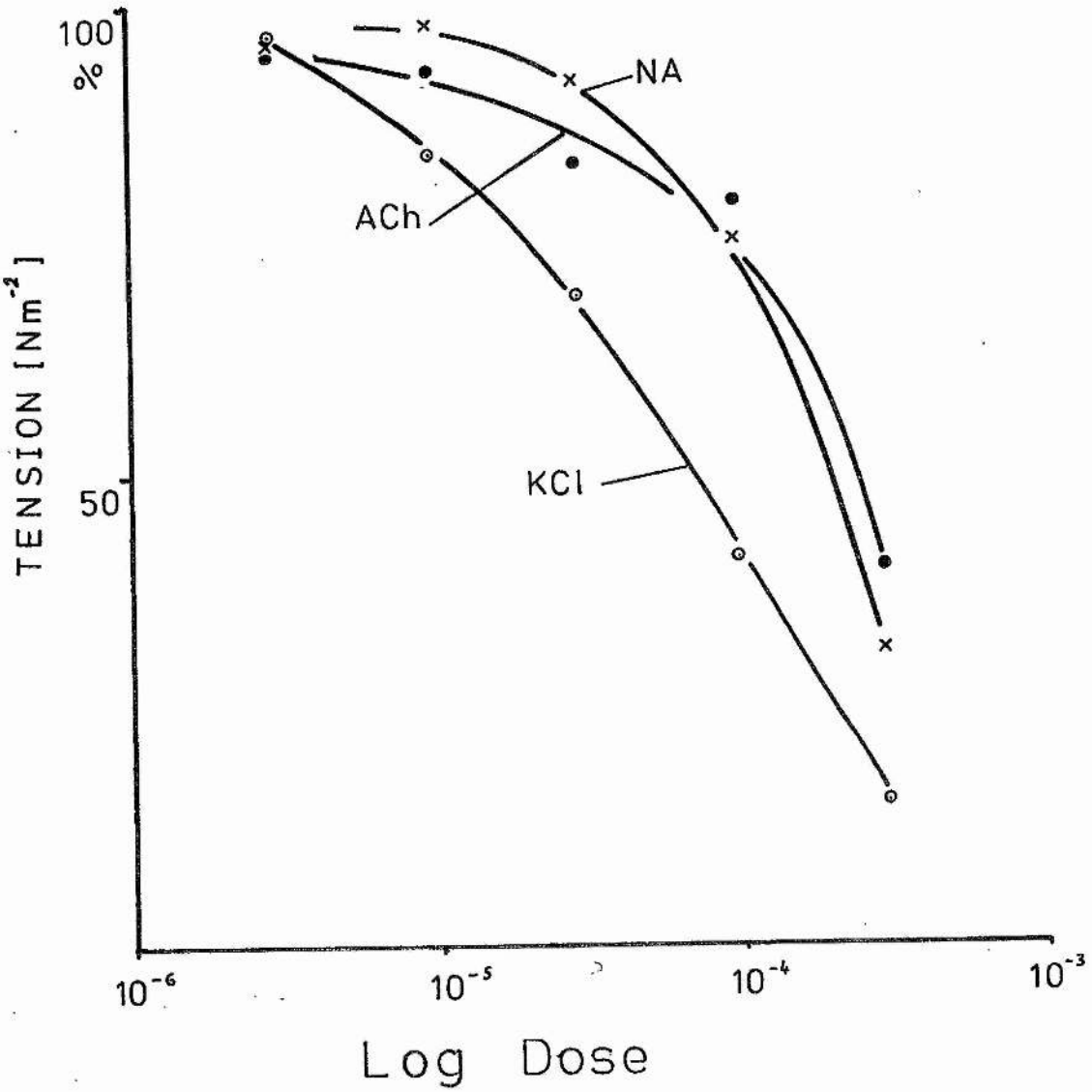
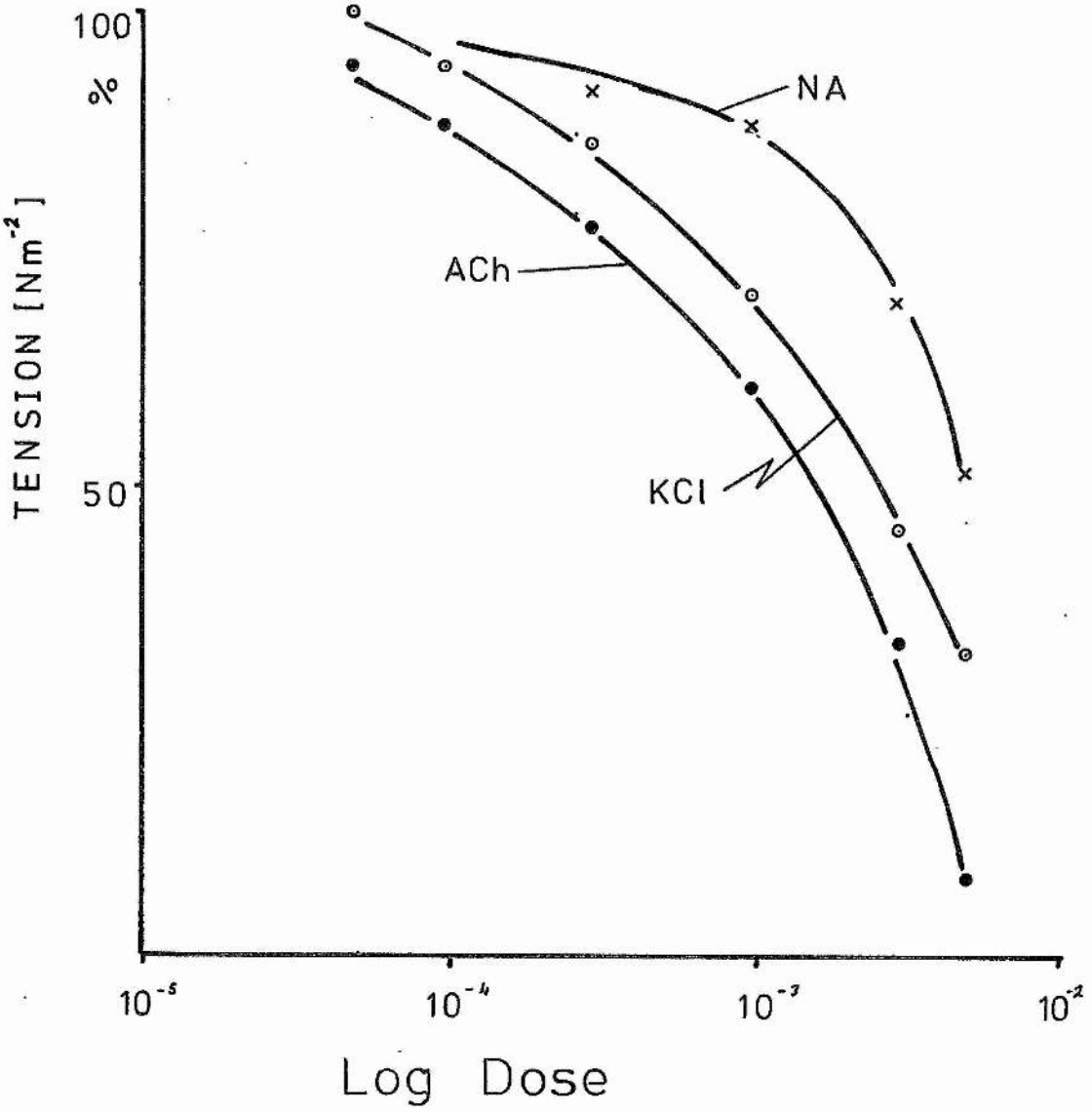

100
%
50
TENSION [Nm^{-2}]
NA
ACh
KCl
10^{-6}
10^{-5}
10^{-4}
10^{-3}
Log Dose

Fig D6 B Effect of Theo

The results of the antagonist dose response curves for Theo for each agonist plotted as % control response for each agonist.


100
%
50
TENSION [Nm^{-2}]
NA
ACh
KCl
10^{-5}
10^{-4}
10^{-3}
10^{-2}
Log Dose

are less sensitive than ACh or KCl induced contractions to both STZ and Theo. Presumably this is due to the more powerfull (or subtly different) activation processes induced by NA. In the case of Theo, it seems reasonable to suppose that the changes induced by Theo will be less effective against the background of the large changes induced by NA itself (see section 1).

The inhibition of MLCK activation produced by STZ cannot be overcome by increasing the calcium concentration (Levin and Weiss 1976). This indicates that, since the contractions induced by ACh or NA are less inhibited by STZ, these agonists are producing a greater contraction than KCl by some other means than by producing a greater rise in intracellular free calcium concentration. ie.- there is some amplification process involved in the activation pathway. All the evidence to date supports the view that NA and ACh produce less rise in intracellular calcium concentration than does KCl.

GENERAL CONCLUSION

The overall scheme for the activation of the Rat anococcygeus by the three agonists which fits the data most consistently is shown in Figs G1 and G2.

Fig G1 shows the proposed scheme for KCl activation. Calcium enters the cells via voltage dependent channels, which are opened when the high extracellular concentration of KCl depolarises the membrane. This influx of calcium is inhibited strongly by Verapamil. The rise in intracellular calcium so produced has two main effects. 1) It activates the contractile proteins (via MLCK) and 2) it causes a fall in C.GMP levels (probably by the calcium- dependent activation of a phosphodiesterase).

The free intracellular calcium is also exchanged with intracellular stores, which produces the charateristic bi-phasic contraction seen with KCl activation. cGMP may act to increase the binding of calcium to the stores and/or to reduce the influx of calcium through the membrane. These effects can account for the observed effects of NP, since this acts to increase cGMP levels. How NP causes a rise in cGMP is not known.

Fig G2 shows the proposed scheme for NA and ACh activation. The activation processes for these two agonists are very similar in principal.

Activation of the muscarinic or alpha adrenergic receptor

Fig G1 General scheme for activation by KCl

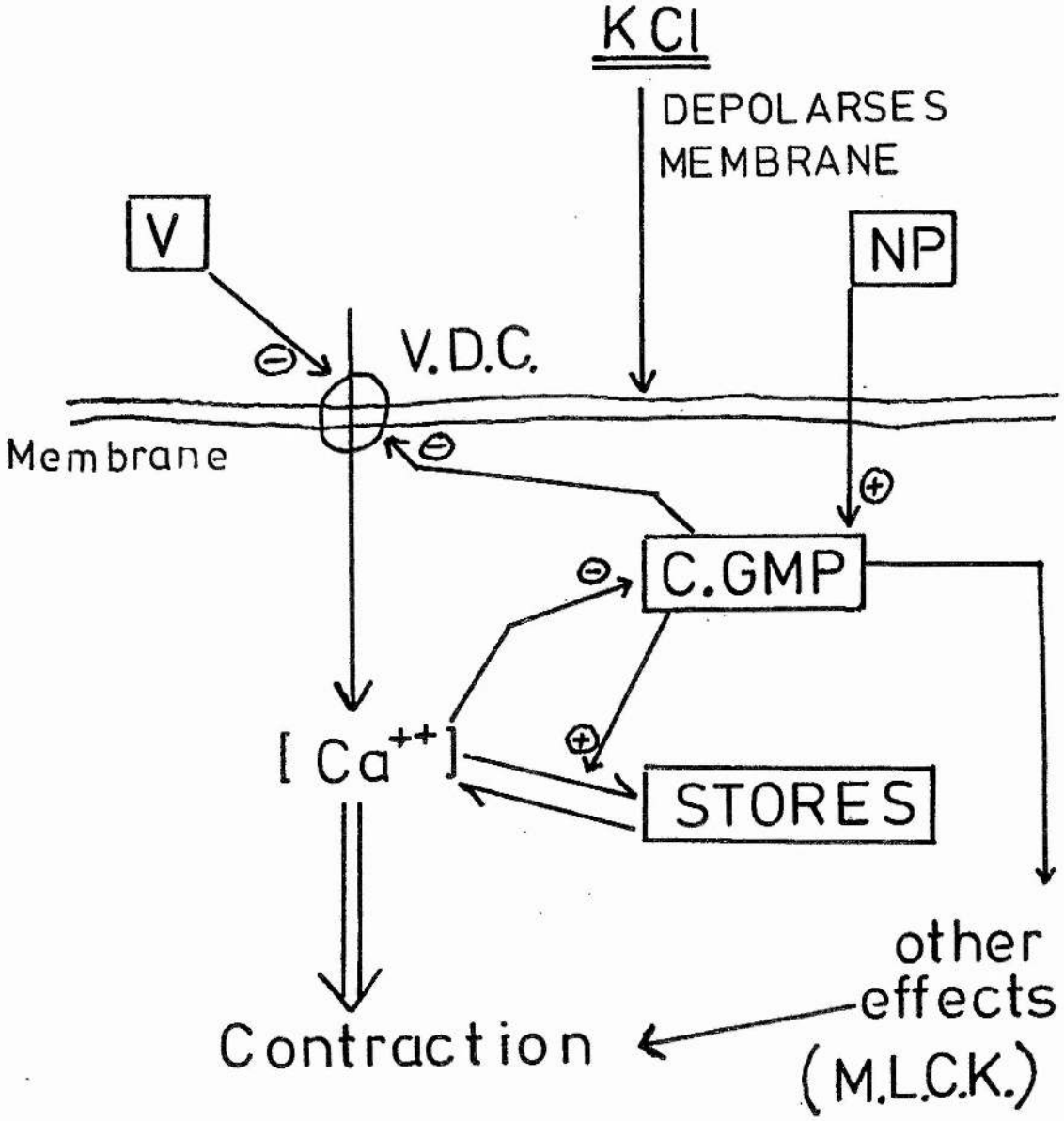
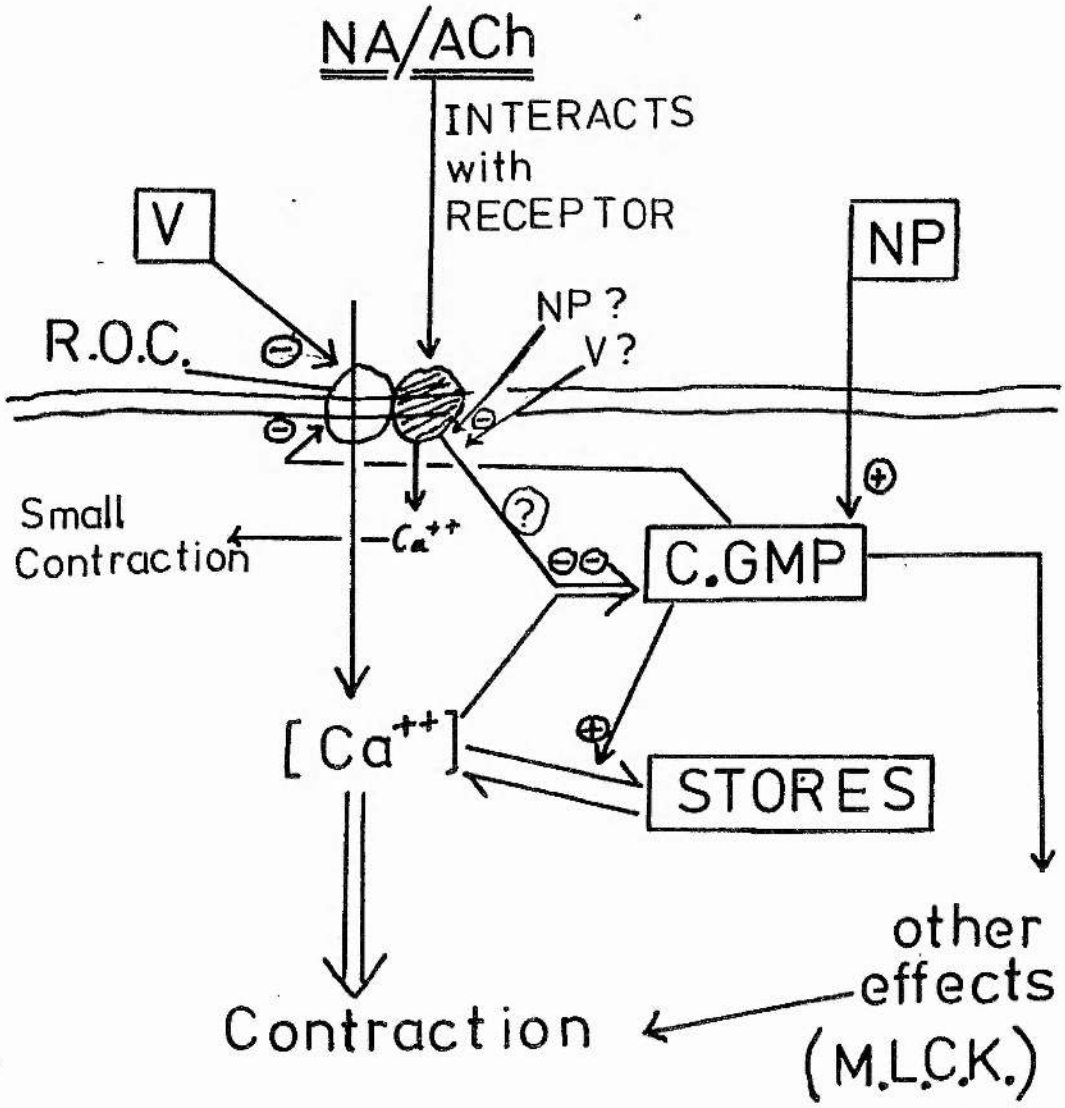

KCl
DEPOLARSES
MEMBRANE
V
NP
V.D.C.
Membrane
C.GMP
[Ca++]
STORES
other
effects
(M.L.C.K.)
Contraction

Fig G2 General scheme for activation by NA and ACh


NA/ACh
INTERACTS
with
RECEPTOR
V
NP
NP ?
V ?
R.O.C.
Small
Contraction
Ca^{++}
?
C.GMP
$[Ca^{++}]$
STORES
other
effects
(M.L.C.K.)
Contraction

initially causes the release of a small amount of intracellularly bound calcium, possibly asociated with the receptor itself. This small amount of calcium can cause a small contracion under certain circumstances (eg in low calcium solutions). Receptor activation also generates some hypothetical signal which acts in conjunction with calcium to cause a fall in cGMP levels. This effect may be due to a calcium- dependent phosphodiesterase associated with the receptor.

Receptor activation also opens ROCs associated with the receptors, so allowing an influx of calcium. This influx raises intracellular free calcium levels, an effect which may be enhanced by the calcium induced release of intracellular calcium stores, which then produces the following effects. 1) It acts with the hypothetical signal from the receptor to lower cGMP levels. 2) It causes the activation of the contractile proteins (via MLCK). This activation is enhanced greatly by the effects on cGMP levels (possibly via an MLCK phosphorylase). This leads to the possiblity that NA and ACh can produce a greater rise in tension, although the rise in free calcium in the cell is smaller, when compared to KCl activation.

NP acts by causing a rise in cGMP levels. This in turn; 1) reduces the "amplification" of the calcium signal due to the fall in cGMP normally seen upon activation, and 2) increases calcium uptake into intracellular stores and/or reduces calcium influx (as for KCl activation). In the case of ACh NP may also affect the coupling of receptor activation to the "amplification" stages of

the activation process (possibly by affecting the hypothetical signal which lowers cGMP levels.)

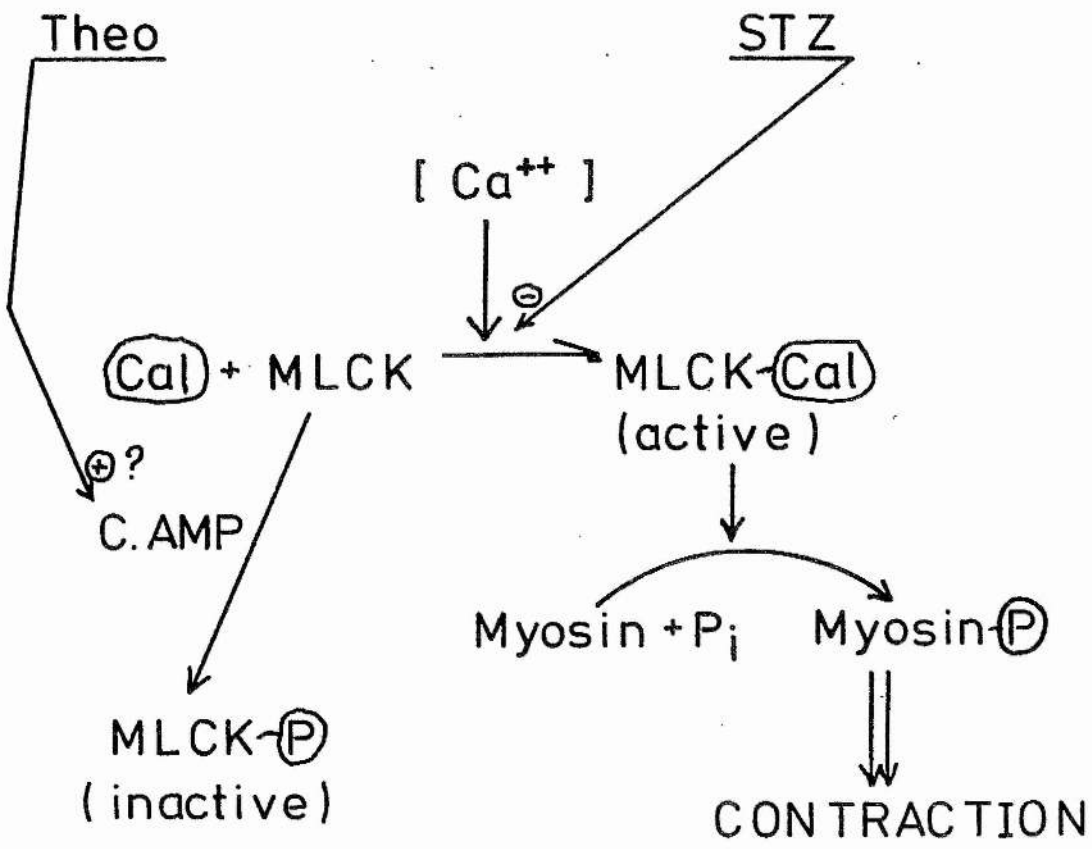
Verapamil also has this type of effect on ACh activation and probably also reduces the influx of calcium through ROCs slightly. The effects of V on NA activation are much less pronounced. These latter effects of NP and V are shown on the figures as NP? and V?.

STZ is proposed to act in the same way for all three agonists. The proposed scheme is shown in Fig G3. STZ prevents the calcium-dependent activation of MLCK by calmodulin, hence causing an ihibition of tension generation. It may also interfere with the calcium- dependent activation of many other enzymes. The fact that NA (and to a lesser extent ACh) activation is less dependent upon a rise in intracellular calcium (due to the "amplification" processes occurring) means that contractions induced by these agonists are less inhibited by STZ.

The effects of Theo are probably due to inhibition of phosphodiesterase, which causes a rise in cGMP levels. This in turn will produce inhibition of tension as seen in the way outlined above. However, Theo undoubtably affects cyclic nucleotides in a more comlicated way than, say, NP, (possibly by effects on a cAMP binding protein), so that the effects on activation by the agonists are different to those produced by NP.

The scheme proposed above is a simplified picture of the activation processes and the effects of the antagonists. In

Fig G3 General scheme for the mode of action of STZ and Theo


Theo
STZ
[Ca++]
⊖
Cal + MLCK
MLCK-Cal
(active)
⊕?
C. AMP
Myosin + Pi
Myosin-P
MLCK-P
(inactive)
CONTRACTION

reality the situation is undoubtably more complex. In particular, some form of compartmentalisation of the activation processes must be proposed if the scheme is to fit all the data. In addition to their effects on contractiliy, cyclic nucleotides can also influence many diverse processes within the cell. Part of the function of the changes in cyclic nucleotide levels produced by activation may be, therefore, to syncronise the events necessary for contraction upon activation. The effects on contracility itself may only be a minor part of their role.

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APPENDIX A – Biuret Reagent