CATECHOLAMINE SECRETION BY ISOLATED BOVINE ADRENAL MEDULLARY CELLS: EFFECTS OF AGENTS WHICH INFLUENCE ACh AND GABA RECEPTORS

NARGIS AKHTER

DECLARATIONS

I, Nargis Akhter, hereby certify that this thesis has been composed by myself, that it is a record of my own work and is submitted in partial fulfilment of the requirements for the degree of M.Sc.

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ABBREVIATIONS

AADC	aromatic L-aminoacid decarboxylase
A-cells	adrenaline-containing cells
ACh	acetylcholine
AChE	acetylcholinesterase
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
BZD	benzodiazepines
CA	catecholamines
CCh	carbachol
cGMP	cyclic guanosine monophosphate
DBH	dopamine β-monooxygenase
DMM	Dulbecco's Modified Medium
DMPP	dimethylphenylpiperazinium
DZ	diazepam
ED	effective dose
e.p.s.p.	excitatory postsynaptic potential
GABA	γ-aminobutyric acid
GTP	guanosine triphosphate
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HPLC	high performance liquid chromatography
i.p.s.c.	inhibitory postsynaptic conductance
N-cells	noradrenaline-containing cells
PNMT	phenylethanolamine-N-methyltransferase
TH	tyrosine hydroxylase
TTX	tetrodotoxin

Units:

g	grams
i.u.	International Units
min	minutes
mg	milligrams
M	molar
mM	millimolar
uM	micromolar
umoles	micromoles
ml	millilitres
ul	microlitres
um	micrometers
N	normal
nM	nanomolar
nmoles	nanomoles
nm	nanometers
pmoles	picomoles

SUMMARY

1. The secretory function of bovine adrenal medullary cells in response to a number of drugs has been investigated.

2. Bovine adrenal medullary cells were isolated and maintained in culture up to 14 days. Catecholamines secreted into the external medium, at 37°C during 10 or 15 min exposure to different agents, were measured fluorometrically. Fluorescence spectra were determined routinely and compared with authentic noradrenaline and adrenaline.

3. High potassium significantly stimulated catecholamine secretion.

4. Nicotine, a selective nicotinic agonist, evoked catecholamine secretion in a dose-dependent manner and the effect was significant over a range of concentrations from 5 to 250 uM. Nicotine at higher levels (>100 uM) exhibited a characteristic inhibition of secretion. The effect of nicotine was antagonized by hexamethonium (1mM).

5. Carbachol, a mixed cholinergic agonist, stimulated catecholamine release in a dose-dependent manner; threshold was about 10uM. There was marked variability from day to day in the concentration of carbachol which produced maximum secretion

(100, 200 and 400 uM). Carbachol at higher concentrations reduced the secretory response.

6. The muscarinic agonist bethanechol had no effect of its own (10-1000 uM), but at 2 and 10uM it significantly reduced the nicotine-stimulated secretion. The effect of bethanechol was antagonized by atropine (0.1uM).

7. GABA (100uM) alone did not influence the background level of catecholamine secretion, but it did significantly depress the secretion evoked by carbachol (200uM) and nicotine (50uM). The effect of GABA (100 uM) on nicotine (50 uM)-evoked secretion was reversed by bicuculline (5uM).

8. Bicuculline (2 uM) itself had no effect on catecholamine secretion but it significantly facilitated the nicotine (50 uM)-evoked secretory response.

9. Diazepam (10uM) alone significantly suppressed the effect of nicotine (50uM). With GABA (10 and 100uM), diazepam (10uM) exerted an additive effect in reducing the catecholamine secretion. There was no interaction between GABA and diazepam (10uM).

10. Alphaxalone (300nM) alone had no effect on nicotine-evoked catecholamine release, but at 100 and 300 nM it significantly potentiated the inhibitory effect of GABA on nicotine-induced

CONTENTS

INTRODUCTION

1.1 Chromaffin Cells

Adrenal chromaffin cells are generally regarded as highly modified postganglionic sympathetic neurones which secrete hormones directly into the circulation rather than to a specific effector cell (Brooks, 1977). The cells synthesize and store the catecholamine hormones, adrenaline and noradrenaline, and secrete them in response to stimulation by a variety of secretagogues (Hochman & Perlman, 1976). Acetylcholine, released from preganglionic sympathetic nerve terminals within the adrenal gland, is thought to be the physiological stimulus for catecholamine secretion (Feldberg, Minz & Tsudzimura, 1934).

The mature adrenal medulla contains two types of histochemically distinguishable chromaffin cells, adrenaline-containing (A-cells) and noradrenaline-containing (N-cells)(Bowman & Rand, 1980). All the cells in medulla can form noradrenaline, while only certain specific cells - whose number varies from none to all - contain methylating enzyme, phenylethanolamine-N-methyltransferase (PNMT), convert noradrenaline to adrenaline and store this hormone (Hillarp & Hokfelt, 1953). The ratio of A-cells to N-cells in the adult human adrenal is about 7:1. The content of catecholamines in the adrenal medulla of various species ranges from 0.3 to 10 mg/g of tissue and the proportion of adrenaline ranges from 40-100% (in man being about 80%)(Bowman & Rand, 1980).

In the fetus, all the adrenal chromaffin cells synthesize and store noradrenaline. The A-cells do not take on their

specialized role until birth. The majority of extra adrenal chromaffin cells are predominantly noradrenaline-containing. Their number decreases with increasing maturity. Chromaffin cells are stained a yellow brown colour by dichromate; on this account the cells are termed chromaffin cells signifying an affinity for chrome salt.

Adrenocortical hormones secreted from the cortex are delivered to the medulla via the intra-adrenal portal vascular system, where they stimulate the synthesis of medullary enzyme PNMT and other medullary proteins. In hypophysectomized animals or in Addison's disease, where the levels of adrenocortical hormones are low, a fall in adrenaline output is evident (Bowman & Rand, 1980). Therefore, adrenaline is the predominant medullary hormone in species where the medulla is completely surrounded by cortical tissue (e.g. man and rat).

1.2 Chromaffin Granule Dynamics

1.2.1 Origin and Structure of Granules

The catecholamine storing organelles of adrenal medulla are called chromaffin granules. It is probable that the chromogranins are synthesized in the rough endoplasmic reticulum which hypertrophies in the chromaffin cells (Smith & Winkler, 1972). Electron microscopists have suggested that chromaffin granules originate in the golgi-apparatus.

The chromaffin granules are membrane-bound spheres containing adrenaline (PNMT-containing cells) or noradrenaline and other components (Ungar & Phillips, 1983). The granular

membrane is characterized by a high lipid to protein ratio (Winkler, 1980).

Energy for transport of granules is derived from ATP hydrolysis catalysed by an H^+-translocating ATPase. The enzyme utilizes cytoplasmic MgATP as a substrate, and hydrolysis is coupled with electrogenic proton transport (Ungar & Phillips, 1983). Chromaffin granules have a high calcium content (Borowitz, Fuwa & Weiner, 1965). About 60% of the total bovine adrenal medullary calcium is found within the granules. Granular calcium probably does not have a role in catecholamine storage but is simply ejected from the cell during catecholamine release.

1.2.2 **Catecholamine Content and Biosynthesis**

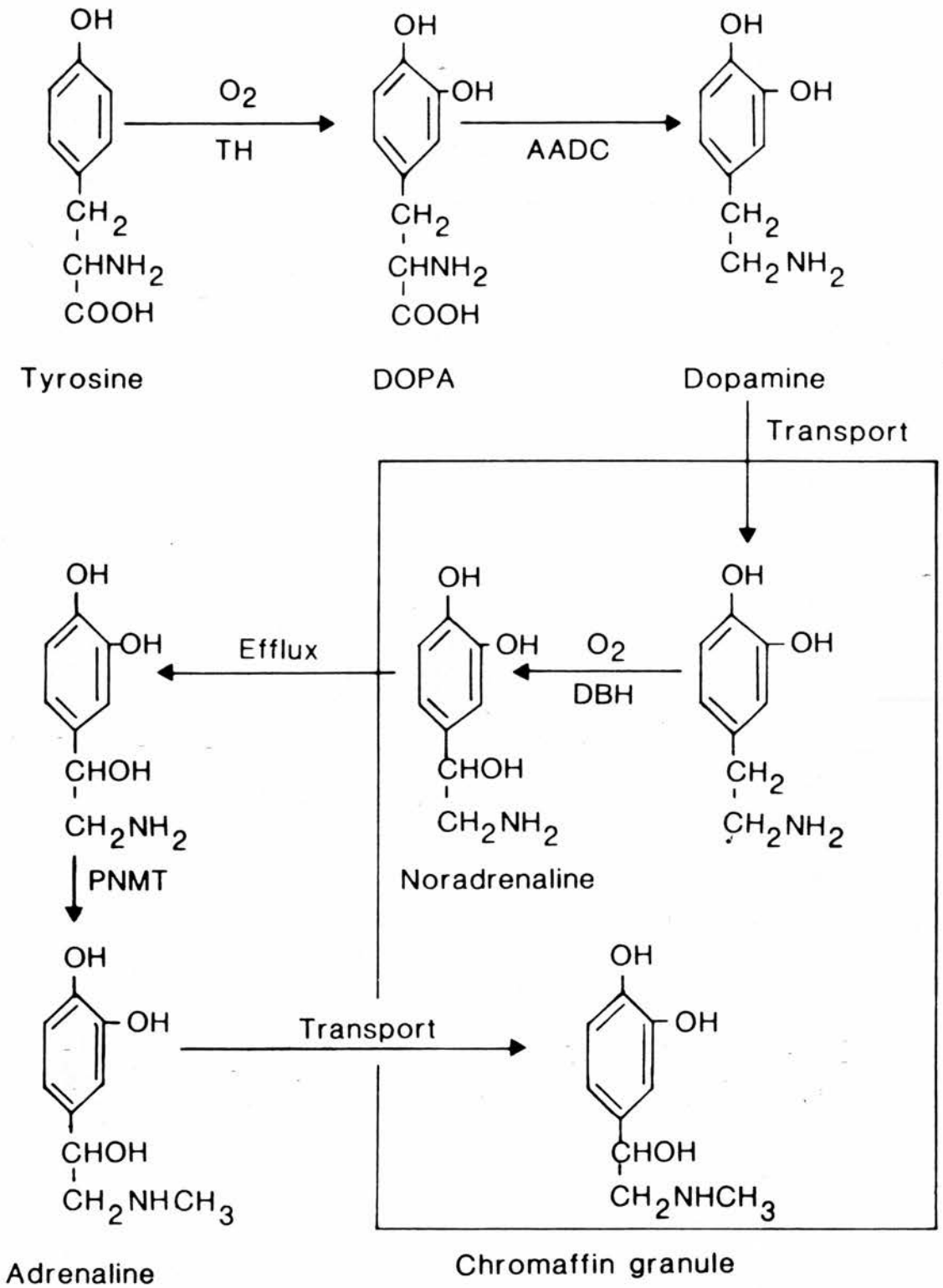
The reported data on the catecholamine concentration of bovine chromaffin granules isolated by density gradient centrifugation fall into a range of 2.0-2.9 umoles/mg protein (Winkler, 1980). In addition to noradrenaline (27% of total CA) and adrenaline (72%), adrenal chromaffin granules also contain trace amounts of dopamine.

The established pathway of noradrenaline and adrenaline biosynthesis is shown in Fig. 1.

The rate-limiting enzyme of the biosynthetic pathway is tyrosine hydroxylase (TH). After prolonged stress, the adrenal medulla contains larger amounts of TH and other biosynthetic enzymes (Ungar & Phillips, 1983).

The final enzyme in the pathway is PNMT, confined to

Fig. 1. Biosynthetic pathway of catecholamines in adrenal medulla. TH, tyrosine hydroxylase; AADC, aromatic L-amino acid decarboxylase; DBH, dopamine β-monooxygenase; PNMT, phenylethanolamine-N-methyltransferase. [Adapted from Ungar & Phillips, 1983.]


OH
O_2
TH
OH
OH
AADC
OH
OH
CH_2
$CHNH_2$
COOH
CH_2
$CHNH_2$
COOH
CH_2
CH_2NH_2
Tyrosine
DOPA
Dopamine
Transport
Efflux
O_2
DBH
CHOH
CH_2NH_2
PNMT
Noradrenaline
Transport
CH_2NHCH_3
Adrenaline
Chromaffin granule

adrenaline-releasing cells in the adrenal medulla and is cytoplasmic (Bowman & Rand, 1980). Consequently noradrenaline formed from dopamine within granules leaks into the cytoplasm for subsequent methylation.

Catecholamine is sequestrated within chromaffin granules and thus is essentially in an extracellular compartment. Stimulation of the gland to release catecholamines therefore does not necessarily lead to a reduction in cytoplasmic amine levels unless such stimulation also leads to an increased synthesis of new granules, which then deplete the cytoplasmic amine (Ungar & Phillips, 1983). Adrenocortical hormones secreted from the cortex stimulate the synthesis of PNMT.

1.2.3 **Other Chromaffin Granule Constituents**

1. Chromogranins and Dopamine β-hydroxylase. The soluble proteins of the chromaffin granules were termed as chromogranins (Blaschko, Comline, Schneider, Silver & Smith, 1967). Antibodies to the major chromogranin, chromogranin A and to dopamine β-hydroxylase have been used to localize these proteins in cultures of bovine chromaffin cells (Fenwick, Fajdiga, Howe & Livett, 1978).

2. ATP. The chromaffin cells of the adrenal medulla store MgATP in specific proportions to catecholamines within the chromaffin granules (1:4), (Winkler, 1980).

Established constituents of bovine chromaffin granules (Winkler, 1980).

Catecholamines:

- adrenaline
- noradrenaline
- dopamine

Nucleotides:

- ATP
- ADP
- AMP
- GTP

Proteins

- Chromogranin A
- Chromomembrin B
- Enzymes:
  - dopamine β-hydroxylase
  - Mg -activated ATP-ase
  - Cytochrome b-561
  - NADH: (acceptor)-oxidoreductase
  - Phosphatidylinositol kinase

Lipids:

- Cholesterol
- Phospholipids

Calcium
Mucopolysaccharides
Ascorbic acid

1.3 Opioid Peptide Content of Chromaffin Cells

The opioid peptide content, enkephalins and dynorphins, in chromaffin cells exhibits great variation among different species. The dog and the ox have the highest tissue levels followed by the human. The enkephalins are localized in the cytoplasm and processes of adrenaline-containing cells in culture. In contrast, the dynorphins are associated with noradrenaline-containing cells. The functional role of those peptides is still not clear (Livett, 1984).

1.4 Stimulus-Secretion Coupling

The release of catecholamines from chromaffin granules is Ca^{2+}-dependent (Douglas, 1968). All the events involved in stimulus-secretion coupling, must occur on, within or very close to plasma membrane (Smith & Winkler, 1972).

The events of stimulus-secretion coupling (Douglas, 1968)

Interaction between acetylcholine and the receptor

↓

Increased permeability of membrane to ions

↓

Depolarization

↓

Electrotonic spread of depolarization around the chromaffin cell

↓

Entry of calcium ions

↓

Attachment of vesicle to the plasma membrane

↓

Fusion of vesicle membrane with plasma membrane and formation of opening into extracellular space

↓

Expulsion or free diffusion of vesicle contents into extracellular space

↓

Fission of vesicular membrane from plasma membrane

[Modified by Smith & Winkler, 1972]

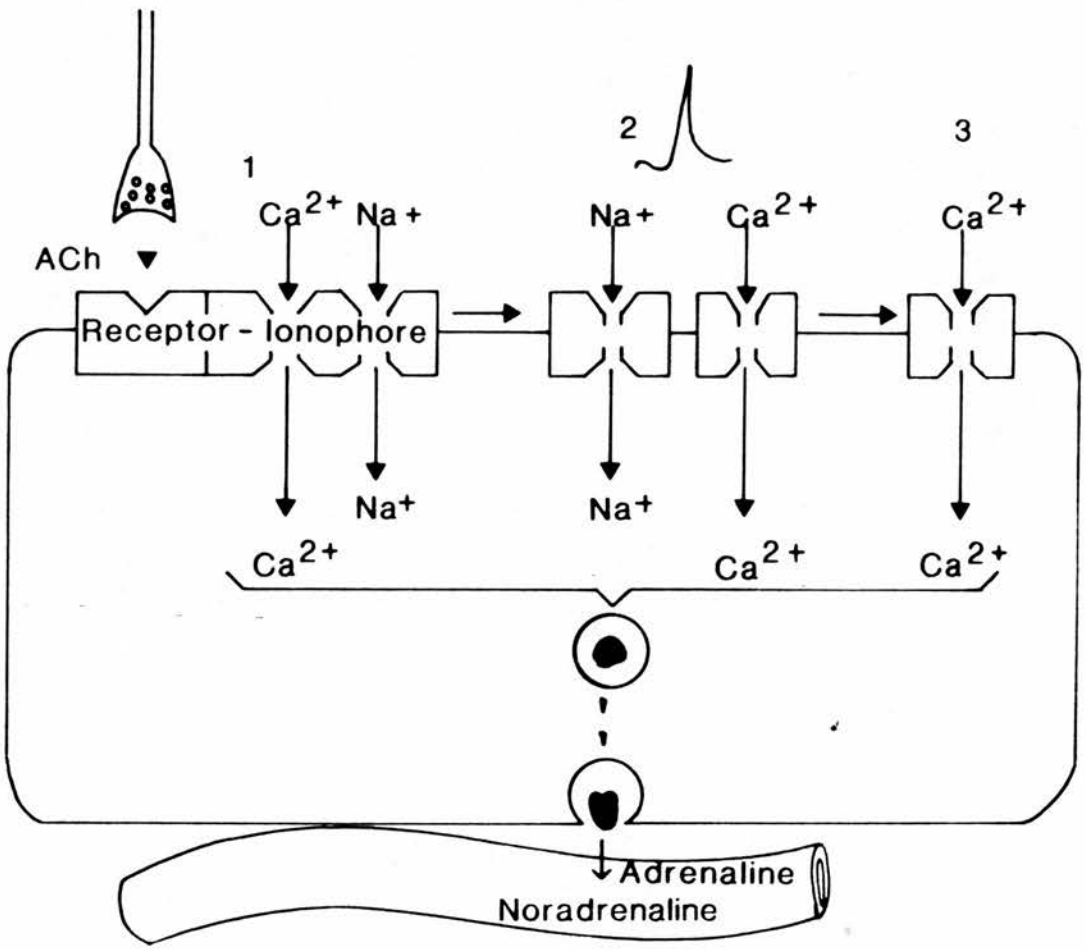
1.5 Ion Channels in Secretion

Acetylcholine released on stimulation from the splanchnic nerve terminals binds to receptor sites on the chromaffin cell membrane (Fig. 2). Binding of ACh to nicotinic receptor (hexamethonium-sensitive) opens a receptor-linked ion channel that is permeable to both Na^+ and Ca^{2+} (insensitive to TTX). ACh also interacts with muscarinic receptors (sensitive to atropine). The binding of ACh to the nicotinic receptor-ionophore complex in step 1 stimulates the entry of Na^+ and Ca^{2+} via separate voltage-sensitive channels. The voltage sensitive Na^+ channel is blocked by TTX (tetrodotoxin). The entry of these positive ions further depolarizes the cell membrane and increases the frequency of action potentials. The depolarization caused by Na^+ and Ca^{2+} flux through the voltage sensitive channels also opens D-600-sensitive Ca^{2+} channels (step 3) through which Ca^{2+} enters and triggers exocytosis and secretion of catecholamines and other vesicular components (ATP, enkephalin, chromogranin etc.)(Schneider, Cline, Rosenheck & Sonenberg, 1981; Kilpatrick, Slepetis & Kirshner, 1981).

1.6 Chromaffin Cells as Modified Neurones

Chromaffin cells of the adrenal medulla share many properties in common with neurones (Fenwick, Marty & Neher, 1982). Their embryological origin is from the neural crest (Bowman & Rand, 1980), they function to release vesicle contained substances in response to specific stimuli, they are

Fig. 2. Normal sequence of events in stimulus-secretion coupling in the bovine chromaffin cell. [Adapted from Livett et al., 1983.]


1
2
3
ACh
Ca2+
Na+
Na+
Ca2+
Ca2+
Receptor - Ionophore
Na+
Na+
Ca2+
Ca2+
Ca2+
Adrenaline
Noradrenaline

electrically excitable and are depolarized by acetylcholine (Brandt, Hagiwara, Kidokoro & Miyazaki, 1976). Furthermore, like neurones these cells have been shown to possess Na^+ and K^+ channels (Kidokoro & Ritchie, 1980). Adrenal medulla is therefore regarded as a model of neurones containing catecholamine (CA) and is the most suitable tissue for studying the regulation of CA release and synthesis (Yanagihara, Isosaki, Ohuchi & Oka, 1979).

1.7 **Different Methods for Study**

The retrogradely perfused adrenal gland maintained *in vitro*, has been used extensively to study the mechanism of secretion from the gland (Banks, 1965; Rubin & Miele, 1968; Douglas & Poisner, 1965; Kidokoro & Ritchie, 1980), but it has a number of limitations. The preparation is not actually suitable for dose-response studies of agonists and antagonists because the secretory response of the perfused gland declines slowly (Livett, Boksa, Dean, Mizobe & Lindenbaum, 1983). In addition, it had limitations for workers who were interested in studying the electrophysiological activity of cells during secretion (Douglas, 1968). Recently many workers have overcome these limitations by using isolated adrenal chromaffin cells (Hochman & Perlman, 1976; Schneider, Herz & Rosenheck, 1977; Brooks, 1977; Fenwick *et al.*, 1978) from different species of animals.

There are several experimental advantages of the use of isolated bovine chromaffin cells over studies *in vivo* or with retrogradely perfused medullae *in vitro*. First, the isolated

cells can be prepared in high yield and maintained in culture free of non-neuronal cells. Second, use of isolated cells avoids any indirect actions of agonists or antagonists on nerve endings that would be present in the intact gland (Livett et al., 1983). Third, the cells are readily accessible to the various agents added to the extracellular medium which can easily be recovered for analysis. Lastly, chromaffin cells can be studied without having any influence of cortical cells which are known to affect for instance the conversion of noradrenaline to adrenaline (Schneider et al., 1977).

1.8. Depolarization of Chromaffin Cells with High K

Evidence has existed for many years that sympathetic nerves innervating the adrenal medulla are cholinergic. Acetylcholine (ACh) released from splanchnic nerve terminals, stimulates catecholamine secretion from the adrenal medulla (Feldberg et al., 1934). Simple depolarization of chromaffin cell membrane is a stimulus for catecholamine secretion (Douglas, 1968). Therefore, secretion can also be evoked by depolarizing concentration of KCl (Douglas & Rubin, 1961). Adrenaline release from the perfused rat adrenal gland was stimulated by raising the concentration of KCl in the perfusion solution and this effect was readily reversible (Kidokoro & Ritchie, 1980). Douglas & Rubin (1963) showed an intense secretion of catecholamine during perfusion of adrenal medulla with high K_2SO_4 Locke's solution. Stimulation with high potassium (65mM KCl) also induced catecholamine secretion

from isolated bovine chromaffin cells but differed from acetylcholine stimulation in being less potent (Schneider et al., 1977). Whatever might be the stimulus for secretion, ACh or KCl, the release requires external Ca^{2+} (Douglas & Rubin, 1961).

1.9 Cholinergic Receptor Activation and Catecholamine Release

Feldberg et al. (1934), in their classical study, found that the action of acetylcholine on the adrenal medulla, although mainly nicotine-like, had a muscarine-like component. This has been confirmed for the cat adrenal medulla (Douglas & Poisner, 1965; Rubin & Miele, 1968) and for the dog adrenal medulla (Critchley, Tibenham, Ungar, Waite & West, 1975; Kayaalp & Turker, 1969). Douglas and Poisner (1965) showed that in the cat adrenal medulla muscarinic agonist pilocarpine released adrenaline with little or no noradrenaline although nicotine released large amounts of both amines. The observation was different in ox adrenal medulla, which when perfused with Tyrode's solution retrogradely in vitro did not release catecholamine even when exposed to high concentrations (0.1-10mM) of two typical muscarinic stimulants: pilocarpine and methylfurmethide (Smith & Winkler, 1972). The role of muscarinic receptors is more complicated. They are present in other species where muscarinic agonists do not selectively release adrenaline (Ungar & Phillips, 1983).

Information on the type of ACh receptor involved in the

activation of isolated chromaffin cells is rather sketchy. Douglas, Kanno & Sampson (1967) found that the depolarization by ACh of isolated gerbil chromaffin cells was reduced by hexamethonium and abolished by the further addition of atropine. The antagonists were acting selectively; the response to nicotine was abolished by hexamethonium and the response to pilocarpine was abolished by atropine alone. Brandt et al. (1976) found that generation of action potentials by ACh in cultured rat chromaffin cells was inhibited by atropine (0.1uM) but not by hexamethonium or d-tubocurarine. They concluded that muscarinic receptors were involved. Working with isolated guinea pig chromaffin cells, Hochman and Perlman (1976) demonstrated that ACh-stimulated catecholamine secretion is inhibited by both atropine and hexamethonium. Atropine is a more potent inhibitor than hexamethonium. The isolated and cultured bovine adrenal chromaffin cells have both nicotinic and muscarinic cholinergic receptors on their plasma membranes (Ungar & Phillips, 1983), but only the nicotinic receptors are involved in ACh-induced secretion of catecholamines (Livett, Kozousek, Mizobe & Dean, 1979; Fenwick et al., 1978; Schneider et al., 1977). Boksa and Livett (1984) demonstrated a dose-dependent release of [^{3}H]-noradrenaline into the medium in response to stimulation by nicotine up to a maximum at 10uM nicotine. Livett et al. (1979) found that methacholine, a muscarinic agonist, did not stimulate the cells to release [^{3}H]-noradrenaline and the ACh-induced release of [^{3}H]-noradrenaline was not inhibited by atropine.

Schneider et al. (1977) reported that the major secretory response of bovine chromaffin cells was found with nicotine; only a small and insignificant response was obtained with muscarine and pilocarpine and that the ACh-stimulated secretion was blocked by hexamethonium. Livett & Boksa (1984) demonstrated the release of [^{3}H]-noradrenaline from the bovine chromaffin cell cultures brought about by nicotine and ACh but not by methacholine, a muscarinic agonist. Mizobe & Livett (1983) found the nicotine (10uM)-induced release of endogenous catecholamine and cellular acetylcholinesterase (AChE) from bovine adrenal chromaffin cells. In contrast, methacholine did not stimulate the release of endogenous catecholamine and AChE. Working with isolated hamster adrenal chromaffin cells, Liang & Perlman (1979) found that in addition to ACh, both nicotine and dimethylphenylpiperazinium (DMPP) stimulate catecholamine secretion. In contrast, neither pilocarpine nor oxotremorine, stimulate catecholamine (CA) release.

Most recently Derome, Tseng, Mercier, Lemaire & Lemaire (1981) have demonstrated the effect of co-stimulation of the muscarinic receptor with low concentration of ACh on nicotine-induced CA secretion from chromaffin cells. The low concentration of ACh is known to stimulate cyclic GMP from the cell (Derome et al., 1981; Yanagihara et al., 1979) but not CA secretion itself. Surprisingly, they found that co-stimulation of the muscarinic receptor reduced the nicotine-induced CA secretion and the inhibitory effect of ACh

was antagonized by atropine. The muscarinic receptors in bovine chromaffin cell membrane might play some physiological role in inhibiting the basal release of catecholamines by nicotinic receptor activation.

1.10 Acetylcholinesterase (AChE) Content of Chromaffin Cells

Mizobe & Livett (1983) demonstrated that nicotinic stimulation released both AChE and CA in a Ca^{2+}-dependent manner from cultured bovine medullary chromaffin cells. This suggests that ACh released by splanchnic nerve terminals or exogenously applied ACh may be hydrolyzed not only by AChE located on the postsynaptic membrane but also by AChE released by nicotinic activation. The concomitant release of AChE and catecholamines on exposure of cells to the nicotinic agonists provides a mechanism by which the chromaffin cell could ensure the termination of the effects of splanchnic nerve stimulation (Mizobe & Livett, 1983), another nice example of an homeostatic mechanism. AChE appears in the cells after 4 days in culture and continues to be synthesized and secreted into the medium (Mizobe & Livett, 1981). The physiological functions of the released AChE are not known but may extend beyond its hydrolysis of acetylcholine (Livett et al., 1983).

1.11 Functional Role of Receptors for Opioids and Substance P

The simplistic view on the neuronal modulation of adrenal medullary function has documented that chromaffin cells can

synthesize, store and release catecholamines (CA) and these processes are modulated by acetylcholine (ACh) released from the splanchnic nerve, which interacts with nicotinic receptors on the chromaffin cell membrane (Livett et al., 1983). However, some recent evidence has complicated this concept of neuronal modulation of medullary function. The evidence includes the presence of receptors for opioids (Costa, Guidotti & Saiani, 1980) and substance P (Livett et al., 1983).

A number of endogenous peptides (substance P, somatostatin, enkephalins) appear to modulate the release of catecholamines from adrenal chromaffin cells. Substance P by itself did not have any effect on catecholamine release, while it produced a dose-dependent inhibition of acetylcholine and nicotine-stimulated [^{3}H]-noradrenaline release from isolated bovine adrenal medullary cells (Livett et al., 1979). Somatostatin at relatively high concentrations, inhibited the release induced by ACh (Mizobe, Kozousek, Dean & Livett, 1979). The evidence thus supports a role for these peptides as inhibitory neuromodulators at nicotinic receptor sites in the nervous system. It has been hypothesized that ACh co-exists with opioid peptides in the splanchnic nerve and are co-released to act on nicotinic and opiate receptors located on chromaffin cell membrane (Costa, Guidotti, Hanbauer, Kageyama, Kataoka, Panula, Quach & Schwartz, 1984). The opioid peptides are shown to reduce the secretion of CA from chromaffin cells evoked by the activation of nicotinic receptors (Kumakura, Karoum, Guidotti & Costa, 1980; Costa et al., 1980; Livett

& Boksa, 1984). Livett, Dean, Whelan, Udenfriend & Rossier (1981) demonstrated the co-release of enkephalins and CA from adrenal chromaffin cells in culture. All the evidence suggests that the occupancy of opioid receptors by endogenous modulators regulates the function of nicotinic receptors by an indirect mechanism.

1.12 **Functional Role of GABAergic Receptors**

Most recent evidence shows that the membranes of chromaffin cells contain a GABA (γ -aminobutyric acid) and benzodiazepine receptor complex (Kataoka, Gutman, Guidotti, Panula, Wroblewski, Cosenza-Murphy, Wu & Costa, 1984). Costa et al. (1984) have also characterized GABA and benzodiazepine (BZD) recognition sites in the membranes of chromaffin cells. γ -aminobutyric acid (GABA) is regarded as the major inhibitory neurotransmitter in the central nervous system of the vertebrates and exerts its effect through increased postsynaptic membrane permeability to chloride ions (McBurney & Barker, 1978; Barker & Ransom, 1978). However, recent evidence has suggested that GABA may be involved in the modulation of hormonal secretion in some of the endocrine glands. Taraskevich & Douglas (1982) have reported that GABA directly affects the electrophysiological properties of endocrine cells isolated from rat pars intermedia and ionic characteristics of this action resemble those seen in central nervous system. Harty & Franklin (1983) concluded that GABA is a potent stimulant of gastrin release from rat antral mucosa. Haber, Kuriyama & Roberts

(1970) have also suggested that GABA may play a role of physiological function in some peripheral non-neural tissue in which the presence of GABA and its synthesizing enzyme, glutamic acid decarboxylase was demonstrated. The recent finding as reported by Kataoka et al. (1984) that primary cultures of chromaffin cells prepared from bovine adrenal medulla can synthesize, store, release and inactivate GABA and possess GABA and benzodiazepine binding sites within their cell membrane, suggests that GABA might modulate the responsiveness of chromaffin cells to neurally released ACh. Using patch clamp technique, GABA dependent Cl^--channels have been demonstrated in the isolated chromaffin cell membrane (Cottrell, Lambert & Peters, 1985; Lambert & Peters, 1985; Bormann & Clapham, 1985). This observation thus supports a physiological role for GABA in chromaffin cell function. Cottrell et al. (1985) have also demonstrated that GABA-gated chloride conductance is antagonized by picrotoxin and bicuculline, two $GABA_A$ receptor antagonists. Kataoka et al. (1984) found that nicotine is more active in releasing catecholamines from chromaffin cells in the presence of bicuculline, thus providing an indirect evidence of the presence of endogenous GABA.

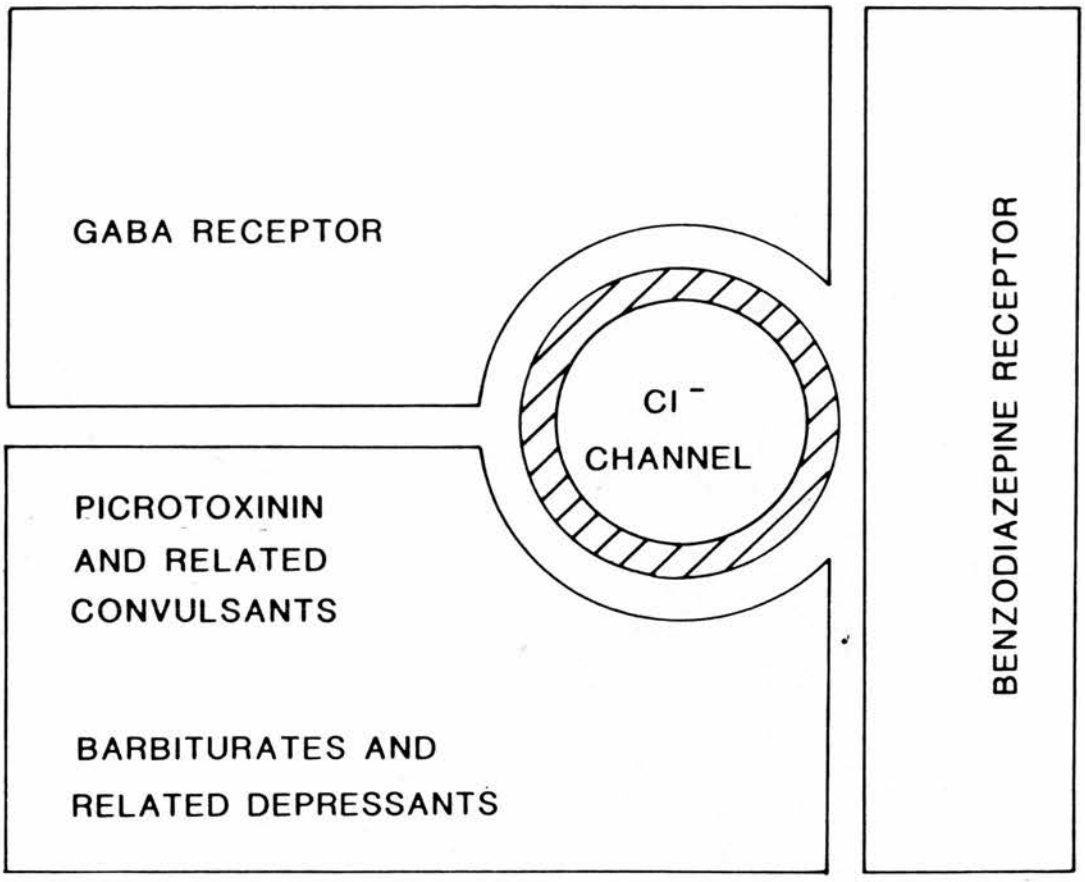
What physiological role does GABA have to play in modulating hormonal secretion from chromaffin cells? Nicotinic receptor stimulation or KCl depolarization releases the [^{3}H]-GABA taken up and stored by cultured chromaffin cells (Kataoka et al., 1984). It might be assumed from this

observation that endogenously released GABA would tend to reduce the nicotine-elicited secretion of catecholamine in a number of cells. Therefore, GABAergic systems *in vivo* would tend to cause an overall reduction of ACh-induced catecholamine release.

1.13 Modulation of GABAergic Receptor Activity by Benzodiazepines

Increasing evidence supports a role for the major inhibitory neurotransmitter, GABA, in the action of many central nervous system depressant and excitatory drugs (Olsen, 1982). Benzodiazepines are commonly used as anticonvulsants, muscle relaxants, antianxiety drugs and hypnotics (Macdonald & Barker, 1978). Although all those clinical actions suggest that benzodiazepines enhance inhibition in the central nervous system the exact cellular mechanisms underlying these clinically important effects have not been well documented. It is now well established that the GABA receptor complex found in the vertebrate central nervous system frequently includes a benzodiazepine binding sub-unit (Olsen, 1981)(Fig. 3). The major type of inhibitory synaptic transmission mediated by GABA involves a rapid (milliseconds) increase in the postsynaptic membrane conductance to chloride ions (McBurney & Barker, 1978) following the interaction of GABA with its recognition site, the receptor. Modulation of the postsynaptic GABA receptor-chloride ionophore appears to mediate many of the sedative, anxiolytic and anticonvulsant actions of benzodiazepines (Olsen, 1981; Simmonds, 1980; Macdonald & Barker, 1978; Simmonds, 1981).

Fig. 3. Theoretical model of the GABA receptor-ionophore complex. The complex consists of three drug receptor sites: the GABA receptor; the benzodiazepine receptor; and the picrotoxinin/barbiturate receptor. Associated with, or part of, these receptors is the chloride ion channel, the opening of which is regulated by GABA agonists binding to the GABA receptor. [Adapted from Olsen, 1981.]


GABA RECEPTOR
Cl⁻
CHANNEL
PICROTOXININ
AND RELATED
CONVULSANTS
BARBITURATES AND
RELATED DEPRESSANTS
BENZODIAZEPINE RECEPTOR

Benzodiazepines potentiated the actions of both synaptically released and exogenously administered GABA on mammalian neuronal preparations (Choi, Farb & Fischbach, 1977; Macdonald & Barker, 1978).

Chromaffin cells (modified sympathetic neurones) are reported to possess high affinity binding sites for [^{3}H]-flunitrazepam, a benzodiazepine ligand (Kataoka et al., 1984). The addition of bicuculline decreases and GABA increases the affinity of these sites for [^{3}H]-flunitrazepam. All the evidence suggests that GABA-benzodiazepine recognition sites are functionally linked (Costa et al., 1984). Workers were interested to know whether the binding sites represent GABA-receptor coupled to chloride channels as in the central nervous system. Using patch clamp technique, a GABA-gated chloride conductance in cultured chromaffin cell membrane is found to be potentiated by diazepam (Cottrell et al., 1985; Bormann & Clapham, 1985). Such enhancement of Cl^- conductance by diazepam might be assumed to influence the catecholamine secretion from adrenal gland. Thus it may be possible to modulate adrenomedullary function in vivo using GABA-mimetic or benzodiazepine like compounds. Such modulation may be of clinical importance in treating catecholamine secreting adrenal medullary tumour e.g. pheochromocytoma.

1.14 Modulation of GABA Activity by Alphaxalone

The concept of steroid anaesthesia has become well

established since the first observation by Cashin & Moravek (1927) that a colloidal suspension of cholesterol when administered intravenously is able to produce deep anaesthesia sufficient to permit major surgery. The development of steroid anaesthetics was stimulated by their favourable therapeutic index (Gillo & Lass, 1984). Alphaxalone, 3 α-hydroxy, 5 α -pregnane-11, 20-dione, is a short-acting synthetic steroid anaesthetic. Its neuropharmacological mechanisms have been studied in central synapses. The molecular mechanisms of action of alphaxalone have been suggested as either due to an effect on membrane fluidity (Torda & Gage, 1977) or a specific block of membrane ion channels (Pennefather & Quastel, 1980; cited by Gillo & Lass, 1984). Scholfield (1980) suggested that the enhancement of postsynaptic inhibition might be the underlying mechanism of action of some anaesthetics including alphaxalone. Harrison & Simmonds (1984) concluded that alphaxalone may modulate the interaction between GABA and its receptor in the rat cuneate nucleus *in vitro*. The isolated bovine chromaffin cells in culture possess GABA and benzodiazepine receptors (Kataoka *et al.*, 1984; Costa *et al.*, 1984) and pharmacologically are similar to those of central neurones (Cottrell *et al.*, 1985). Cottrell, Lambert & Peters (1986) demonstrated that alphaxalone modulates the GABA receptor activity by potentiating GABA-evoked responses and by directly activating the GABA receptor itself in the bovine chromaffin cells. Alphaxalone, because of its potentiating and stimulating effect on GABA receptor, might modulate the catecholamine

secretion from chromaffin cells and this may partly account for its underlying mechanism of action.

1.15 Objectives of Study

The primary cultures of bovine chromaffin cells provide a useful experimental system for investigating the evoked release of catecholamines. In the present study, adrenal medullary cells have been dissociated by retrograde perfusion of bovine adrenal medulla with a solution containing collagenase. The chromaffin cells are functionally characterized in an *in vitro* system free of splanchnic nerve elements and essentially free of cortical cells. The cultured cells are readily accessible to the reagents added to the incubation medium which can easily and rapidly be changed. Moreover, release is not complicated by a diffusion barrier present in the intact tissue. The present work describes the dependence of secretory function on nicotine and carbachol concentration and K depolarization and the modulation of agonist-evoked catecholamine secretion by hexamethonium, bethanechol and atropine, γ-aminobutyric acid (GABA), bicuculline, benzodiazepines and alphaxalone.

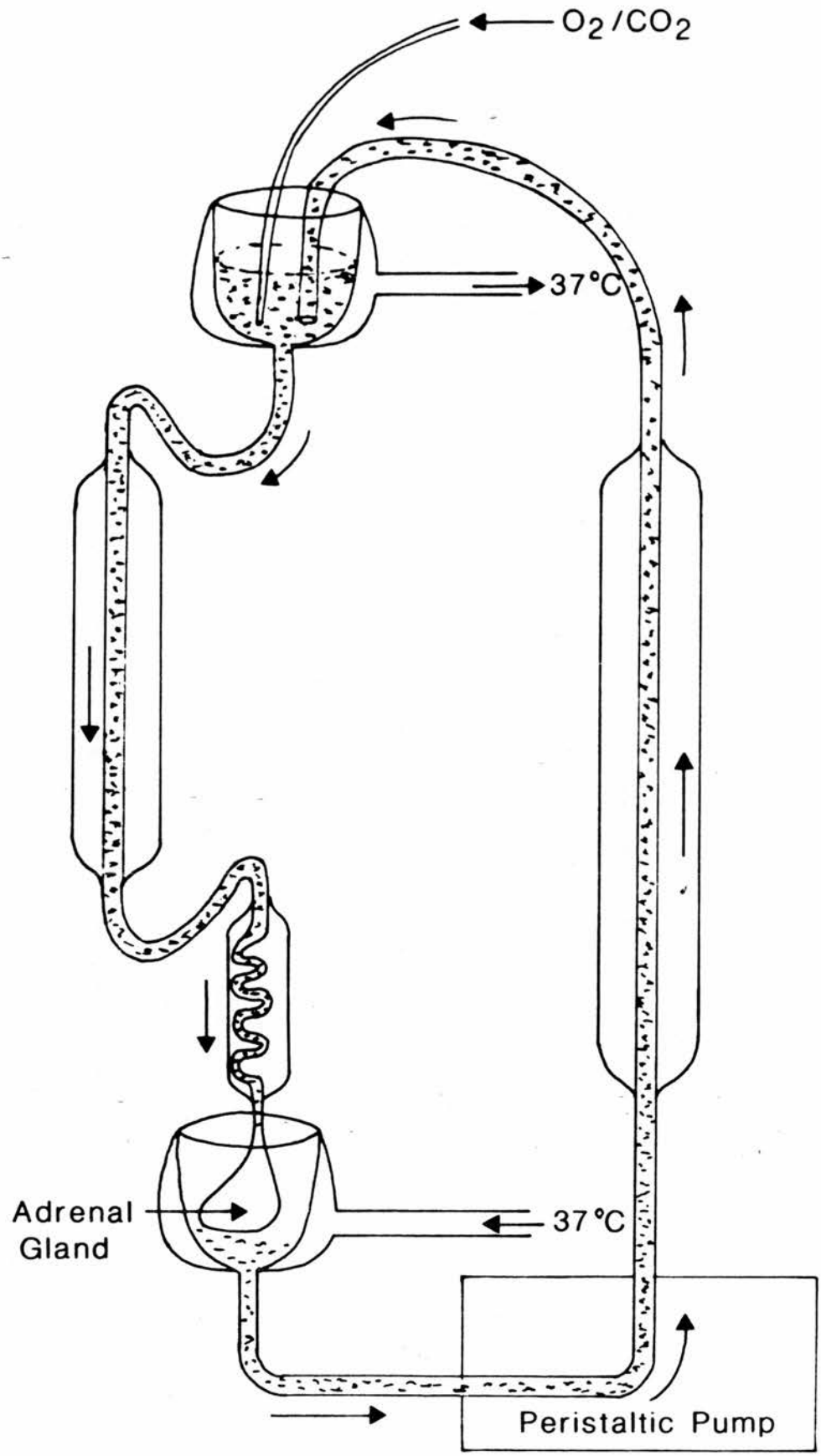
MATERIALS AND METHODS

METHODS

2.1 **Primary Cultures of Chromaffin Cells**

The isolation procedure was adopted from Cottrell et al. (1985) which was a modification of that reported by Fenwick et al. (1978). Bovine adrenal glands were rapidly removed from the animals after decapitation in a local abattoir. The fat surrounding the gland was removed as far as possible and a series of incisions were made through the cortex with a scalpel blade. A volume of 20 ml of ice-cold Ca-free Krebs containing 10 units/ml heparin was perfused through the adrenal vein with a syringe. The glands were wrapped in a foil, kept on ice and brought to the laboratory in less than 1 hour after their removal. In the laboratory all the remaining periadrenal fat was dissected away and an incision was made along the edge of the cortex to facilitate perfusion. Chromaffin cells were isolated from the gland by enzymatic dissociation of the adrenal medulla. The gland was cannulated through the adrenal vein and flushed with 100 ml of heparinized Krebs. The adrenal gland was then retrogradely perfused with Krebs solution containing 0.1 mM Ca; 1 mg/ml collagenase and 5 mg/ml bovine serum albumin (BSA) equilibrated with 95% O_2; 5% CO_2 continuously (37^{o} C) at a rate of 3 ml/min (Fig. 4). The perfusion was continued for 30 min and the perfusate was saved. The cortex was then sliced open and the partially digested medulla was dissected away from the cortex and minced by means of iridectomy

Fig. 4. Apparatus for retrograde perfusion of adrenal medulla with collagenase solution. The solution is recirculated by means of a peristaltic pump. The incubation chamber is maintained at 37°C by water pumped around the chamber from a heated water bath. The Krebs solution is equilibrated with 95% O_2/5% CO_2 continuously.


O_2/CO_2
37°C
Adrenal Gland
37°C
Peristaltic Pump

scissors and placed in a 50 ml siliconised conical flask. 8 ml of enzyme solution (perfusate) was added to the chopped medulla and incubated at 37° C for 30 min with shaking. The flask was flushed with 95% O_2/5% CO_2 throughout the incubation and triturated after 15 min. Following further trituration, the medullary suspension was filtered through "Nytex" gauze (pore size 250 um) to remove undigested material, yielding a final crude suspension of medullary cells. Undissociated tissue trapped on the gauze was again incubated with 8 ml of perfusate for another 30 min. Two successive 30 min incubations were done to dissociate the medullary tissue. The dissociated cells obtained from each incubation were collected by centrifugation (100 g for 10 min). The supernatant was discarded and the cell pellets were resuspended in 2 ml of Ca-free Krebs supplemented with 5 mg/ml BSA. Cells were again pelleted by centrifugation (100g for 5 min), resuspended in 2 ml of fresh Ca-free Krebs solution with 5 mg/ml BSA and finally centrifuged at 20g for 5 min. Dissociated cells in each tube were resuspended in 2 ml Ca-free Krebs solution and the two suspensions were pooled and carefully placed on a cushion of Ca-free Krebs solution containing 25 mg/ml BSA. The cells were allowed to sediment under gravity for 2 hours. After this time, the chromaffin cells appeared as a buff pellet while erythrocytes and cellular debris remained in suspension above. The purified chromaffin cell fraction was then thoroughly mixed with 10 ml culture medium containing antibiotics (see later). Cells were counted on a coulter counter and plated in 2 ml of

Fig. 5. Flow diagram for the preparation of enzymatically dispersed chromaffin cells from bovine adrenal medulla.

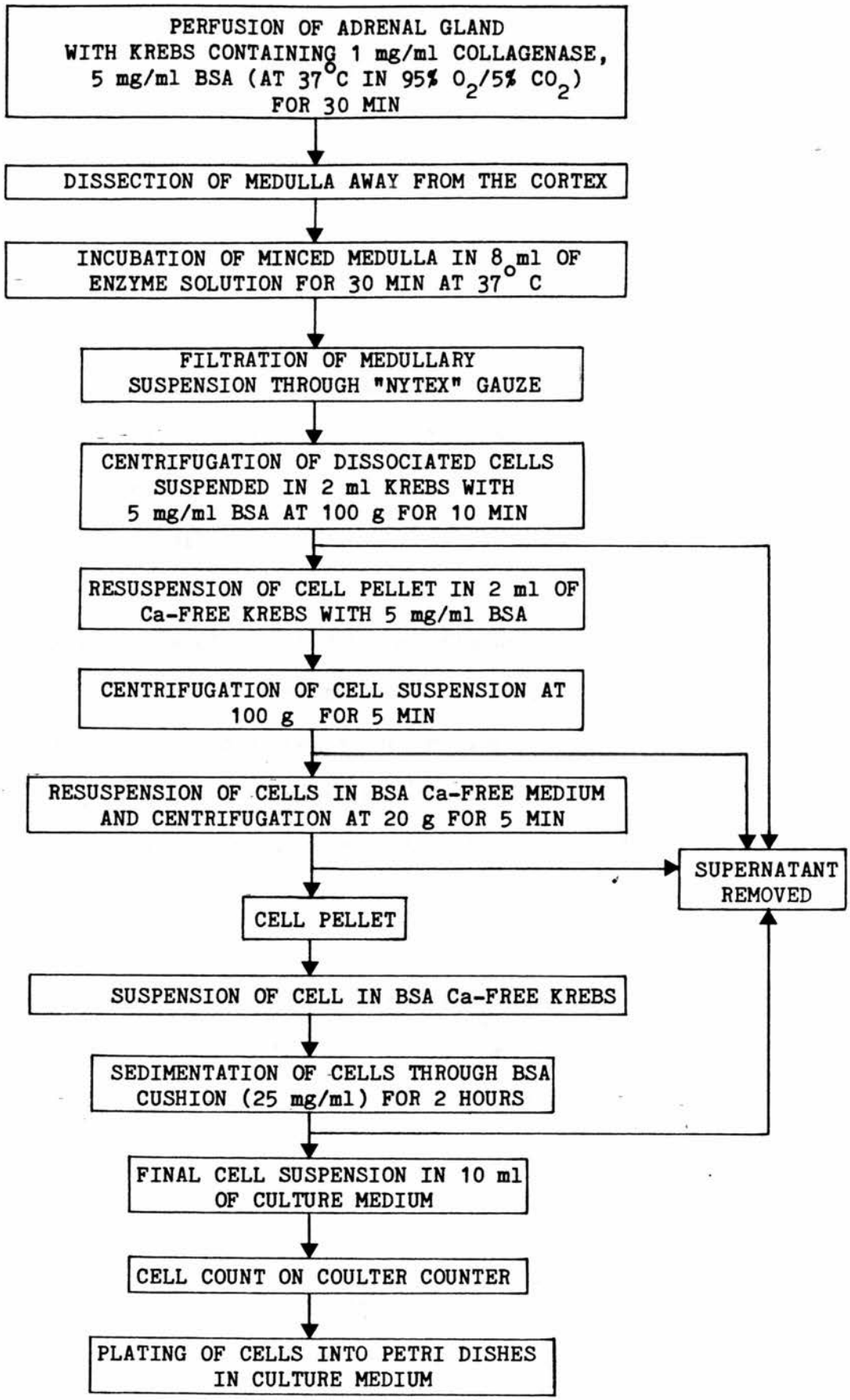
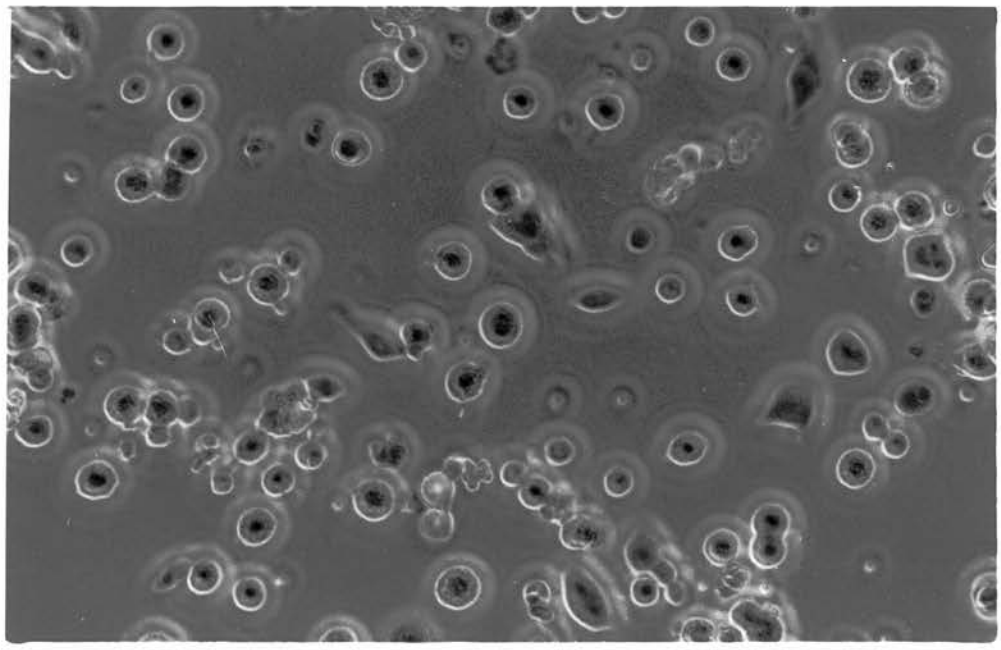
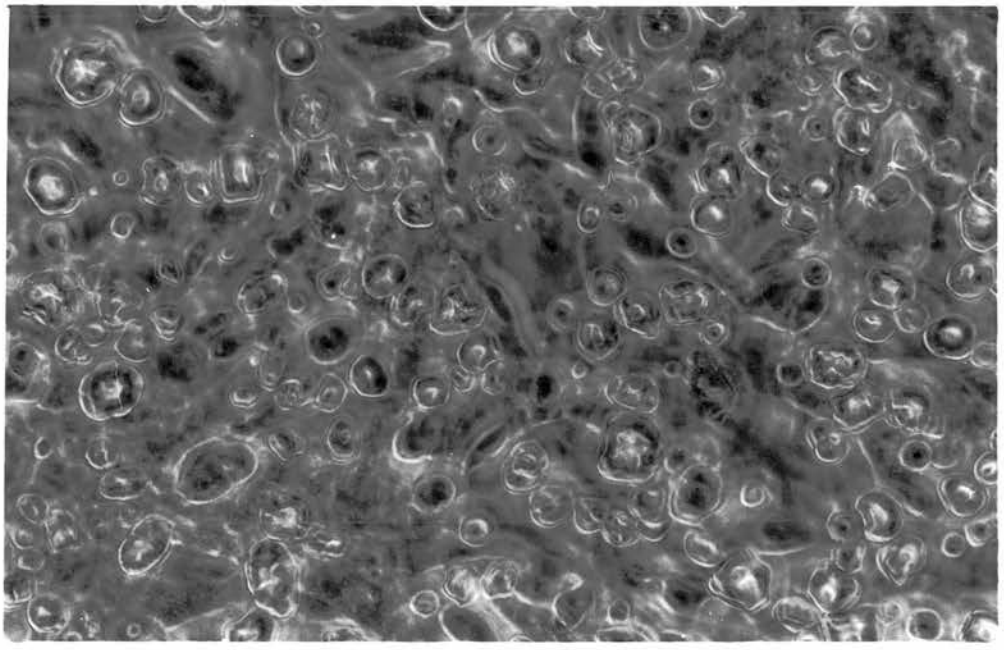

PERFUSION OF ADRENAL GLAND WITH KREBS CONTAINING 1 mg/ml COLLAGENASE, 5 mg/ml BSA (AT 37°C IN 95% O_2/5% CO_2) FOR 30 MIN
DISSECTION OF MEDULLA AWAY FROM THE CORTEX
INCUBATION OF MINCED MEDULLA IN 8 ml OF ENZYME SOLUTION FOR 30 MIN AT 37° C
FILTRATION OF MEDULLARY SUSPENSION THROUGH "NYTEX" GAUZE
CENTRIFUGATION OF DISSOCIATED CELLS SUSPENDED IN 2 ml KREBS WITH 5 mg/ml BSA AT 100 g FOR 10 MIN
RESUSPENSION OF CELL PELLET IN 2 ml OF Ca-FREE KREBS WITH 5 mg/ml BSA
CENTRIFUGATION OF CELL SUSPENSION AT 100 g FOR 5 MIN
RESUSPENSION OF CELLS IN BSA Ca-FREE MEDIUM AND CENTRIFUGATION AT 20 g FOR 5 MIN
SUPERNATANT REMOVED
CELL PELLET
SUSPENSION OF CELL IN BSA Ca-FREE KREBS
SEDIMENTATION OF CELLS THROUGH BSA CUSHION (25 mg/ml) FOR 2 HOURS
FINAL CELL SUSPENSION IN 10 ml OF CULTURE MEDIUM
CELL COUNT ON COULTER COUNTER
PLATING OF CELLS INTO PETRI DISHES IN CULTURE MEDIUM

Fig. 6. The freshly isolated chromaffin cells were round cells, containing dark granules, with a diameter of 15-20um (above). As the cells grew older, they lost their normal shape and began to flatten out (below: cells on 6th day in culture).



50 µm



Modified Medium (DMM) or GIBCO 199 and was kept at 37°C in 5% CO_2/95% air. The cells were tightly attached to the plastic after 2-3 days in culture. At this time dishes were taken out, the culture medium was aspirated and the cells were washed once with physiological saline. The cells were then incubated at 37°C for 10 min in 1 ml of physiological saline with or without drugs in 5% CO_2/95% air. In the case of drugs inhibitory to CA secretion, the cells were preincubated for 5 min with these drugs and then incubation was continued for another 10 min with stimulatory drugs. The CA secreted by the drugs would be released into the suspending medium. A measured portion of supernatant (500 ul) was carefully drawn off and assayed for catecholamine content.

Secretory experiments were performed at least in triplicate in each set of experiments. Controls were obtained by incubation of cells in physiological saline in the absence of drugs at 37°C.

2.5 **Methods Used for Analysis of Data**

In some experiments, statistical analysis of significance of the difference of means was carried out with the Student t test, while in others levels of significance of data were determined by analysis of variance.

2.6 **Fluorometric Assay of Catecholamines**

Catecholamines were measured by a fluorescence development of the hydroxyindole type, after the method of

Ansell & Beeson (1968), using noradrenaline as a standard. After adjusting the pH of the samples with EDTA reagent (6.5), they were subjected to the oxidation procedure. Fluorescence was monitored on an Aminco-Bowman Spectrophotofluorometer at excitation/emission wave lengths of 385/485 nm respectively (slit scheme 4).

2.7 **Fluorometric Method** (Ansell & Beeson, 1968)

Reagents:

EDTA Reagent, pH 6.5, 0.1M EDTA. Disodium salt of ethylenediamine-tetra-acetic acid (0.372 g) was dissolved in 95 ml 1M sodium acetate and adjusted to pH 6.5 with 10N NaOH. The total volume was adjusted to 100 ml.

Iodine, 0.1N. This was prepared by dissolving 1.27 g iodine in 100 ml of absolute ethanol.

Alkaline Sulphite. Immediately before use 4.5 ml 5N NaOH was mixed with 0.5 ml sodium sulphite solution. The latter solution was prepared by dissolving 50 g of the hydrated salt (Na_2SO_3, $7H_2O$) in 100 ml.

Method: The supernatant (500 ul) drawn from the suspending medium of chromaffin cells was mixed with 50 ul of 0.1M EDTA reagent, pH 6.5. 50 ul of 0.1 N iodine reagent was then added. After exactly 2 min 100 ul freshly prepared alkaline sulphite was added, followed by 100 ul 6N acetic acid 2 min later.

Each mixture was immediately heated at 100°C for 2 min and then cooled rapidly to room temperature before adding 1

ml water. The fluorescence due to noradrenaline was measured at 485 nm, with activation at 385 nm. In measuring the catecholamines present in the cell extract, fluorescence spectra were done routinely and compared with authentic noradrenaline and adrenaline (Fig. 7). The noradrenaline content of each sample was then calculated out from standard curve (Fig. 8).

2.8 Possible Problems with Fluorometric Assay

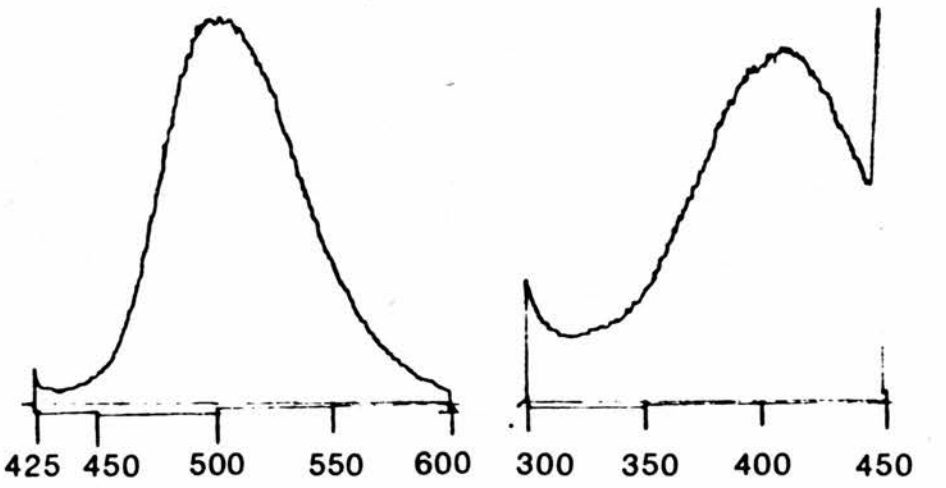
The background level of noradrenaline in the sample was low and it was close to the minimum amount detectable by the method. For this reason background levels were not very accurate and tended to be overestimated.

2.9 Electrochemical Detection of Catecholamines

The only method available to measure catecholamines in our laboratory was fluorometry. This method was more sensitive to noradrenaline than adrenaline. During this project I had an opportunity to use a better method, i.e. high performance liquid chromatography (HPLC) coupled to electrochemical detection. With this method the samples were separated chromatographically and CA was measured electrochemically. Comparison of the results obtained by the two methods showed that when CA levels were low, high values were obtained by fluorescence technique, for instance the background level of catecholamine detected by fluorescence technique was 640 pmoles, whereas the level detected by electrochemical method was 130 pmoles. As there was a rise in catecholamine level the amount

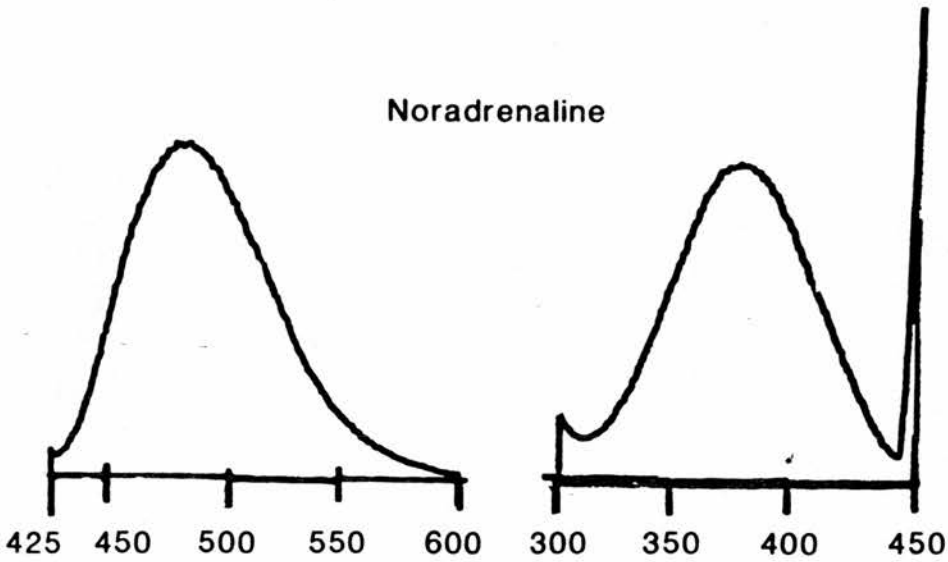
Fig. 7. Fluorescence spectrum of adrenaline at varying excitation and emission wave lengths (above). Fluorescence spectrum of noradrenaline (middle). Fluorescence spectrum of catecholamines released from bovine adrenal chromaffin cells (below).

Adrenaline



Emission Wavelength (nm) Excitation Wavelength (nm)

Noradrenaline



Cell Extract

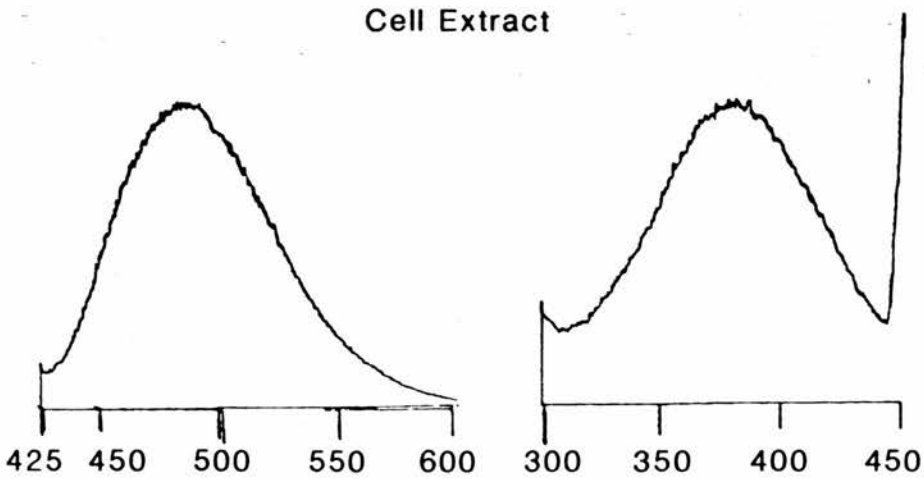
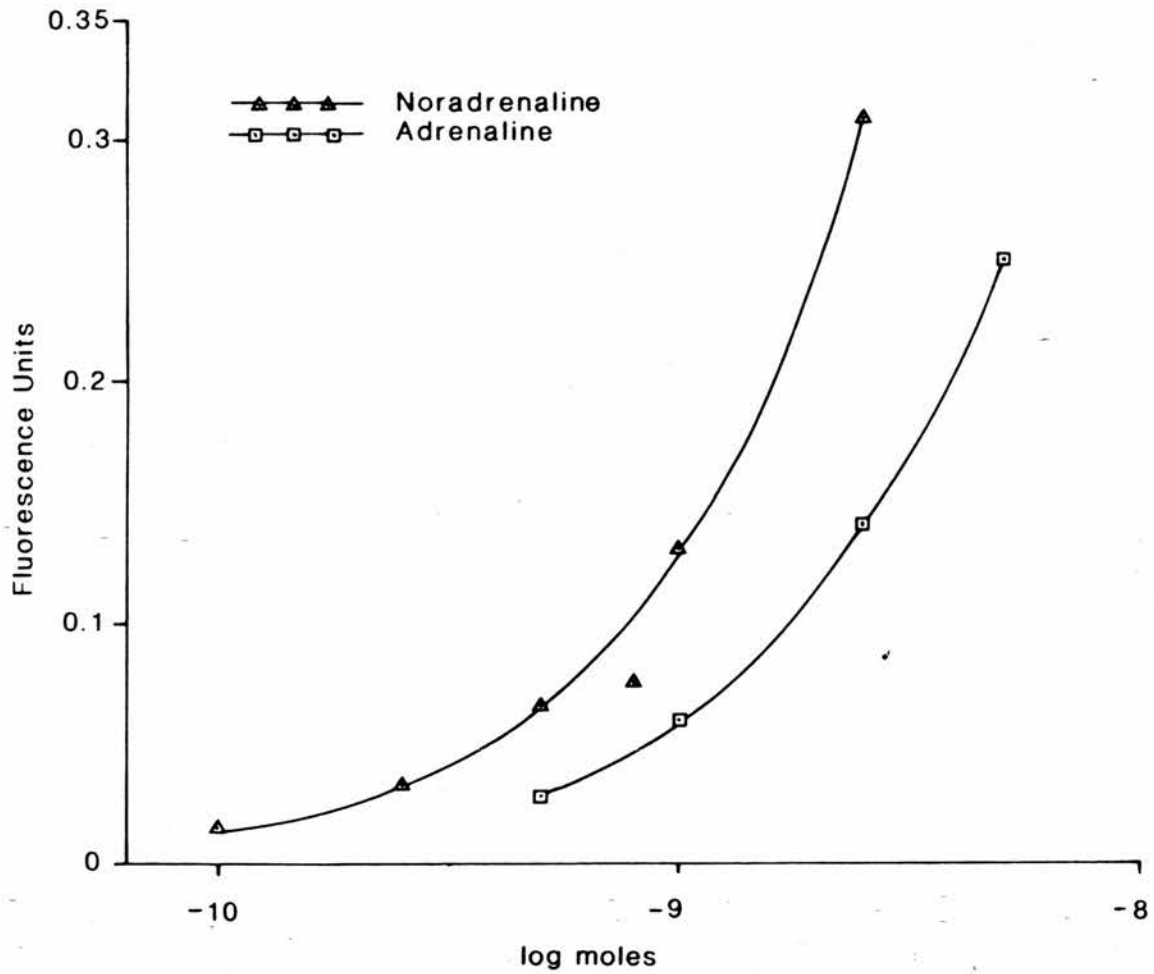


Fig. 8. Standard curve of adrenaline and noradrenaline.


0.35
0.3
0.2
0.1
0
Fluorescence Units
Noradrenaline
Adrenaline
-10
-9
-8
log moles

measured by both methods was almost the same. With this method adrenaline release from the cell could also be detected (Fig. 9). Comparison of the results obtained by the two methods implied that basically the fluorescence technique was reliable (see Figure legend 9).

MATERIALS

2.10 **Chemicals**

Collagenase (Type I) and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. Dulbecco's Modified Medium (DMM), GIBCO 199, heat-inactivated foetal calf serum, penicillin-streptomycin and gentamycin were obtained from GIBCO. Heparin sodium was obtained from Evans Medical Ltd. All the drugs employed in the study, L-noradrenaline (L-arterenol HCl), L-adrenaline bitartrate, carbamyl β-methyl choline chloride (bethanechol), atropine sulphate, carbamylcholine chloride (carbachol), hexamethonium bromide, nicotine hydrogen tartrate, (-) and (+) bicuculline methiodide, γ-amino-n-butyric acid, were purchased from Sigma Chemical Company. Diazepam was obtained from Hoffman-La-Roche. Alphaxalone (a GLAXO product) was a gift from Dr. J. Peters, Department of Pharmacology and Clinical Pharmacology, Ninewells Medical School, University of Dundee. All other reagents and salts were purchased from BDH Chemicals Ltd.

Fig. 9. Tracing showing the peaks of noradrenaline and adrenaline present in the extract of bovine medullary chromaffin cells detected with an HPLC coupled to electrochemical detection. (1) Standard, (2) Control I, (3) Nicotine (5uM), (4) Nicotine (50uM) + GABA (1uM); 5 times diluted, (5) Control II, (6) Nicotine (50uM); 2 times diluted and (7) Nicotine (50uM) + GABA (1uM); 5 times diluted.

Sample	Fluorometric Method CA release (pmoles) per 0.5ml of cell extract	Electrochemical Method CA release (pmoles) per 0.5ml of cell extract
Control 1	320	65.4
Control 2	290	64.8
Nicotine (5uM)	460	175.2
Nicotine (50uM)	1820	2172.0
Nicotine (50uM) + GABA (1uM)	2400	2352.0
Nicotine (50uM) + GABA (1uM)	1740	1566.0

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	6.533	48230	S	3	8	NA-MHPG
2	7.6	71519	T	4	10	AD
	TOTAL	119749			18	

ANAL 2
86/02/06 15:26:12
955.7

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	6.5	29137	S	3	4.8329	NA-MHPG
2	7.6	6473	T	4	0.9051	AD
	TOTAL	35610			5.7381	

ANAL 3
86/02/06 15:26:43
1025.45

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	6.5	55886	S	3	9.2698	NA-MHPG
2	7.6	36926	T	4	5.1632	AD
3	8.733	802	T	5	4.2847	DOPAC
	TOTAL	93614			18.7177	

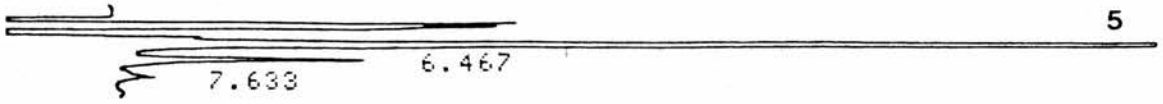
ANAL 5
86/02/06 15:28:18
1285.1

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	6.5	158032	S	3	26.2128	NA-MHPG
2	7.567	106830	T	4	14.9374	AD
	TOTAL	264862			41.1502	

ANAL 7

86/02/06 15:28:47

1078.85



PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	6.467	25841	S	3	4.2863	NA-MHPG
2	7.633	5768	T	4	0.8066	AD
	TOTAL	31610			5.0929	

ANAL 8

86/02/06 15:29:15

1127.55

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	6.5	271506	S	3	45.0349	NA-MHPG
2	7.567	302253	T	4	42.262	AD
	TOTAL	573758			87.2969	

ANAL 9

86/02/06 15:29:51

1221.85

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	6.5	82713	S	3	13.7197	NA-MHPG
2	7.567	77828	T	4	10.8821	AD
	TOTAL	160541			24.6018	

2.11 Solutions

Ca-free Krebs solution contained 119 mM - NaCl; 4.7 mM - KCl; 1.2 mM - $MgSO_4$, $7H_2O$; 25 mM - $NaHCO_3$; 1.2 mM - KH_2PO_4; 11 mM - glucose. The buffer was equilibrated with 95% O_2/5 % CO_2, and had a pH of 7.4. Cells were bathed in a solution (Fenwick et al., 1982) containing 140 mM - NaCl; 2.8 mM - KCl; 2 mM - $MgCl_2$, $6H_2O$; 1 mM - $CaCl_2$, $2H_2O$; 10 mM - HEPES - NaOH; pH 7.2. In experiments in which the KCl concentration of this buffer was increased to 30 mM, the NaCl concentration was reduced in order to maintain a constant osmolarity.

The culture medium comprised of GIBCO 199 or DULBECCO's MODIFIED MEDIUM (1X) supplemented with 10% heat-inactivated foetal calf serum, 50 i.u. /ml penicillin-streptomycin and 200 ug/ml gentamycin.

With the exception of diazepam and alphaxalone, which were prepared as concentrated stock solution in 95% ethanol, all other drugs were dissolved into the physiological saline. (+) Bicuculline and L-adrenaline were dissolved in 1N and 0.01N HCl respectively. The stock solution of noradrenaline was prepared with distilled water. The serial dilutions of adrenaline and noradrenaline were made in distilled water, while all other drugs were prepared with physiological saline.

RESULTS

In the present study, catecholamine (CA) secretion from isolated and cultured bovine adrenal medullary cells was stimulated with high K, nicotine or carbachol and the influence of different modulators on this secretory response was investigated.

3.1. The Secretory Response to High K

To assess the influence of depolarization on catecholamine secretion, chromaffin cells were exposed to physiological saline with high K (30mM KCl) for 10 min. High K stimulated the release of catecholamines from isolated cells (Table 1).

TABLE 1. EFFECT OF HIGH K ON CATECHOLAMINE RELEASE FROM ISOLATED BOVINE ADRENAL CHROMAFFIN CELLS.

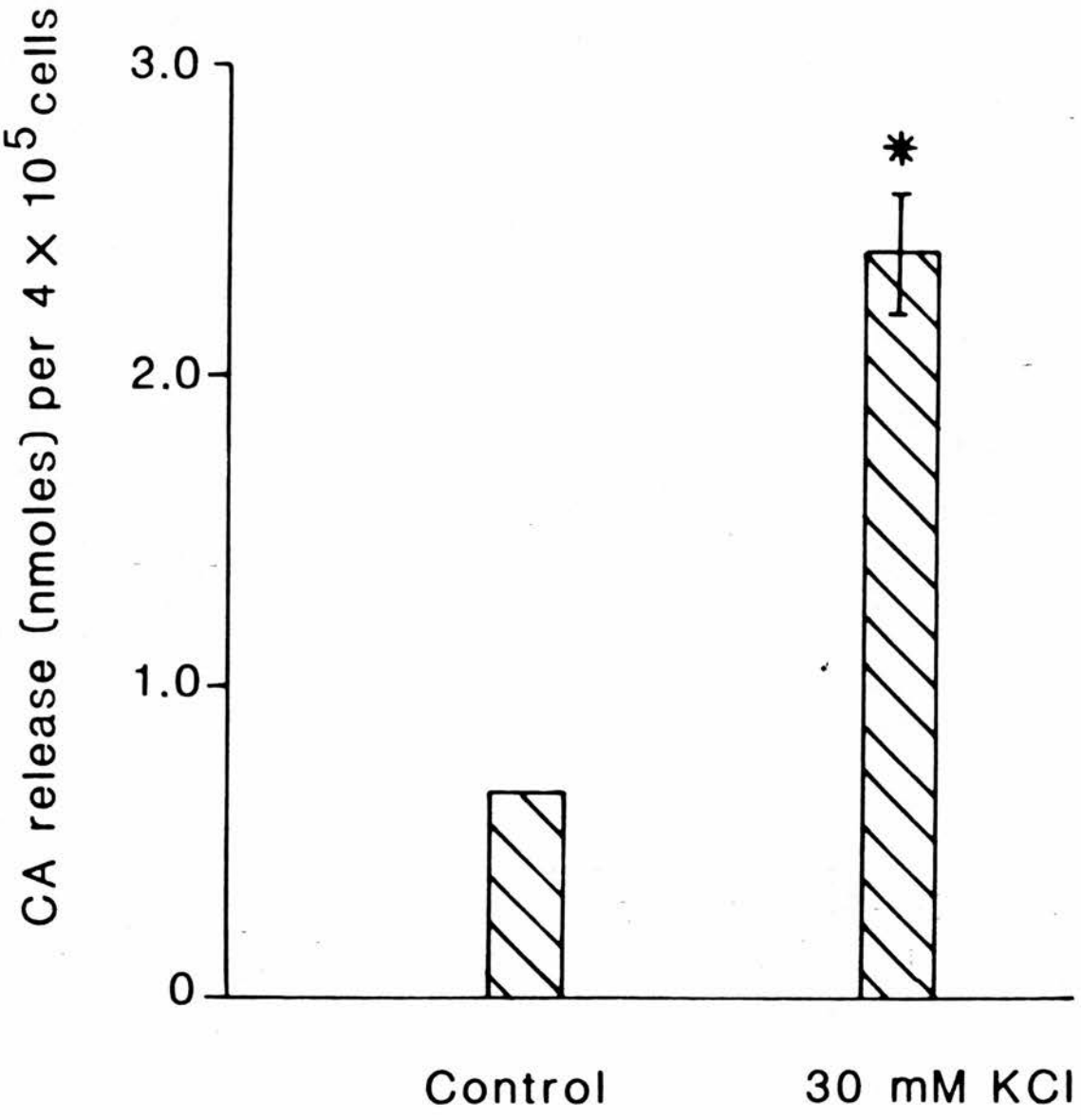
Treatment	CA release (nmoles) mean ± S.E per 4×10^5 cells
None (control)	0.65 ± 0.01
High K (30mM)	2.40 ± 0.20*

Cells were incubated for 10 min at 37° C in physiological saline with normal K (control) and in saline modified to contain 30 mM KCl. The results represent the mean ± S.E of duplicate assays.
*Significantly different from control at $P<0.05$.

30mM KCl produced about a 4 fold increase in catecholamine secretion. The amount of release is shown in Fig. 10 for cells stimulated with high K and for unstimulated control cells

Fig. 10. Effect of high K on catecholamine release from bovine medullary chromaffin cells. The incubation was carried out in medium containing 30mM KCl. The bars represent the mean and the vertical lines are S.E.

*Significantly different from control at $P<0.05$.


CA release (nmoles) per 4 X 10^5 cells
3.0
2.0
1.0
0
*
Control
30 mM KCl

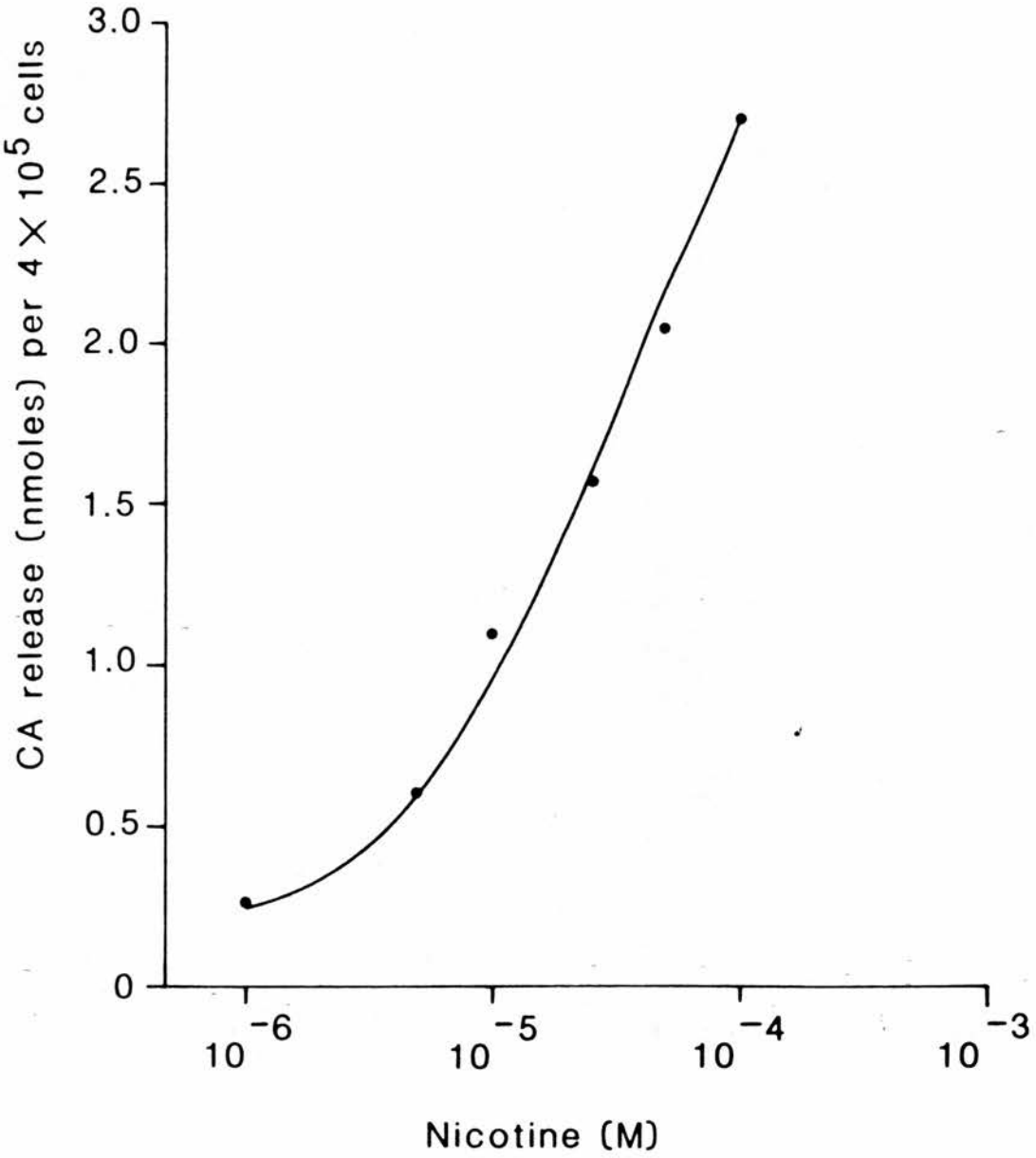
which represent the basal level of secretion.

3.2 Cholinergic Receptor Activation of Catecholamine Release

3.2.1 Characterization of Nicotinic Response

Since the response to acetylcholine (ACh) in the bovine adrenal medulla appears to be mediated by nicotinic receptor (Livett et al., 1979), secretory responses to different concentrations of nicotine, a selective agonist, were studied. When isolated bovine medullary chromaffin cells were incubated with normal saline at 37°C, they released a small amount of their stored catecholamine into the incubation medium, but the addition of nicotine to chromaffin cells caused a great increase in catecholamine secretion. The nicotine-stimulated release was dependent on the extracellular concentration of secretagogue (Fig. 11) and was shown to increase with increasing concentrations of nicotine. A wide range of variation was marked in experimental results. In six separate assays, 100 uM nicotine stimulated a catecholamine secretion of 1.26 to 5.0 nmoles per 4×10^5 cells. A dose-response curve is shown in Fig. 11 for nicotine-stimulated secretion where the cells were incubated for 10 min at 37°C with saline containing nicotine at different concentrations in individual plates. The stimulation of secretion was evident at 1uM, was half maximal at about 15uM and was maximal at 100uM. The effect of nicotine (5-250 uM) was significant (**$P<0.01$). Nicotine at high concentrations (>100uM) showed a characteristic inhibition ("desensitization") of catecholamine

Fig. 11. Catecholamine secretion from isolated medullary chromaffin cells in response to varying concentrations of nicotine. Cells were incubated for 10 min at 37°C in saline containing nicotine. Following incubation, catecholamine secretion was measured. CA levels were calculated out after subtraction of basal secretion.


3.0
2.5
2.0
1.5
1.0
0.5
0
CA release (nmoles) per 4 X 10^5 cells
10^-6
10^-5
10^-4
10^-3
Nicotine (M)

release. Results are shown in Table 2 and Fig. 12.

TABLE 2. NICOTINE-EVOKED RELEASE OF CATECHOLAMINES FROM BOVINE ADRENAL CHROMAFFIN CELLS.

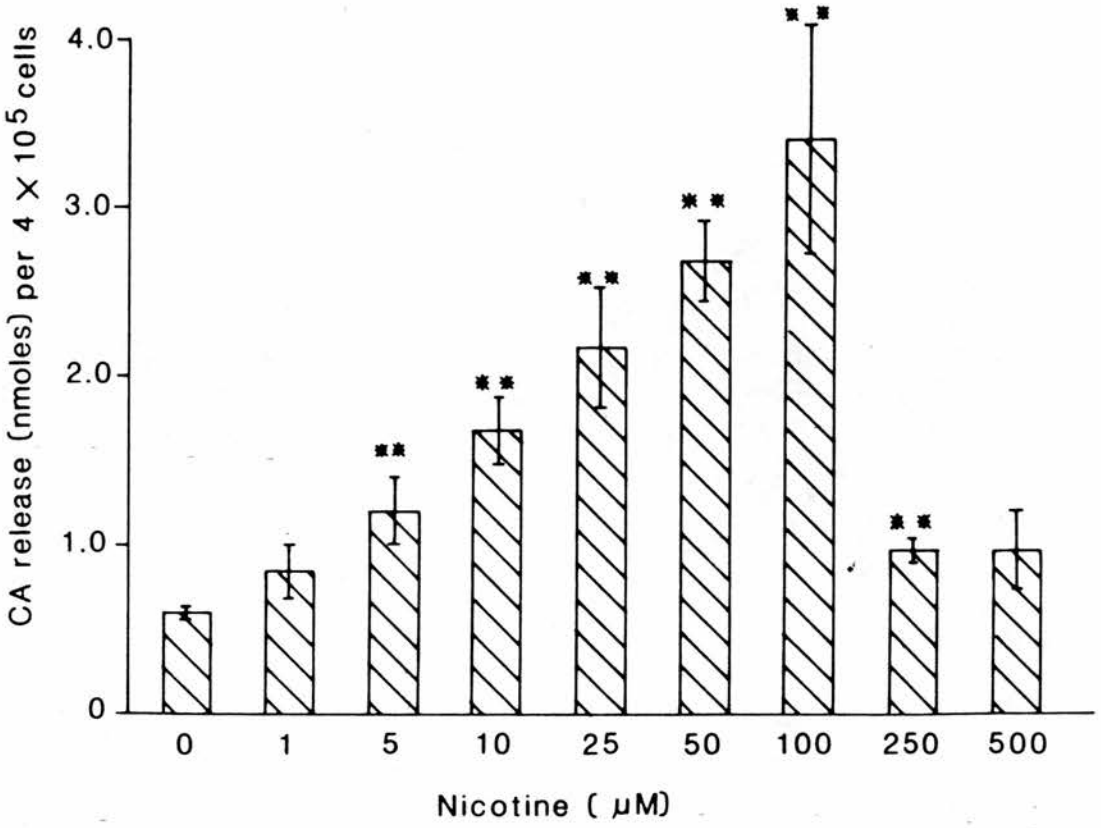
Treatment	CA secretion (nmoles) mean ± S.E per 4 x 10^5 cells
Control	0.58 ± 0.03
Nicotine 1uM	0.84 ± 0.16
5uM	1.18 ± 0.20**
10uM	1.68 ± 0.20**
25uM	2.15 ± 0.35**
50uM	2.63 ± 0.25**
100uM	3.41 ± 0.69**
250uM	0.96 ± 0.07**
500uM	0.96 ± 0.23

Chromaffin cells were exposed to nicotine for 10 min.
Data represent the mean ± S.E of six assays.
**Significantly different from control at P<0.01.

Carbachol is a non-selective cholinergic agonist acting on both nicotinic and muscarinic receptors. The stimulation of catecholamine (CA) secretion with carbachol (CCh) was found to be concentration-dependent, but there was marked variation from day to day. In the experiment depicted in Fig. 13, cells were incubated at 37°C for 10 min in the presence of various concentrations of CCh. The stimulation of catecholamine release was detectable at 10uM in two experiments and at 50uM in another experiment. The concentration of CCh for maximal stimulation

Fig. 12. Nicotine-evoked catecholamine release from bovine medullary chromaffin cells. The bars represent the mean and the vertical lines are S.E. Total number of experiments in each bar is 6.

**Significantly different from control at $P<0.01$.


CA release (nmoles) per 4 X 10^5 cells
4.0
3.0
2.0
1.0
0
0
1
5
10
25
50
100
250
500
Nicotine (μM)

was also variable, for instance in three different experiments maximal release was obtained at 100, 200 and 400 uM respectively. Results are summarized in Table 3.

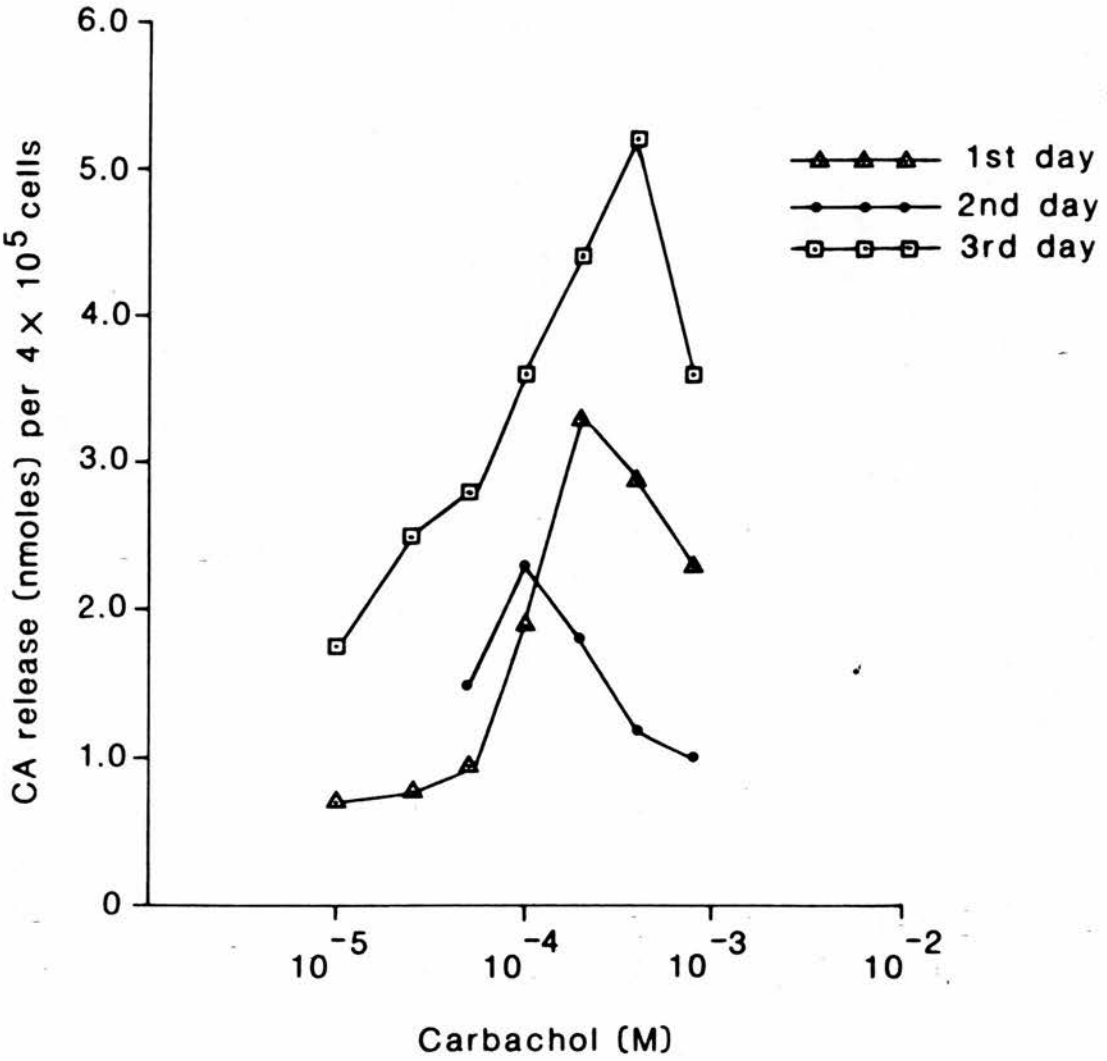
TABLE 3. STIMULATION OF CATECHOLAMINE SECRETION BY CARBACHOL FROM BOVINE MEDULLARY CHROMAFFIN CELLS.

	CA secretion (nmoles) per 4 x 10^5 cells			
Treatment	1st day	2nd day	3rd day	Mean
Control	0.66	0.56	0.70	0.64
CCh 10uM	0.70		1.74	1.22
25uM	0.76		2.50	1.63
50uM	0.92	1.52	2.80	1.75
100uM	1.90	2.30	3.60	2.60
200uM	3.30	1.82	4.40	3.17
400uM	2.90	1.20	5.20	3.10
800uM	2.30	0.96	3.60	2.28

Bovine adrenal medullary cells were incubated for 10 min at 37° C in physiological saline containing CCh at different concentrations as indicated in the table. Following incubation, catecholamine secretion was measured.

Although the stimulation of CA secretion by CCh was reproducible, there was a great variation from day to day in experimental results. On the first day, there was a slow rise in CA release with increasing concentrations of CCh, whereas on the third day with the same concentrations of CCh there was a sharp rise in CA release (Fig. 13). Fig. 13 shows the dose-dependent release of catecholamines by CCh in three separate experiments. At higher concentrations of CCh, there

Fig. 13. Dependence of catecholamine secretion on carbachol concentration. The ordinate shows the CA released during 10 min exposure to carbachol. Three dose-response curves represent three separate experiments.


6.0
5.0
4.0
3.0
2.0
1.0
0
CA release (nmoles) per 4 X 10^5 cells
1st day
2nd day
3rd day
10^-5
10^-4
10^-3
10^-2
Carbachol (M)

was a decrease in secretion. The concentrations for desensitization of response also varied in three different experiments, for instance >100, >200 and >400 uM respectively.

The bar diagram (Fig. 14) shows the levels of catecholamine released from chromaffin cells when incubated at 37°C for 10 min with different concentrations of CCh (3rd day).

In order to compare the effects of a mixed cholinergic agonist and a selective nicotinic agonist on catecholamine secretion from adrenal chromaffin cells, carbachol and nicotine were used respectively. Nicotine showed a consistent effect on CA release and a clear dose-response relationship. Although carbachol stimulated a dose-dependent release, the effect varied greatly from day to day. Nicotine-induced secretion was detectable at a lower concentration (1uM). The phenomenon of "desensitization" was more marked with higher concentrations of nicotine (>100uM) than carbachol.

3.2.2 **Blockade of Nicotinic Response**

In order to test further whether the agonist-stimulated catecholamine release is mediated by the activation of nicotinic receptors on the chromaffin cell membrane, the influence of hexamethonium was studied. Cells were preincubated with hexamethonium (1mM) for 5 min and then the secretion was stimulated with nicotine (50uM) for 10 min at 37°C. Hexamethonium pretreatment significantly reduced the secretory response of nicotine (**P<0.01). Results are summarized in

Fig. 14. Carbachol-stimulated catecholamine secretion from bovine adrenal chromaffin cells. The diagram represents the catecholamine levels of one experiment (3rd day).

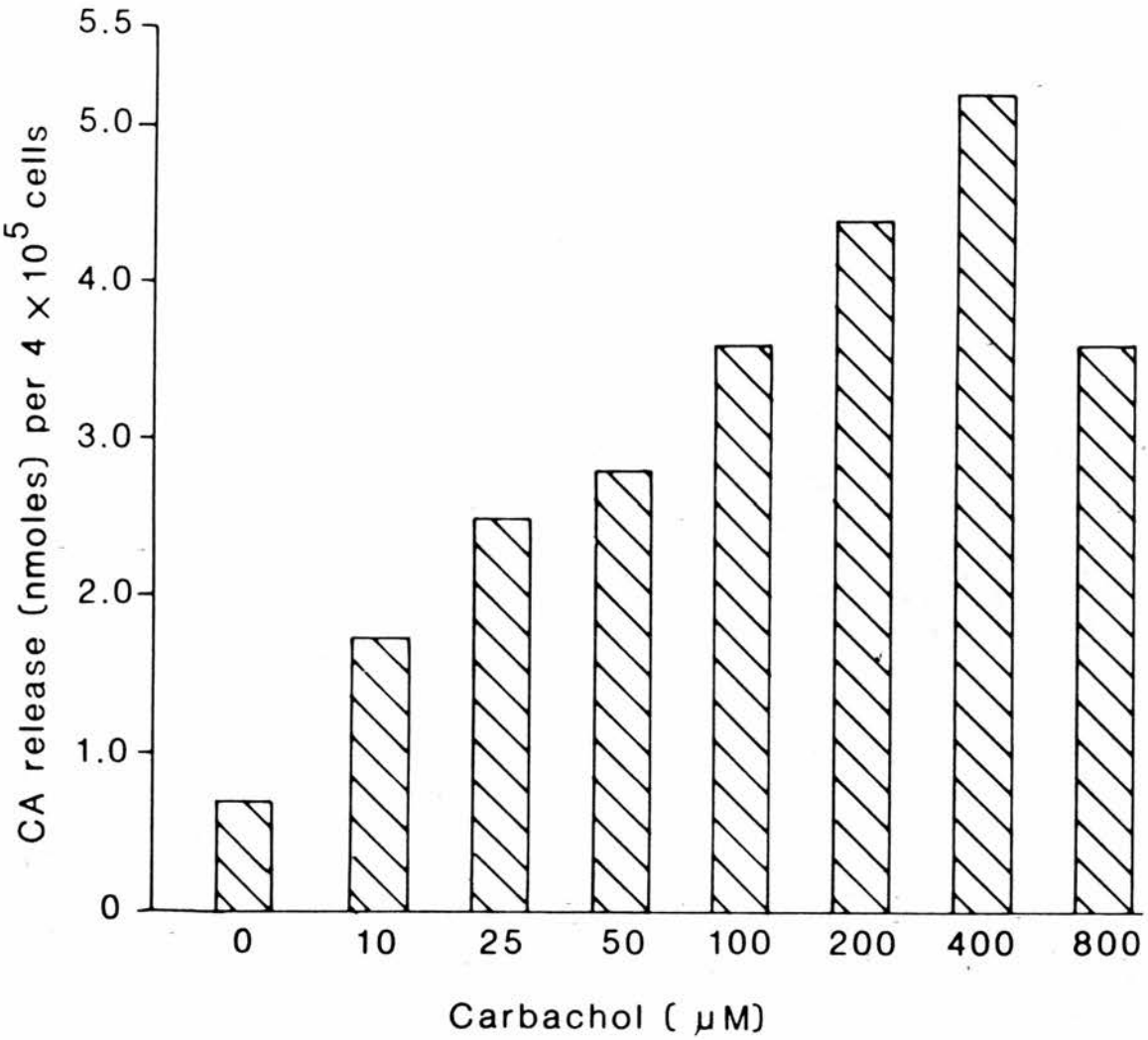

5.5
5.0
4.0
3.0
2.0
1.0
0
CA release (nmoles) per 4 X 10^5 cells
0
10
25
50
100
200
400
800
Carbachol (μM)

Table 4 and Fig. 15.

TABLE 4. EFFECT OF HEXAMETHONIUM PRETREATMENT ON NICOTINE-STIMULATED CATECHOLAMINE RELEASE FROM BOVINE MEDULLARY CHROMAFFIN CELLS.

Treatment	CA secretion (nmoles) mean ± S.E per 3 x 10^5 cells	% of Response
Control	0.68 ± 0.02	28
Nicotine (50uM)	2.44 ± 0.21^{+}	100
Hexamethonium(1mM) + Nicotine(50uM)	0.84 ± 0.05**	34

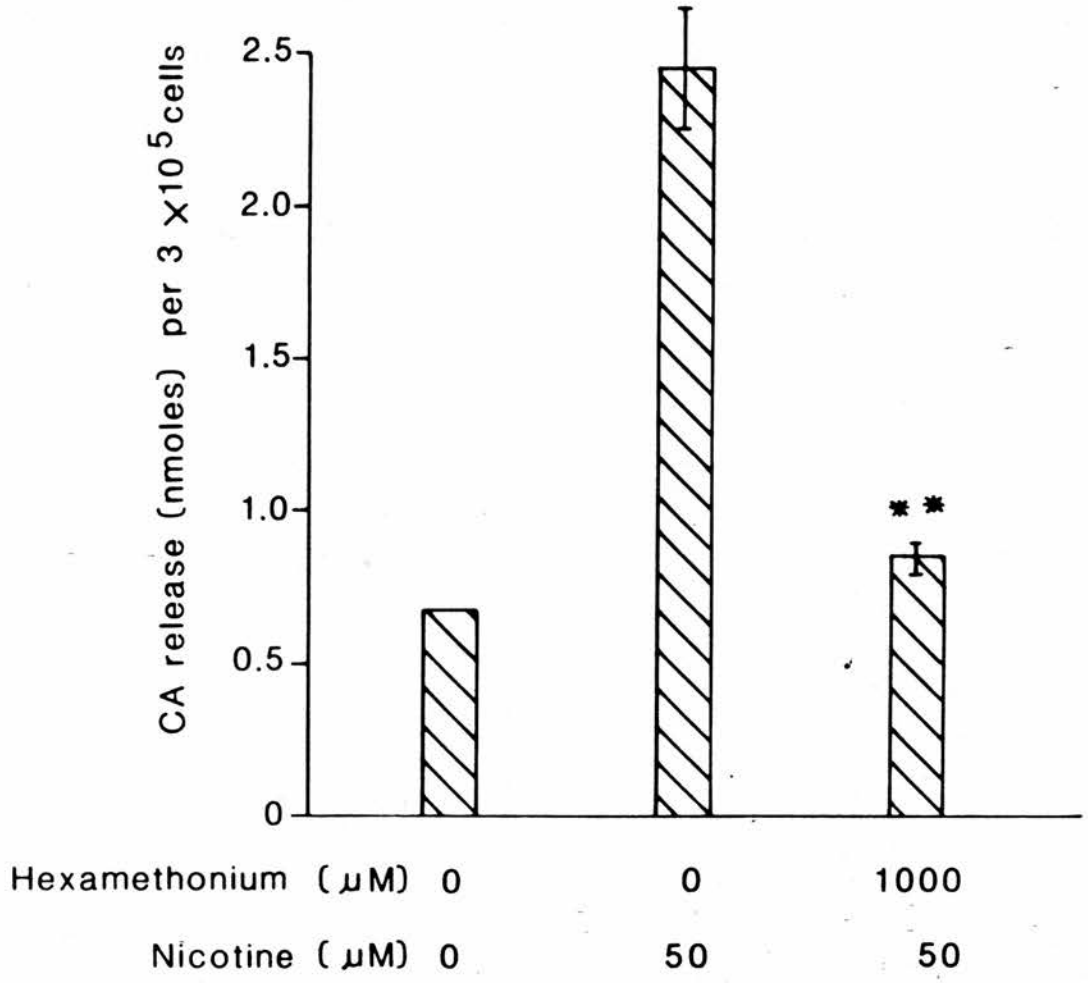
Cells were preincubated for 5 min with hexamethonium and then nicotine was added and the incubation was continued for 10 min. The data represent the mean and S.E. of 4 assays.
$^{+}$Significantly different from control at $p<0.01$.
**Significantly different from nicotine at $P<0.01$.

3.2.3 **Characteristics of Muscarinic Response**

In addition to the nicotinic receptor, the bovine chromaffin cell membrane also possesses muscarinic cholinergic receptors (Livett, 1984). In order to test for muscarinic receptor function, bethanechol (carbamyl- β -methyl choline) was used because it is a specific muscarinic receptor agonist and not hydrolyzed by acetylcholinesterase (AChE). Bethanechol (10-1000 uM) itself did not stimulate the release of endogenous catecholamines. Some experiments were done to determine the effect of muscarinic receptor activation on nicotine-stimulated catecholamine release. Chromaffin cells were preincubated for 5 min with two concentrations of bethanechol (2 and 10uM) at 37°C, then nicotine (50uM) was

Fig. 15. Effect of hexamethonium on nicotine-stimulated catecholamine release from bovine medullary chromaffin cells. The bars represent the mean and the vertical lines are S.E. The number of experiments in each bar is 4.

**Significantly different from nicotine at $P<0.01$.


CA release (nmoles) per 3 X10 5 cells
2.5
2.0
1.5
1.0
0.5
0
* *
Hexamethonium (μM) 0 0 1000
Nicotine (μM) 0 50 50

added and the incubation was continued for 10 min to determine CA release. Bethanechol (2 and 10uM) significantly reduced the nicotine-evoked CA release (*P<0.05 and **P<0.01 respectively). Results are shown in Table 5 and Fig. 16.

TABLE 5. EFFECT OF BETHANECHOL PRETREATMENT ON NICOTINE-EVOKED CATECHOLAMINE SECRETION FROM BOVINE ADRENAL CHROMAFFIN CELLS

Treatment	CA secretion (nmoles) mean ± S.E. per 10^5 cells	% of Response
Control	0.55 ± 0.01	56
Nicotine (50uM)	0.97 ± 0.01+	100
Bethanechol (2uM) + Nicotine (50uM)	0.60 ± 0.12*	62
Bethanechol (10uM) + Nicotine (50uM)	0.46 ± 0.06**	47

Cells were preincubated for 5 min with bethanechol before the addition of nicotine. The data represent the mean ± S.E of duplicate assays.
+Significantly different from control at P<0.01.
*Significantly different from nicotine at P<0.05.
**Significantly different from nicotine at P<0.01.

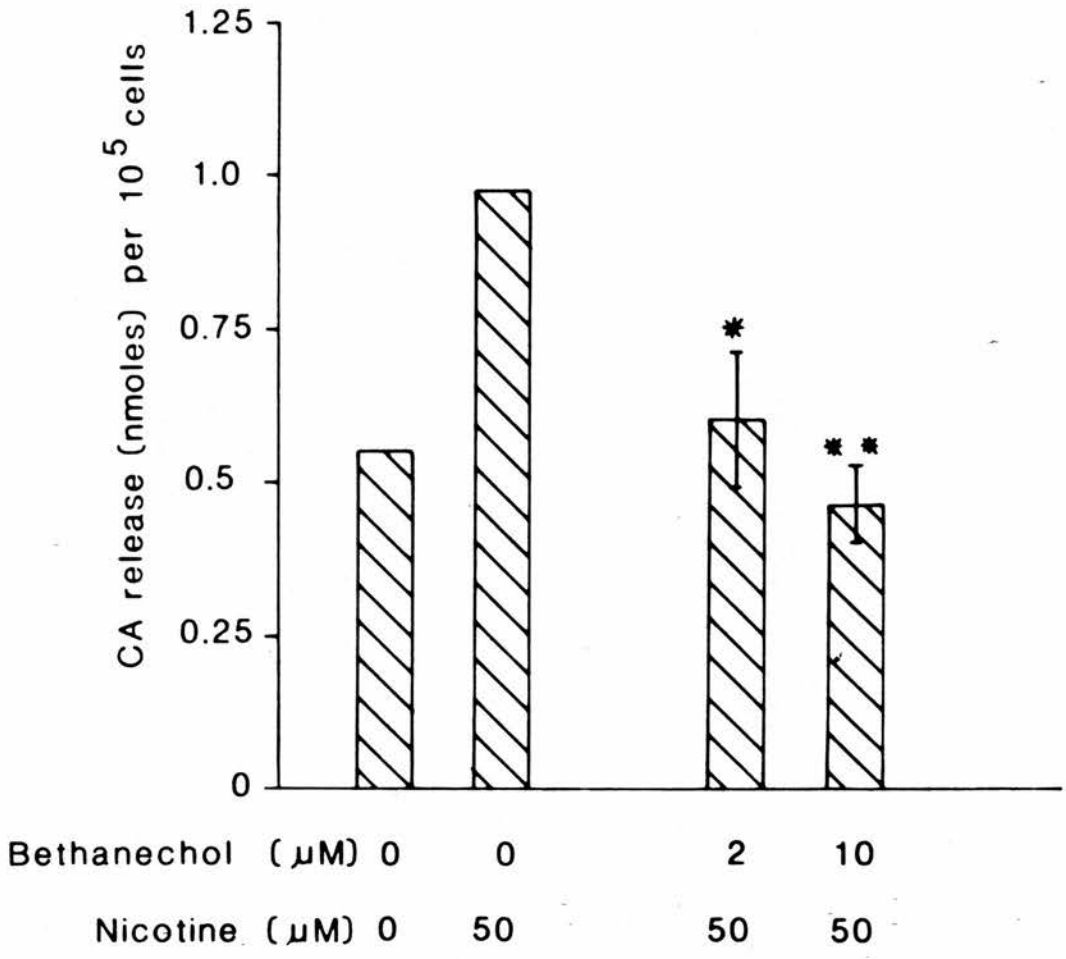
3.2.4 Blockade of Muscarinic Response

The effect of the cholinergic antagonist, atropine, on catecholamine secretion was studied to test whether the bethanechol-induced inhibition on the secretory response of nicotine was due to stimulation of muscarinic receptors. Cells were preincubated with atropine (0.1uM) and bethanechol (2uM) for 5 min and then nicotine (50uM) was added for a further incubation of 10 min at 37° C. Atropine pretreatment

Fig. 16. Effect of bethanechol on nicotine-evoked catecholamine secretion from chromaffin cells. The bars represent the mean and the vertical lines are S.E.

*Significantly different from nicotine at $P<0.05$

**Significantly different from nicotine at $P<0.01$.


1.25
1.0
0.75
0.5
0.25
0
CA release (nmoles) per 10^5 cells
Bethanechol (μM) 0 0 2 10
Nicotine (μM) 0 50 50 50

significantly reversed the inhibitory action of bethanechol on the secretory response of nicotine (*P<0.05). Results are summarized in Table 6 and Fig. 17.

TABLE 6. EFFECT OF ATROPINE AND BETHANECHOL PRETREATMENT ON CATECHOLAMINE RELEASE FROM BOVINE ADRENAL CHROMAFFIN CELLS IN RESPONSE TO NICOTINE.

Treatment	CA secretion (nmoles) mean ± S.E per 2 x 10^5 cells	% of Response
Control	0.68 ± 0.02	35
Nicotine (50uM)	1.96 ± 0.24+	100
Bethanechol (2uM) + Nicotine (50uM)	0.80 ± 0.06**	41
Atropine (0.1uM) + Bethanechol (2uM) + Nicotine (50uM)	1.10 ± 0.03*	56

Cells were preincubated for 5 min with atropine and bethanechol, then nicotine was added to stimulate the secretion for 10 min. The data represent the mean and S.E. of triplicate assays.
+Significantly different from untreated control group at P<0.01.
**Significantly different from nicotine at P<0.01.
*Significantly different from bethanechol plus nicotine at P<0.05.

3.3 GABAergic Receptor Activation of Catecholamine Release

A possible functional role of GABAergic receptors located on adrenal medullary chromaffin cell membranes was investigated by studying whether GABA (γ -amino butyric acid), bicuculline, benzodiazepines and alphaxalone can modulate the release of catecholamines evoked by cholinergic agonists.

Fig. 17. Effect of atropine and bethanechol pretreatment on nicotine-stimulated catecholamine release from chromaffin cells. The bars represent the mean and the vertical lines are S.E. The number of experiments in each bar is 3.

**Significantly different from nicotine at $P<0.01$.

*Significantly different from bethanechol plus nicotine at $P<0.05$.

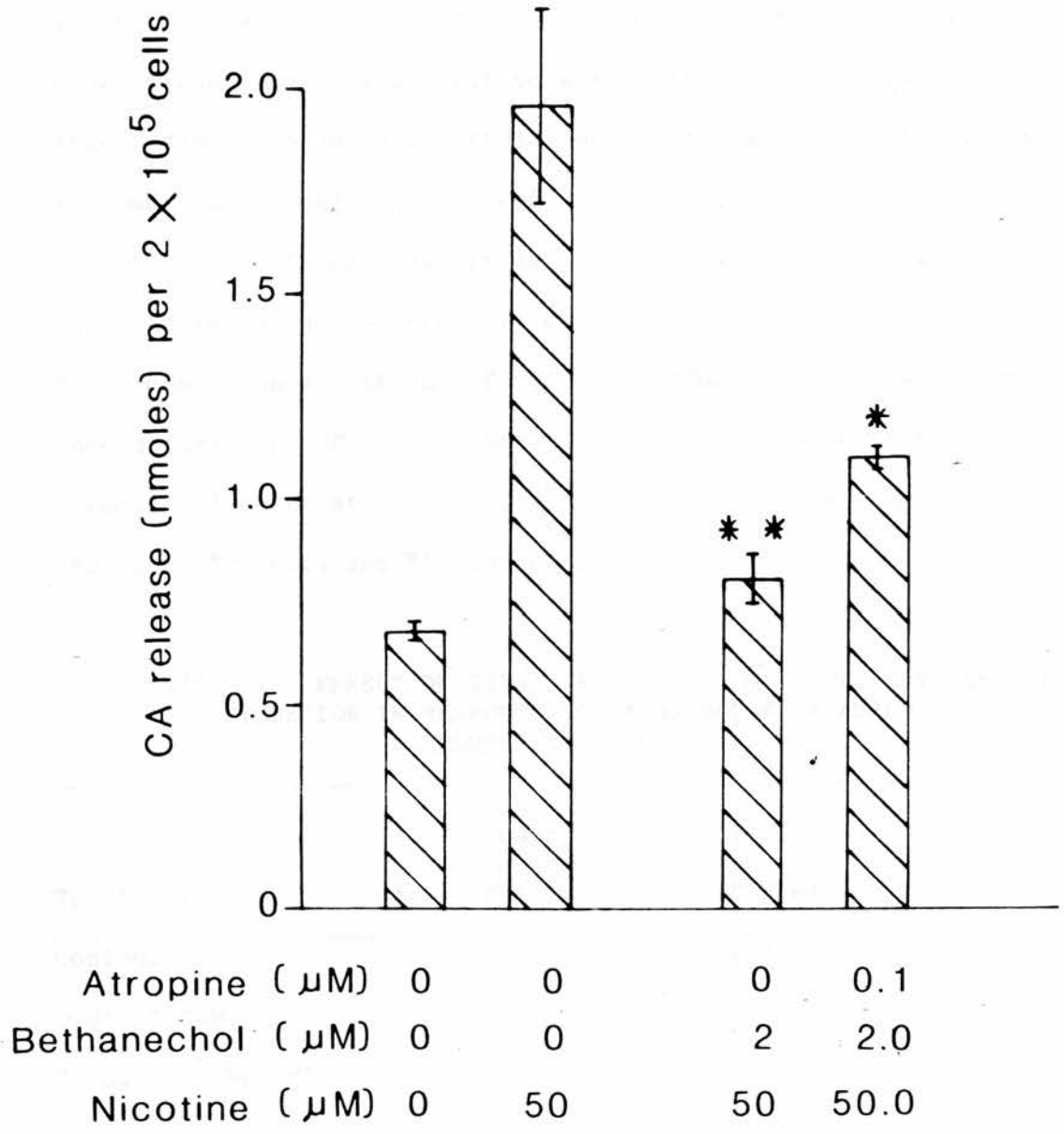
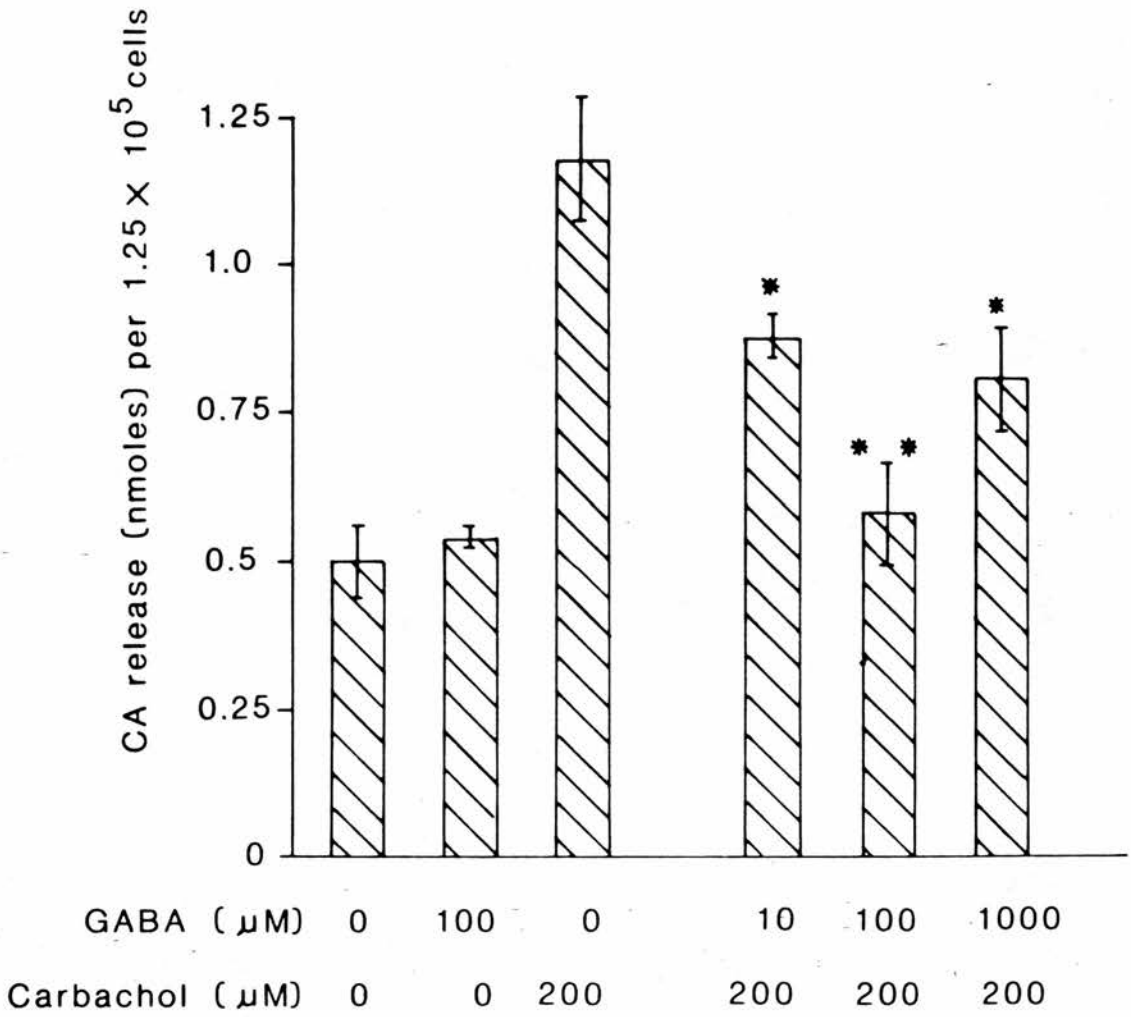

CA release (nmoles) per 2 X 10^5 cells
2.0
1.5
1.0
0.5
0
* *
*
Atropine (μM) 0 0 0 0.1
Bethanechol (μM) 0 0 2 2.0
Nicotine (μM) 0 50 50 50.0

Fig. 18. Effect of GABA pretreatment on catecholamine release from chromaffin cells in response to carbachol. The bars represent the mean and the vertical lines are S.E. The number of experiments in each bar is 4.

*Significantly different from carbachol at $P<0.05$.

**Significantly different from carbachol at $P<0.01$.


CA release (nmoles) per 1.25 X 10^5 cells
1.25
1.0
0.75
0.5
0.25
0
GABA (μM) 0 100 0 10 100 1000
Carbachol (μM) 0 0 200 200 200 200

The effect of GABA on catecholamine secretion in response to a selective nicotinic agonist, nicotine, has also been studied. Chromaffin cells were pretreated with GABA (10 and 100uM) and then stimulated with nicotine (50uM) as described previously. In this experiment also, GABA (10 and 100 uM) decreased the nicotine-stimulated catecholamine release from cells and the effect of GABA, at a concentration of 100uM, was significant (**P<0.01 in an analysis of variance). Results are shown in Table 8 and Fig. 19.

TABLE 8. EFFECT OF GABA PRETREATMENT ON NICOTINE-EVOKED CATECHOLAMINE RELEASE FROM BOVINE ADRENAL CHROMAFFIN CELLS.

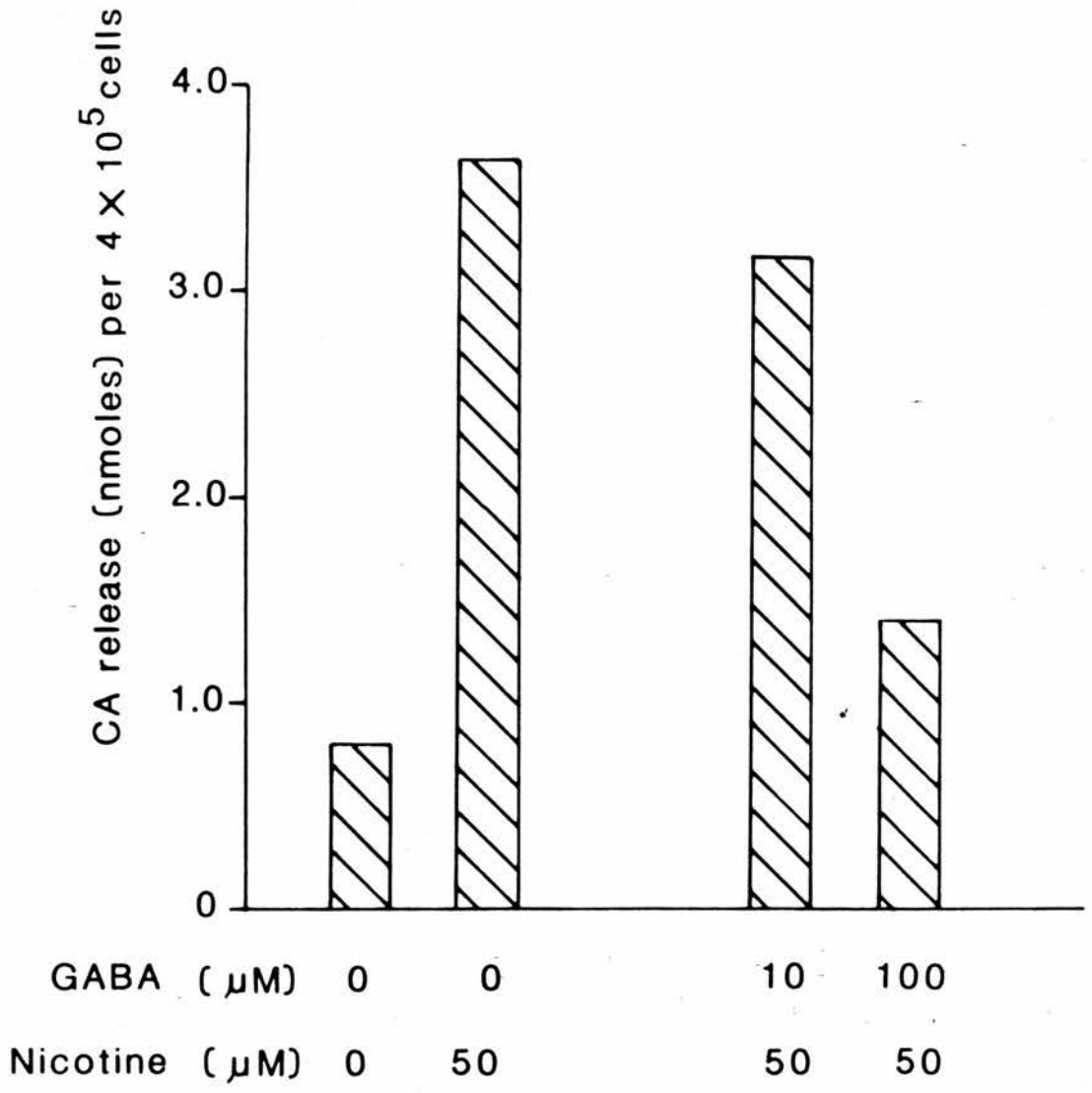
Treatment	CA release (nmoles) per 4 x 10^5 cells				
	1st day	2nd day	3rd day	Mean	% of Response
Control	0.80	0.84	0.96	0.86	24
Nicotine (50uM)	3.64	2.76	4.10	3.50	100
GABA (10uM) + Nicotine (50uM)	3.16	1.90	3.80	2.95	84
GABA (100uM) + Nicotine (50uM)	1.38	2.00	2.40	1.93	55

Cells were preincubated with GABA for 5 min and then nicotine was added and the incubation was continued for a further 10 min. The data represent the levels of catecholamine in three separate experiments. The effect of GABA (100uM) was significant (**P<0.01), determined by an analysis of variance.

3.3.2 Effect of Bicuculline on GABA Response

The effect of bicuculline, a $GABA_A$ receptor antagonist, on catecholamine secretion was investigated to test whether the GABA-induced inhibition on the secretory response of

Fig. 19. Effect of GABA on nicotine-stimulated catecholamine release from bovine chromaffin cells. The bars represent the catecholamine levels of one experiment (1st day).


CA release (nmoles) per 4 X 10^5 cells
4.0
3.0
2.0
1.0
0
GABA (μM) 0 0 10 100
Nicotine (μM) 0 50 50 50

nicotine was mediated by stimulation of $GABA_A$ receptors in the chromaffin cell membrane. Cells were preincubated with bicuculline (5uM) and GABA (100uM) for 5 min at 37°C and then nicotine (50uM) was added and the incubation was continued for another 10 min. Bicuculline pretreatment blocked the inhibitory effect of GABA on the secretory response of nicotine. Results are shown in Table 9.

TABLE 9. EFFECT OF BICUCULLINE AND GABA PRETREATMENT ON NICOTINE-EVOKED CATECHOLAMINE SECRETION FROM BOVINE ADRENAL MEDULLARY CELLS.

	CA secretion (nmoles) per 3 x 10^5 cells					
Treatment	1st day	2nd day	3rd day	4th day	Mean	% of Response
Control	0.66	0.72	0.66		0.68	28
Nicotine (50uM)	2.64	2.52	2.76	1.82	2.44	100
GABA(100uM) + Nicotine (50uM)	2.00	1.82	1.66	1.38	1.72	70
Bicuculline (5uM) + GABA (100uM) + Nicotine (50uM)	2.40	2.00	2.76	1.74	2.23	91

Cells were preincubated for 5 min with bicuculline and GABA and then nicotine was added. The data represent the levels of CA released in four different experiments. The effect of GABA was significantly different from nicotine only at *P<0.05. The effect of bicuculline was significant at *P<0.05, determined by an analysis of variance.

GABA (100uM) caused a significant inhibition of nicotine-evoked secretion (*P<0.05). Bicuculline (5uM) reversed the effect of GABA (100uM). The effect of bicuculline (5uM) was also significant (*P<0.05, determined by an analysis of variance). Fig. 20 shows the effect of bicuculline plus GABA on nicotine-stimulated CA release (3rd day).

3.3.3 Effect of Bicuculline on Nicotine-Evoked Secretion

Recent evidence suggests that chromaffin cells can synthesize, store, release and inactivate GABA and nicotinic receptor stimulation may release GABA from chromaffin cells which in turn may reduce the nicotine-evoked secretion of CA (Kataoka et al., 1984). To examine intrinsic GABA activity, if any, chromaffin cells were preincubated with bicuculline (2uM) for 5 min at 37°C and then the cells were stimulated with nicotine, 5 and 50 uM, for another 10 min. Bicuculline (2uM) did not change the secretory response of 5uM nicotine. However, bicuculline (2uM) significantly facilitated the CA secretion evoked by 50uM nicotine (*P<0.05, determined by an analysis of variance). Bicuculline (2uM) itself did not have any influence on catecholamine secretion. Results are summarized in Table 10.

Fig. 20. Effect of bicuculline plus GABA on nicotine-stimulated catecholamine release from bovine adrenal medullary chromaffin cells. The bars represent the CA levels of one experiment (3rd day).

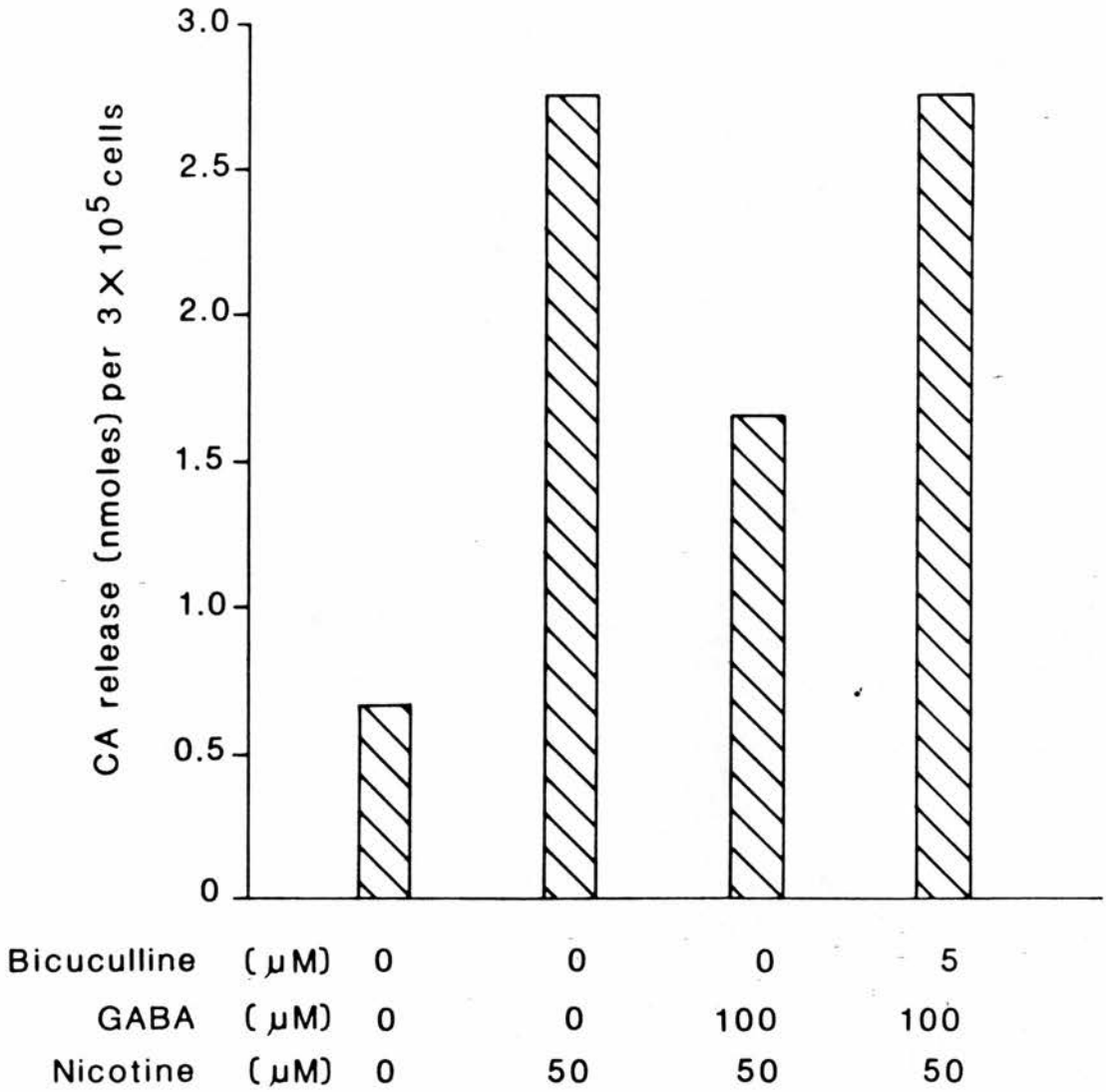

3.0
2.5
2.0
1.5
1.0
0.5
0
CA release (nmoles) per 3 X 10^5 cells
Bicuculline (μM) 0 0 0 5
GABA (μM) 0 0 100 100
Nicotine (μM) 0 50 50 50

TABLE 10. EFFECT OF BICUCULLINE ON NICOTINE-STIMULATED CATECHOLAMINE RELEASE FROM BOVINE ADRENAL MEDULLARY CELLS.

Treatment	CA release (nmoles) per 10^5 cells			
	1st day	2nd day	3rd day	Mean
Control	0.56	0.56	0.60	0.57
Nicotine (5uM)	1.10	1.26	0.80	1.05
Bicuculline (2uM) + Nicotine (5uM)	1.00	1.20	0.70	0.96
Nicotine (50uM)	0.96	1.20	1.44	1.20
Bicuculline (2uM) + Nicotine (50uM)	1.26	1.82	1.90	1.66

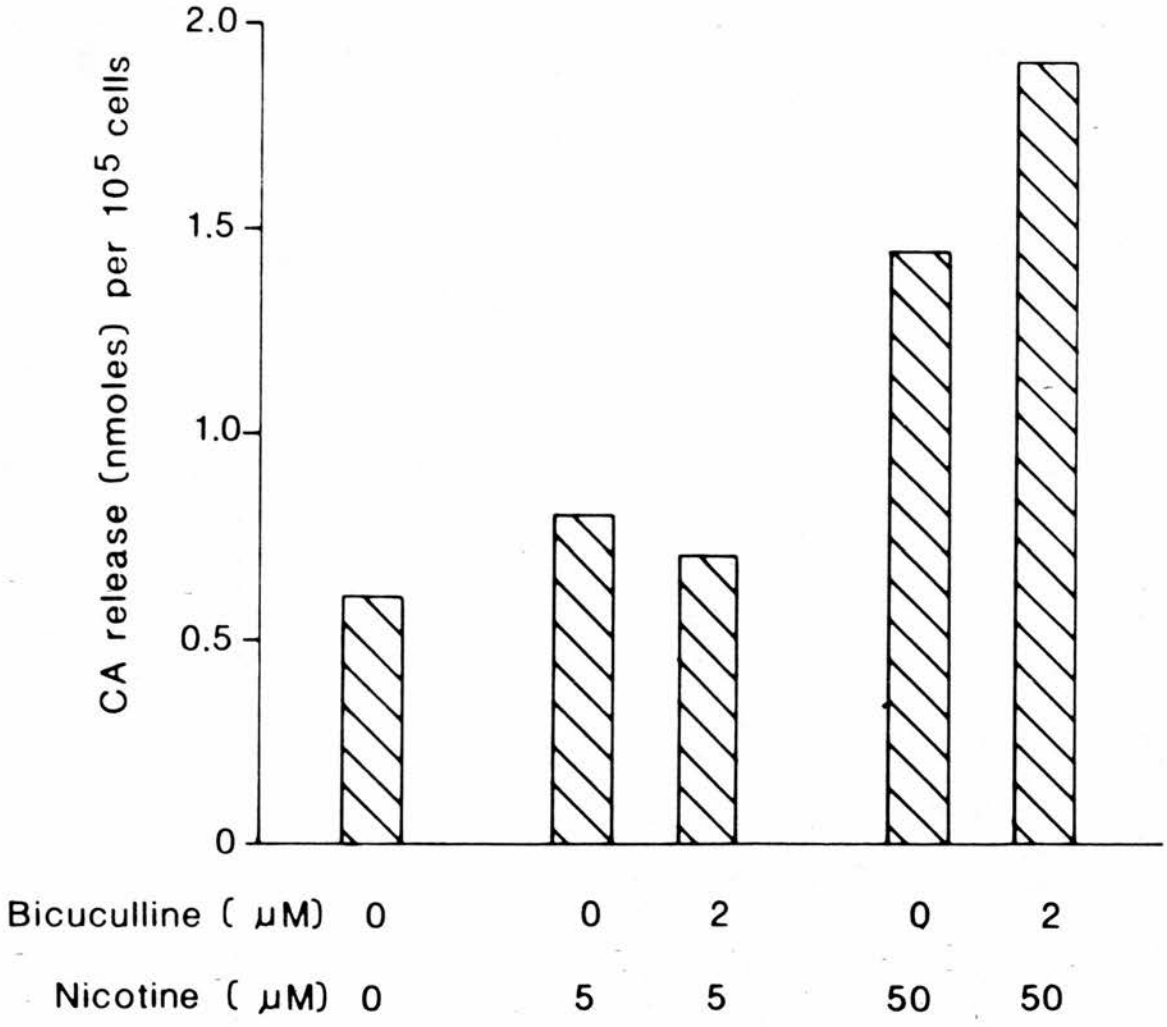
Cells were preincubated with bicuculline (5 min) and then stimulated with nicotine for a further 10 min. The data represent the levels of CA released in three separate experiments. Bicuculline (2uM) significantly increased the nicotine (50uM)-evoked CA release (*P<0.05, determined by an analysis of variance).

Fig. 21 shows the effect of bicuculline on nicotine-evoked CA release (3rd day).

3.4 Modulation of Catecholamine Secretion by Diazepam

Ligand binding techniques have revealed the existence of recognition sites for GABA and benzodiazepines within the chromaffin cell membrane (Kataoka et al., 1984). Functionally, GABA receptors of adrenal medulla resemble those of the brain with regard to their interaction with benzodiazepine receptors. Experiments were done to examine the influence of benzodiazepines on catecholamine secretion. Chromaffin cells were preincubated for 5 min with diazepam

Fig. 21. Effect of bicuculline pretreatment on catecholamine release from chromaffin cells in response to nicotine. The bars represent the levels of CA secreted from the cells (3rd day).


2.0
1.5
1.0
0.5
0
CA release (nmoles) per 10^5 cells
Bicuculline (μM) 0 0 2 0 2
Nicotine (μM) 0 5 5 50 50

(10uM) and GABA (10 and 100uM) and then the CA secretion was stimulated with nicotine (50uM) for 10 min. Results are shown in Table 11.

TABLE 11. EFFECT OF DIAZEPAM AND GABA PRETREATMENT ON CATECHOLAMINE SECRETION FROM BOVINE ADRENAL CHROMAFFIN CELLS IN RESPONSE TO NICOTINE.

Treatment	CA release (nmoles) per 4 x 10^5 cells 1st day	2nd day	3rd day	Mean	% of Response
Control	0.72		0.72	0.72	21
Nicotine (50uM)	3.98	3.00	3.32	3.43	100
Diazepam (10uM) + Nicotine (50uM)	2.40		1.90	2.06*	60
GABA (10uM) + Nicotine (50uM)	3.64	1.90	2.60	2.70	79
Diazepam (10uM) + GABA (10uM) + Nicotine (50uM)	1.38	1.10	0.44	0.97	28
GABA (100uM) + Nicotine (50uM)	2.40	2.40	1.58	2.10	61
Diazepam (10uM) + GABA (100uM) + Nicotine (50um)	1.74	1.20	0.76	1.23	36

Bovine chromaffin cells were preincubated for 5 min at 37°C in saline containing GABA or diazepam or both and then stimulated with nicotine for 10 min. The data represent the CA levels of three different experiments. The effects of GABA, 10 and 100 uM, were significant at *P<0.05 and **P<0.01 respectively. The effect of diazepam was significant at **P<0.01 (determined by analysis of variance). *The mean of two values in the table and an estimated value of 1.89.

Levels of significance of data were determined by analysis of variance. Both concentrations of GABA, 10 and 100 uM, significantly inhibited the effect of nicotine (*P<0.05 and

P<0.01 respectively), but the effect of GABA did not significantly increase with concentration from 10 to 100 uM. Diazepam alone (10uM) significantly inhibited the effect of nicotine (P<0.01). The effect of diazepam is stronger than that of GABA. From this result it is evident that there is no interaction between the effects of GABA and diazepam i.e. effect of GABA 10 and diazepam 10 = effect of GABA 10 + effect of diazepam 10. There was a significant difference between the experimental results obtained on different days.

Fig. 22 shows the effect of GABA plus diazepam on nicotine-stimulated catecholamine secretion (1st day).

3.5 **Modulation of Catecholamine Secretion by Alphaxalone**

Alphaxalone, a steroid anaesthetic, has been reported to modulate the interaction between GABA and its receptor in the rat cuneate nucleus <u>in vitro</u> (Harrison & Simmonds, 1984). This enhancement of interaction between GABA and its receptor might modulate the agonist-evoked catecholamine secretion. Cells were preincubated for 5 min with alphaxalone (0.1uM) and GABA (100uM) or only alphaxalone (10uM) and then the secretion was stimulated with nicotine for 10 min. Results are shown in Table 12.

Fig. 22. Effect of diazepam and GABA pretreatment on nicotine-stimulated catecholamine release from bovine adrenal chromaffin cells. The bars represent the results of one experiment (1st day).

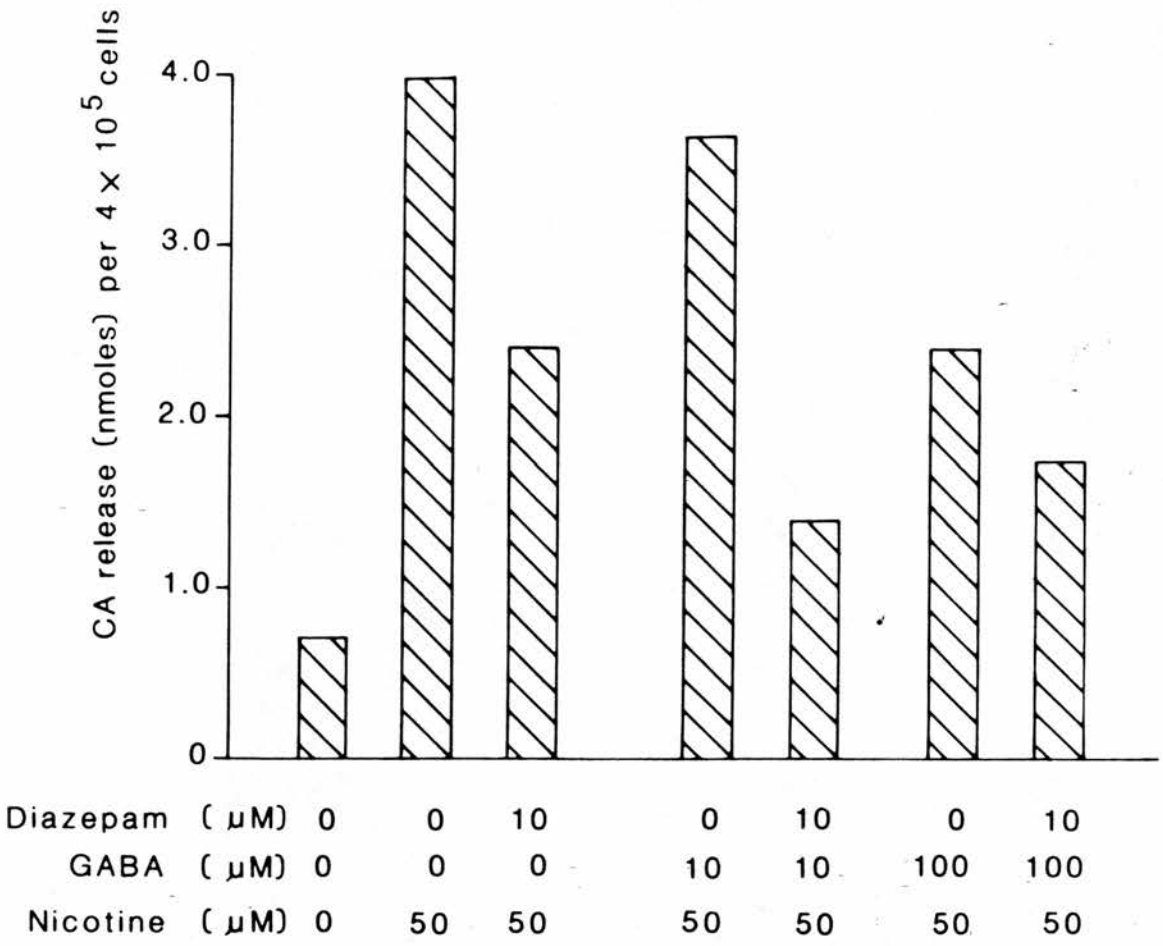

CA release (nmoles) per 4 X 10^5 cells
4.0
3.0
2.0
1.0
0
Diazepam (μM) 0 0 10 0 10 0 10
GABA (μM) 0 0 0 10 10 100 100
Nicotine (μM) 0 50 50 50 50 50 50

TABLE 12. EFFECT OF ALPHAXALONE AND GABA PRETREATMENT ON CATECHOLAMINE SECRETION IN RESPONSE TO NICOTINE FROM BOVINE MEDULLARY CHROMAFFIN CELLS.

Treatment	CA release (nmoles) per 4 x 10^5 cells				% of Response
	1st day	2nd day	3rd day	Mean	
Control	0.64	0.64	0.72	0.67	16
Nicotine (50uM)	4.38	4.20	3.64	4.07	100
GABA (100uM) + Nicotine (50uM)	3.98	3.64	2.40	3.34	82
Alphaxalone (0.1uM) + GABA (100uM) + Nicotine (50uM)	3.48	2.80	2.20	2.83	69
Alphaxalone (10uM) + Nicotine (50uM)	2.30	2.00	1.26	1.85	45

Cells were preincubated for 5 min with GABA or alphaxalone or both and then nicotine was added. The data represent the levels of CA released in three different experiments. The effect of alphaxalone (0.1uM) was significant at *P<0.05 (determined by analysis of variance). The effect of alphaxalone (10uM) was significant at **P<0.01.

In the experiment, GABA (100uM) appeared to reduce the nicotine-evoked CA release but the effect was not significant. Alphaxalone at low concentration (0.1uM) potentiated the effect of GABA on nicotine-stimulated secretion and this effect was significant (*P<0.05, in an analysis of variance). Alphaxalone alone at high concentration (10uM) caused a significant reduction in nicotine-evoked CA release (**P<0.01).

Fig. 23 shows the effect of alphaxalone plus GABA on catecholamine secretion (2nd day).

The same experiment was done with alphaxalone at a concentration of 0.3uM. Cells were preincubated in the

Fig. 23. Effect of alphaxalone (0.1uM) and GABA pretreatment on catecholamine secretion from chromaffin cells in response to nicotine. The diagram represents the results of one experiment (2nd day).

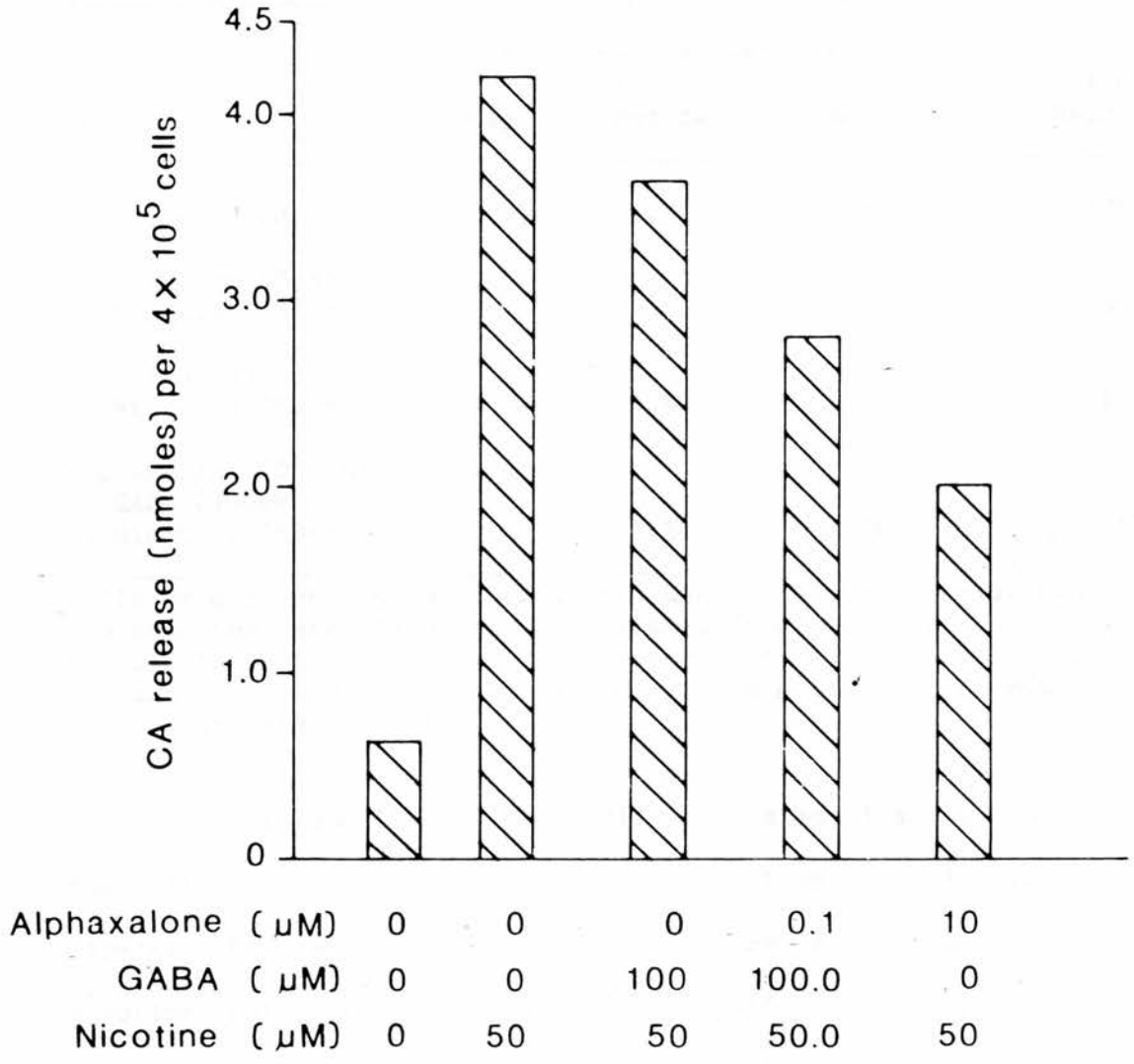
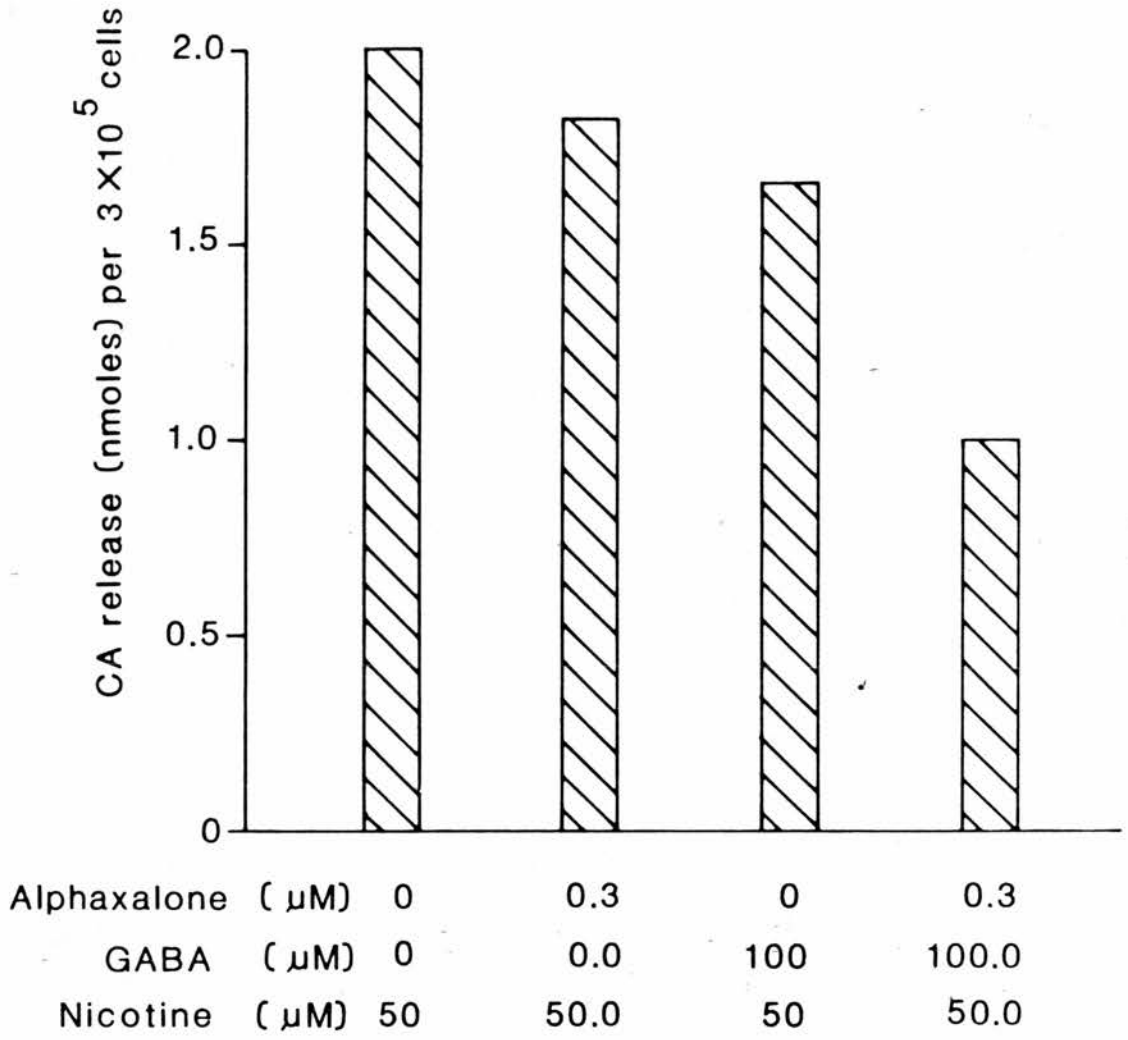

4.5
4.0
3.0
2.0
1.0
0
CA release (nmoles) per 4 X 10^5 cells
Alphaxalone (μM) 0 0 0 0.1 10
GABA (μM) 0 0 100 100.0 0
Nicotine (μM) 0 50 50 50.0 50

Fig. 24. Effect of alphaxalone (0.3uM) plus GABA on nicotine-evoked secretion from bovine adrenal medullary cells. The bars represent the levels of CA released in one experiment (1st day).


CA release (nmoles) per 3 X10 5 cells
2.0
1.5
1.0
0.5
0
Alphaxalone (μM) 0 0.3 0 0.3
GABA (μM) 0 0.0 100 100.0
Nicotine (μM) 50 50.0 50 50.0

In order to test whether the effect of alphaxalone alone at high concentration is mediated through GABA receptor activation, cells were pre-exposed to bicuculline and alphaxalone for 5 min and then catecholamine secretion was stimulated with nicotine for 10 min (Table 14).

TABLE 14. EFFECT OF BICUCULLINE AND ALPHAXALONE PRETREATMENT ON NICOTINE-EVOKED CATECHOLAMINE SECRETION FROM BOVINE ADRENAL MEDULLARY CELLS.

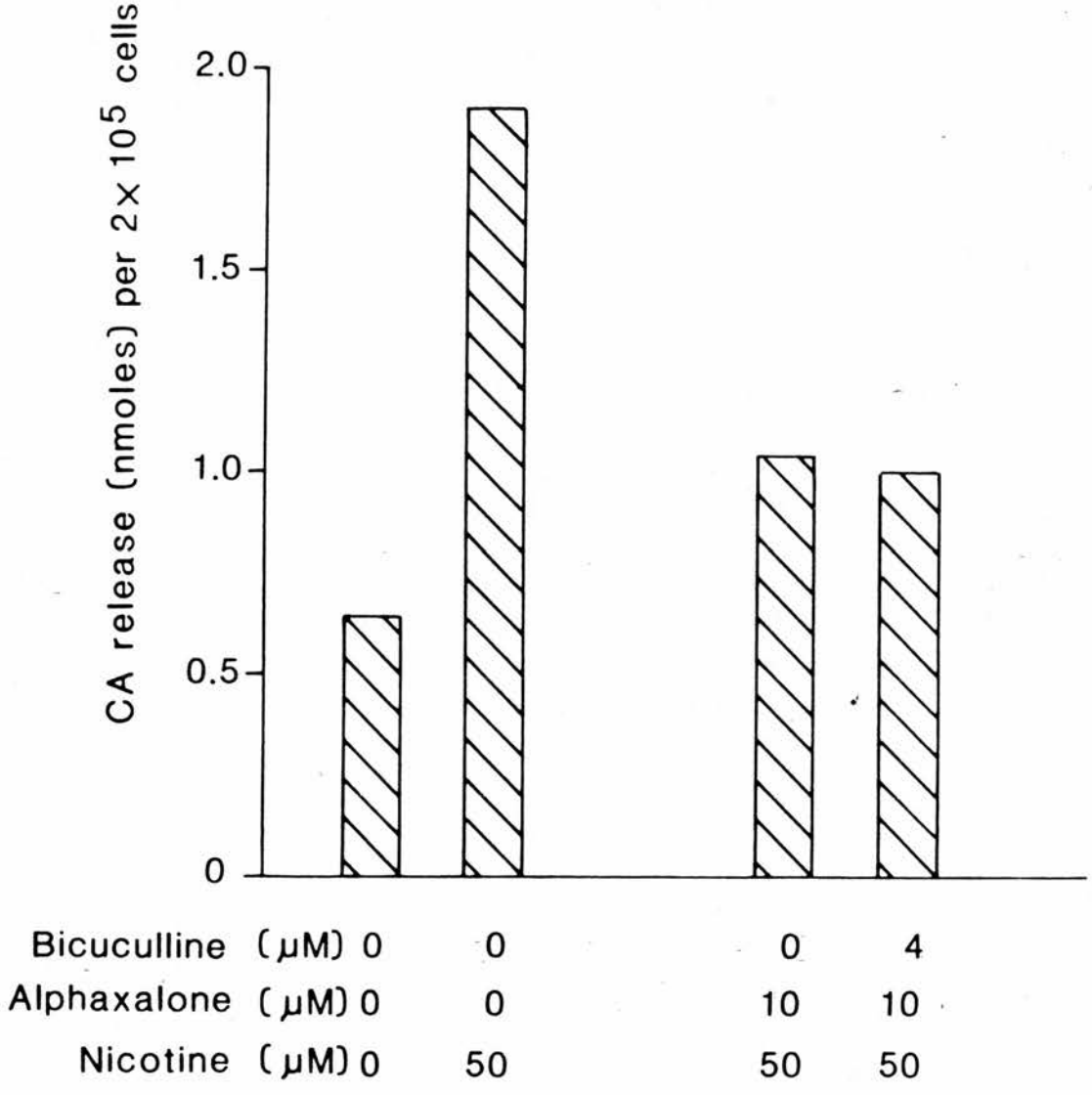
	CA release (nmoles) per 2 x 10^5 cells			
Treatment	1st day	2nd day	3rd day	Mean
Control	0.72	0.70	0.64	0.68
Nicotine (50uM)	2.40	1.58	1.90	1.96
Alphaxalone (10uM) + Nicotine (50uM)	1.58	0.84	1.04	1.15
Bicuculline (4uM) + Alphaxalone(10uM) + Nicotine (50uM)	1.52	0.96	1.00	1.16

Cells were preincubated with bicuculline and alphaxalone for 5 min before nicotine was added. The data represent the levels of CA released in three separate experiments. The effect of alphaxalone on nicotine-evoked CA release was significant (***$P<0.001$), determined by an analysis of variance.

Bicuculline pretreatment did not reverse the inhibitory effect of alphaxalone on catecholamine secretion stimulated by nicotine.

Fig. 25 shows the effect of bicuculline plus alphaxalone on nicotine-stimulated catecholamine release (3rd day).

Fig. 25. Effect of bicuculline and alphaxalone pretreatment on nicotine-evoked catecholamine release from bovine adrenal medullary cells. The bars represent the levels of CA released in one experiment (3rd day).


CA release (nmoles) per 2 × 10^5 cells
2.0
1.5
1.0
0.5
0
Bicuculline (μM) 0 0 0 4
Alphaxalone (μM) 0 0 10 10
Nicotine (μM) 0 50 50 50

3.6 Characteristics of Catecholamines Secreted from Cultured Bovine Adrenal Chromaffin Cells.

The method of electrochemical detection could detect both noradrenaline and adrenaline in the extract of chromaffin cells. At basal secretion, adrenaline to noradrenaline ratio was 1:5. When the secretion was stimulated with nicotine (5uM), the ratio became about 1:2 and at higher concentration of nicotine (50uM) the ratio was almost 1:1 (Fig. 9).

DISCUSSION

Bovine adrenal chromaffin cells have been isolated in large quantity, shown to be viable and maintained in culture. A pharmacological study was made of the effects of various nicotinic and muscarinic agonists and their antagonists on the release of catecholamines from these cells. The influence of GABA and its antagonist and its modulators, diazepam and alphaxalone, on the secretion of catecholamines evoked by nicotinic agonist was studied. In addition, the effect of K depolarization on the secretory response was investigated.

4.1 Effect of High Levels of K on Catecholamine Secretion

Catecholamine secretion from the adrenal medulla can be stimulated by concentration of potassium sufficient to depolarize chromaffin cell membranes (Baker & Rink, 1975). In the present study, when cells were exposed to high K (30mM KCl) containing physiological saline for a period of 10 min, catecholamine secretion was stimulated. 30mM KCl induced a 4-fold increase in the secretory response of chromaffin cells. This observation correlates with the finding of Kidokoro & Ritchie (1980) who showed that 5-20mM KCl progressively depolarized chromaffin cells, increased spike frequency and released catecholamines from perfused rat adrenal glands. Douglas & Rubin (1961) demonstrated that high potassium (56mM) caused considerable depolarization of chromaffin cells and thus excited medullary secretion which was dependent on the presence

of Ca in Locke's solution. Transient increase in catecholamine secretion has also been demonstrated by Baker & Rink (1975) after prolonged exposure of venous perfused bovine adrenal glands to high K in the presence of external Ca. Schiavone & Kirpekar (1982) showed that 70 mM K stimulated catecholamine release from cat adrenal medulla. Catecholamine secretion from isolated guinea pig adrenal cells is also stimulated by 56mM K which is not inhibited by atropine or hexamethonium (Hochman & Perlman, 1976).

Boksa & Livett (1984) hypothesised that stimulation of catecholamine release with a high K concentration occurs as a result of direct depolarization of the chromaffin cell membrane and does not involve activation of the ACh-receptor. Depolarization of the cell membrane facilitates the movement of Na^+ and Ca^{2+} (Perlman, Cossi & Role, 1980). Although the response to high K indicates that depolarization is an adequate stimulus for secretion, depolarization is clearly not by itself a sufficient stimulus. Douglas (1968) showed that omission of Ca does not prevent high K from depolarizing the chromaffin cells yet it blocks secretion. Since the stimulation is dependent on the presence of external Ca and is thought to involve entry of Ca^{2+} into the cells, through a potential dependent Ca^{2+} channel, the consequent rise in intracellular Ca^{2+} stimulates catecholamine release (Douglas & Rubin, 1961).

4.2 Cholinergic Receptor Activation of Catecholamine Release

4.2.1 Functional Role of Nicotinic Agonists

The nature of the cell surface cholinergic receptor with respect to nicotinic and muscarinic agents has been examined. Isolated cells exhibited a secretory response to carbachol, a synthetic analogue of acetylcholine, and nicotine, a selective nicotinic agonist.

Exposure of chromaffin cells to nicotine, in this investigation, caused a great increase in CA release. The response to nicotine was dose-related. The secretion was evident at 1uM, was half maximal at about 15uM and was maximal at 100uM. Nicotine at high concentrations (>100uM) exhibited a marked inhibition of CA secretion. Mizobe et al. (1979) demonstrated a characteristic activation of secretion of [^{3}H]-noradrenaline at low levels of nicotine (0.1-10uM) and an inhibition ("desensitization") at higher levels (>100uM) from bovine chromaffin cells. Mizobe & Livett (1983) also showed dose-response curves with nicotine-evoked release of endogenous catecholamines and acetylcholinesterase (AChE) with inhibition at higher concentrations of the agonist. In this study, the cell needed a nicotine concentration one order of magnitude higher for its secretory response (1-100uM). This might be due to some difference in the secretory mechanism of endogenous and exogenous catecholamines. The processes of uptake, storage and release of exogenously

administered catecholamines might be different from that of endogenous catecholamines. The concentration of nicotine for desensitization of secretory response (>100uM) is in agreement with other workers' findings.

In the present study, carbachol (CCh) stimulated catecholamine secretion from chromaffin cells in a dose-dependent manner, but there was a great variation in secretion from experiment to experiment. The secretion was detectable at a range of concentrations (10-50uM) and the maximal release was obtained at concentrations of 100, 200 and 400 uM respectively in three separate experiments. The maximum amount of catecholamines released by carbachol also varied from 2.3 to 5.2 nmoles/4 x 10^5 cells in different experiments. There was a reduction in secretion with higher concentrations of the drug.

A number of workers reported a dose-dependent catecholamine (CA) release by acetylcholine (ACh), a mixed cholinergic agonist, from bovine chromaffin cells. Schneider et al. (1977) demonstrated that ACh stimulates CA release from bovine chromaffin cells, which has its half maximal value at 10uM and its maximum at 100uM. Livett et al. (1979) showed that the half maximal concentration for ACh-stimulated release of [^{3}H]-noradrenaline was 50uM. At concentrations of ACh >100uM, there was a reduction in CA secretion (Livett & Boksa, 1984; Liang & Perlman, 1979).

4.2.2 **Inhibition of Nicotinic Response**

In the present investigation, the nicotine (50uM)-stimulated secretion was blocked by the nicotinic antagonist hexamethonium (1mM) by 66%, as shown by Schneider et al. (1977). Hexamethonium was also effective in blocking the ACh-stimulated release of catecholamines from bovine chromaffin cells (Fenwick et al., 1978). Hochman & Perlman (1976) showed that hexamethonium (100uM) inhibits the secretory response of acetylcholine in guinea pig adrenal chromaffin cells. Liang & Perlman (1979) demonstrated that hexamethonium and tubocurarine produce a concentration-dependent inhibition of ACh-stimulated CA secretion in hamster adrenal cells.

4.2.3 **Role of Muscarinic Agonist**

In the present study, bethanechol (10-1000uM), a selective muscarinic agonist, did not stimulate the release of endogenous CA from bovine chromaffin cells. Livett et al. (1979) demonstrated that methacholine (100pM-100uM), a muscarinic agonist, did not stimulate the bovine chromaffin cells to release [^{3}H]-noradrenaline. Mizobe & Livett (1983) also showed that methacholine (1-1000uM) did not cause release of endogenous CA. Muscarine, a potent muscarinic agonist, did not evoke any secretion of CA from bovine chromaffin cells (Derome et al., 1981). On the other hand Schneider et al. (1977) found only a small and insignificant response with muscarine and pilocarpine. Smith & Winkler (1972) have also noted the lack of effect of muscarinic agents on

perfused bovine adrenal medullary secretion.

4.2.4 Receptors Involved in Catecholamine Secretion

In the intact animal, activation of ACh receptors on adrenal chromaffin cells is brought about by a release of ACh from splanchnic nerve terminals, resulting in a release of CA into the circulation (Mizobe & Livett, 1983). In a similar manner, the present study on bovine adrenal chromaffin cells showed that cholinergic receptor activation stimulated the CA release. The cholinergic receptors were found to be predominantly nicotinic based on (i) the ability of nicotine to stimulate secretion, (ii) the lack of secretion induced by muscarinic agonist and (iii) the blockade of secretion by hexamethonium. In this respect, too, the properties of the cholinergic receptor in bovine adrenal cells are similar to those previously reported for hamster adrenal cells (Liang & Perlman, 1979). This observation correlates with the finding obtained by Schneider et al. (1977); Mizobe et al. (1979); Fenwick et al. (1978); Livett et al. (1979); and Derome et al. (1981). Wilson & Kirshner (1977) have similarly confirmed the dependence of secretory function of bovine medullary cells on nicotinic ACh receptors. This may be a peculiarity of the species, because muscarinic agents are known to be active in other mammals. In the cat adrenal medulla, pilocarpine released adrenaline preferentially, while nicotine released large amounts of both amines (Douglas & Poisner, 1965; Rubin & Miele, 1968). These findings suggested that muscarinic

receptors are located on adrenaline containing cells and nicotinic receptors are on both adrenaline and noradrenaline containing cells. Such a correlation was not found in studies on the dog adrenals (Critchley et al., 1975). The cat is the only studied species whose adrenals contain equal amounts of adrenaline and noradrenaline. Dual control of adrenaline and noradrenaline release could thus be peculiar to cats (Ungar & Phillips, 1983).

4.2.5 **Nicotine-Induced Desensitization of Secretory Response**

A characteristic feature of the nicotinic response exhibited by most tissues is the induction by agonists of nicotinic receptor desensitization (Livett & Boksa, 1984). This study showed a characteristic reduction in CA release with higher concentrations of nicotine (>100uM) and CCh (>200uM). Nicotine-induced inhibition of secretion at concentrations >100uM has also been reported by a number of workers (Boksa & Livett, 1984; Mizobe et al., 1979; Mizobe & Livett, 1983). The likely explanation for this decline in nicotine-evoked secretion might be a consequence of receptor desensitization (Kirpekar & Prat, 1978). At present, the most widely accepted model of desensitization is the receptor model proposed by Katz & Thesleff (1957); according to this hypothesis, an activated agonist-receptor complex is slowly converted to an inactive agonist-receptor complex during prolonged exposure of the cells to high concentration of agonist. However, recent evidence also points to a change in

membrane ion conductance as a possible mechanism of action for the desensitization process (Magazanik & Vyskocil, 1970, 1975). Nicotine at high concentrations might also induce persistent depolarization of chromaffin cell membrane that would inactivate the voltage-dependent fast Na^+ channels which would normally generate an action potential. The other possible explanation might be (i) inactivation of voltage-dependent Ca^{2+} entry or (ii) inactivation of the stimulus-secretion coupling process at some steps later than voltage dependent Ca^{2+} entry (Schiavone & Kirpekar, 1982). This phenomenon of "nicotinic receptor desensitization" might play some physiological role in vivo in reducing the CA release in conditions of excessive firing of splanchnic nerve.

4.2.6 Muscarinic Receptor Activation and Catecholamine Secretion

Muscarinic receptors on chromaffin cells appear to exert an inhibitory rather than a facilitatory function (Livett & Boksa, 1984). In the present investigation, bethanechol, a selective muscarinic agonist, at a concentration of 2 and 10 uM induced a respective 38 and 53% reduction of nicotine (50uM)-stimulated CA release. The effect of bethanechol (2uM) was antagonized by atropine (0.1uM). This finding correlates the observation of Derome et al. (1981) where they showed that co-stimulation of the muscarinic receptor with ACh (0.5uM) caused a decrease of 33% of nicotine (50uM)-evoked CA release. The inhibitory effect of ACh on CA secretion was

antagonized by atropine (0.1uM). The antagonism of the effect of bethanechol with atropine, in the present study, clearly suggests the involvement of the muscarinic receptor in the inhibitory action of bethanechol. Derome et al. (1981) and Yanagihara et al. (1979) showed that muscarinic receptors in bovine adrenal cells are fully activated by ACh at <0.1uM elevating the intracellular cyclic GMP level that is inhibitory to ACh-evoked CA release. At concentrations of ACh >1uM, this inhibition is overcome by activation of nicotinic receptors. They have also demonstrated that nicotine, a potent stimulator of CA secretion, had no stimulatory effect on cyclic GMP level. Conversely, muscarine, a potent stimulator of cyclic GMP level, did not evoke any secretion of CA. Therefore, the inhibitory effect of bethanechol on agonist-evoked CA secretion in this study might be due to a rise in cGMP levels as a consequence of stimulation of muscarinic receptors in bovine chromaffin cells.

The variable effect of carbachol on catecholamine secretion, in this investigation, might be because of co-stimulation of muscarinic receptors. The variability was more marked with low concentrations of carbachol. This may be correlated with the observation that ACh at low concentration elevates cGMP level that is inhibitory to agonist-evoked catecholamine secretion (Derome et al., 1981). Carbachol, a mixed cholinergic agonist, might increase the cGMP level in the cell, which might complicate the nicotinic receptor-stimulated catecholamine release.

A physiological role for this dual mechanism of cholinergic receptors has been proposed. Under basal conditions, low levels of ACh released from the splanchnic nerve fully activate the muscarinic receptor, thereby inhibiting the basal release of CA. When the splanchnic nerve is firing at a higher rate, greater amounts of ACh will then be released from nerve terminals and will activate nicotinic receptors, overcoming the muscarinic blockade causing release of CA (Derome et al., 1981).

Indirect evidence also comes from work on ganglion cells (Brown & Constanti, 1980) which shows that depolarization in response to muscarinic agonists involves blockade of a voltage-sensitive K^+ channel, with a fall in membrane conductance. In contrast the observed effects of ACh on isolated chromaffin cells appear to resemble the effects of nicotinic activation at other sites, with the opening of channels and a rise in membrane conductance (Ungar & Phillips, 1983).

4.2.7 Characteristics of Catecholamines Released from Cultured Bovine Chromaffin Cells

The bovine adrenal medulla has been reported to contain 75% adrenaline and 25% noradrenaline (Schneider et al., 1977). The electrochemical method, used in this investigation, could detect both noradrenaline and adrenaline in the extract of chromaffin cells. At basal secretion, adrenaline to noradrenaline ratio was 1:5 (i.e. adrenaline 16%), in

nicotine (5uM)-stimulated secretion, the ratio was about 1:2 (adrenaline 33%), whereas with 50uM nicotine, the ratio became about 1:1 (adrenaline about 50%)(Fig. 9). Schneider et al. (1977) showed that isolated cultured bovine chromaffin cells have a lower adrenaline content (44%) and in ACh (100uM)-stimulated secretion adrenaline accounts for 42% of the total CA. Rubin & Miele (1968) showed that in cat adrenals low concentration of nicotine (1.2uM) elicited 70% noradrenaline release and at higher concentrations (12-60 uM) evoked a release of 53% adrenaline. Livett et al. (1981) suggested that nicotine and other agonists are more effective at inducing noradrenaline release from isolated and cultured cells. It may indicate that noradrenaline containing chromaffin cells have a high density of nicotinic receptors (Livett, 1984). Schneider et al. (1977) suggested that there might be increased lability of the adrenaline-containing cell during tissue disaggregation. The other possibility might be the absence of cortical cells in culture, which are known to influence the synthesis of phenylethanolamine-N-methyltransferase (PNMT), the converting enzyme from noradrenaline to adrenaline.

4.3 GABAergic Receptor Activation and Catecholamine Release.

4.3.1 Functional Role of GABA in Catecholamine Secretion

The recent finding that adrenal chromaffin cells can synthesize, store, release and inactivate GABA, suggests that γ -aminobutyric acid (GABA) might have some physiological role

in modulating the responsiveness of chromaffin cells to neurally-released ACh (Kataoka et al., 1984). The effect of GABA receptor activation on the secretory response of adrenal chromaffin cells has been studied. Carbachol (200uM), in the medium, caused secretion of catecholamines (CA). Exposure of cells to GABA at a concentration of 10, 100 and 1000 uM caused a reduction of carbachol (200uM)-stimulated CA release by 26, 51 and 33% respectively. The effect of GABA at 10 and 100 uM was dose-related but some desensitization of response was evident at higher concentration of GABA (1000uM). GABA at a concentration of 10 and 100 uM also caused a respective 16 and 45% reduction of nicotine (50uM)-induced CA release. GABA alone at a concentration of 100 uM did not stimulate the secretion of CA. Bicuculline (5uM), a $GABA_A$ antagonist, reversed the effect of GABA (100uM) on nicotine-evoked catecholamine release.

Using patch clamp technique, Cottrell et al. (1985) and Lambert & Peters (1985) showed that chromaffin cells (86%) responded to locally applied GABA (100uM) by increasing chloride currents associated with conductance increase. GABA-activated whole cell currents were reversibly antagonized by the $GABA_A$ receptor antagonists, picrotoxin (3uM) and bicuculline (3uM). Bormann and Clapham (1985) also demonstrated the GABA (10uM)-activated chloride currents in bovine chromaffin cell membrane. Bicuculline (5uM) reduced the response of GABA to 22% and 25uM bicuculline almost completely blocked the GABA-activated current. These electrophysiological findings correlate well with the observations on the effect of GABA on

agonist-evoked catecholamine release.

On the other hand, Kataoka et al. (1984) demonstrated that muscimol (1nM-1uM), a $GABA_A$ agonist, partially reduced the ACh-induced CA release, but they could not reproduce this inhibition consistently. Costa et al. (1984) also obtained some variable results with muscimol where they detected some activity only at very high concentrations; moreover, this activity was not dose-related. In this investigation, the inhibitory effect of GABA (100uM) on nicotine-evoked catecholamine secretion varied from 18 to 45% in different experiments. This variability might reflect occupancy of GABA recognition sites by endogenous GABA (Kataoka et al., 1984). GABA receptor stimulation has also been reported to modify the spontaneous secretion of opioid peptides (Costa et al., 1984). The concomitant release of opioid peptides might complicate the effect of GABA on secretory response.

4.3.2 **Intrinsic GABA Activity in Chromaffin Cells**

Kataoka et al. (1984) in a recent report suggested the presence of endogenous GABA in cultured chromaffin cells. In the present investigation, bicuculline (2uM) facilitated (by 38%) the CA release stimulated by 50uM nicotine. However, the same concentration of bicuculline did not increase the secretion evoked by 5uM nicotine. Bicuculline (2uM) itself did not change the background level of CA secretion. Kataoka et al. (1984) showed the facilitation of nicotine-induced release of CA by bicuculline, where the dose-response curve of CA release

elicited by various doses of nicotine was shifted to the left by 1uM bicuculline. Bicuculline itself failed to change the spontaneous release of CA. In the present study, the facilitation of CA release by bicuculline could only be demonstrated when the secretion was stimulated with high concentration of nicotine, but this effect of bicuculline at various doses of nicotine could not be reproduced consistently. This variability might reflect the variation in endogenous GABA content from cell to cell and condition of cells in culture. Lambert & Peters (1985) found that only 86% of their isolated chromaffin cells responded to GABA.

4.3.3 Possible Physiological Role of GABA in Chromaffin Cell Function

It is thought that under basal conditions, i.e. resting conditions, there is some spontaneous release of ACh from the splanchnic nerve terminals. Fenwick et al. (1982) showed that chromaffin cells in culture possess a relatively high input resistance and the opening of a single ACh-activated ion channel may initiate the firing of an action potential. If chromaffin cells also possess a high input resistance in vivo and there is spontaneous release of ACh from nerve terminals, there must be some homeostatic mechanism for inhibiting the basal release of catecholamines. Kataoka et al. (1984) demonstrated that bicuculline increases the nicotine-evoked CA release. This investigation also showed that bicuculline facilitated the nicotine-evoked CA release. All these findings imply that

nicotinic receptor activation might stimulate the release of endogenous GABA from chromaffin cells. This conclusion is consistent with the finding that nicotinic receptor stimulation or K depolarization releases the [^{3}H]-GABA taken up by these cells (Kataoka et al., 1984). The presence of endogenous GABA is also indicated by the detection of glutamic acid decarboxylase (GAD) immunoreactivity in cultured bovine chromaffin cells (Fujimoto, Hanbauer, Alho & Guidotti, 1985). However, the source of GABA might also be the terminal bits of GABAergic nerve fibres supplying chromaffin cells, since histochemical evidence indicates that the adrenal medullae of several mammalian species contain GABAergic nerve fibres (Fujimoto et al., 1985). GABA is the major inhibitory neurotransmitter in the central nervous system. Adrenal medullary chromaffin cells are modified sympathetic neurones because they share many properties in common with neurones (Fenwick et al., 1982). This study demonstrated that GABA exerts an inhibitory effect on nicotinic receptor mediated catecholamine release and the effect is reversed by $GABA_A$ antagonist, bicuculline. Electrophysiological experiments (Cottrell et al., 1985; Bormann & Clapham, 1985) also suggest that GABA-activated Cl^- currents conduct currents in the opposite direction to ACh receptor channels and are therefore inhibitory. All these findings suggest that GABA probably acts as an inhibitory neurotransmitter in adrenal chromaffin cells as in central neurones. The presence of endogenous GABA suggests a receptor-receptor interaction between GABA and ACh (Kataoka et

al., 1984). It might be assumed that nicotinic receptor stimulation releases endogenous GABA from chromaffin cells in vivo which would tend to oppose the ACh-depolarization and cause an overall reduction in catecholamine release (Fig. 26).

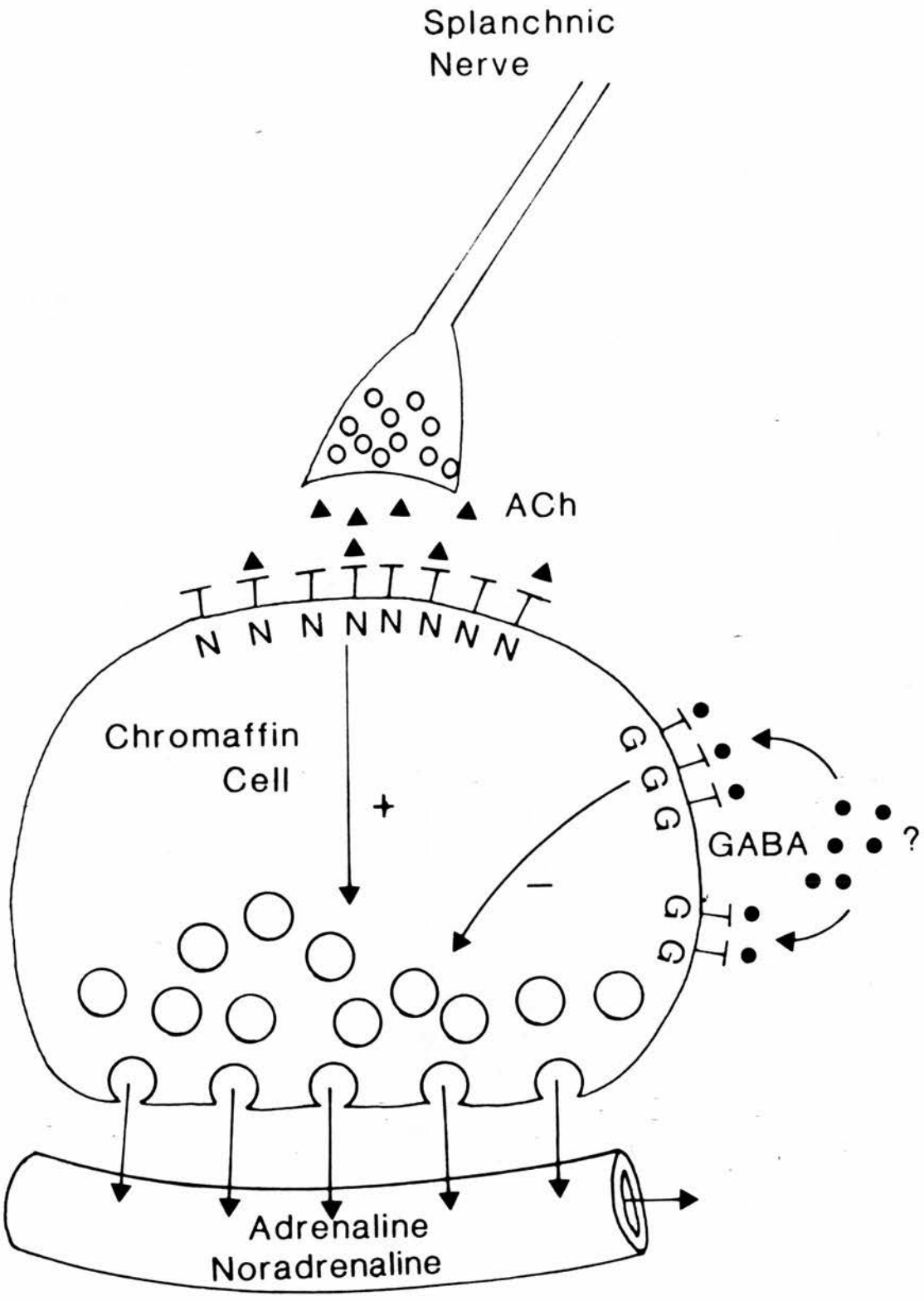
In contrast to these studies, Sangiah, Borowitz & Yim (1974) demonstrated that GABA releases catecholamines from isolated perfused bovine adrenal glands and that this effect is blocked by bicuculline. GABA has also been reported to stimulate CA release from isolated perfused dog adrenals in a dose-dependent manner (Kitayama, Morita, Dohi & Tsujimoto, 1984). It is possible that in the intact gland GABA evokes CA release by interacting with its receptor located on splanchnic nerve terminals. GABA might exert its effect by stimulating ACh release (Kitayama et al., 1984).

Although the physiological role of GABA in chromaffin cells is yet to be evaluated, it can be concluded that GABA might play some role in modulating the catecholamine secretion from those cells.

4.4 Diazepam, an Inhibitory Modulator of Catecholamine Secretion

In the present study, diazepam (10uM) alone reduced the nicotine-evoked catecholamine secretion by 40%. GABA (10uM) caused a decrease of 21% and both the drugs together reduced the secretion by 72%. GABA at a concentration of 100uM decreased the secretory response by 39% while GABA (100uM) and diazepam (10uM) both reduced the release by 64%. Both GABA and diazepam

Fig. 26. Possible functional significance of GABA at its receptor site in bovine medullary chromaffin cells. ACh released from splanchnic nerve terminals acts on nicotinic (N) receptors. The nicotinic receptor stimulation causes release of catecholamines and possibly GABA (Kataoka et al., 1984). The GABA receptor (G) activation may exert an inhibitory effect causing an overall reduction of catecholamine release.


Splanchnic
Nerve
ACh
N N N N N N N
Chromaffin
Cell
+
–
G G G G G
GABA
?
Adrenaline
Noradrenaline

significantly inhibited the effect of nicotine. The effect of diazepam was stronger than GABA. Both drugs exerted an additive effect when used together, and there was no interaction between the effects of GABA and diazepam. Diazepam at a concentration of 1uM did not have any effect on secretion.

Electrophysiological experiments demonstrated that diazepam (DZ) at a concentration of 1uM enhanced the GABA-induced current on chromaffin cells and the potentiation produced by 1uM diazepam was abolished by the benzodiazepine receptor antagonist RO 15-1788 (1uM) (Cottrell et al., 1985). Bormann & Clapham (1985) also reported that GABA (10uM)-activated currents were strongly potentiated by diazepam (10uM). Diazepam alone (0.1-1uM) had no direct effect on the whole cell (Cottrell et al., 1985).

Costa et al. (1984) found that the addition of muscimol to a membrane suspension of chromaffin cells increases the affinity of benzodiazepine (BZD) recognition sites for [^{3}H]-flunitrazepam, whereas the addition of bicuculline decreases the affinity. They concluded that GABA-BZD recognition sites are linked. In their study, to determine whether GABA and BZD can change the secretion of CA elicited by ACh, they obtained some variable results.

The GABA receptor complex found in the vertebrate central nervous system includes a benzodiazepine (BZD) binding sub-unit (Olsen, 1982). BZD have been reported to potentiate the GABA-gated Cl^- currents in spinal neurones (Study & Barker, 1981; Macdonald & Barker, 1978; Macdonald & Barker, 1979; Choi

et al., 1977). On the other hand, Macdonald & Barker (1978) obtained a direct effect of BZD on Cl^- current, consisting of depolarization and increase in conductance. Suria & Costa (1975) demonstrated that the addition of diazepam (1uM) evoked a depolarization of preganglionic axon terminals similar to the action of GABA and this effect was antagonized by picrotoxin (10uM).

This study shows that diazepam has a specific inhibitory effect on agonist-evoked catecholamine secretion but no potentiating effect of GABA. These findings, however, are not consistent with the observations of Cottrell et al., 1985; Bormann & Clapham, 1985; Study & Barker, 1981. The direct effects of BZD reported by Macdonald & Barker (1978) were observed at BDZ currents higher than those required for GABA response augmentation. Diazepam at a concentration of 100 uM has been shown to suppress ACh-currents in bovine chromaffin cells (J. Peters, personal communication). Thus, if diazepam augments GABA response at low concentrations, the direct effect of it may be relevant only at toxic or non-therapeutic doses (Macdonald & Barker, 1978).

The possibility that chromaffin cells might possess specific BZD receptor sites, not associated with GABA receptor, is also likely. Evidence shows that rat brain possesses specific receptors for BZD which mediate their pharmacological actions (Squires & Braestrup, 1977). Mohler & Okada (1977) also demonstrated a specific [^{3}H]-diazepam binding site at the rat cerebral cortex. They showed that GABA agonists and

antagonists failed to influence diazepam binding and benzodiazepines failed to influence GABA receptor binding. The other possibility is that the action of diazepam is mediated through release of endogenous GABA. It has been reported that picrotoxin which inhibits the depolarization induced by GABA, antagonizes the depolarization elicited by diazepam (Suria & Costa, 1975). This suggestion is further supported by the presence of an endogenous pool of GABA in cultured chromaffin cells (Kataoka et al., 1984).

The exact mechanisms by which benzodiazepines exert their characteristic pharmacological and clinical effects are yet to be evaluated, but it can be speculated that the regulation of anxiety states by benzodiazepines may not be restricted to central GABAergic system, they might have some peripheral component of action. The modulation of adrenal medullary secretory function by benzodiazepines may be useful in the treatment of pheochromocytoma, an excessive catecholamine secreting adrenal medullary tumour.

4.5 Modulation of Catecholamine Secretion by Alphaxalone

Alphaxalone, a short acting intravenous steroid anaesthetic, has recently been reported to modulate the interaction between GABA and its receptor in isolated bovine chromaffin cells (Cottrell et al., 1986). In the present work, alphaxalone at a concentration of 0.1 and 0.3 uM enhanced the effect of GABA on nicotine-evoked catecholamine secretion by 13 and 25% respectively. Alphaxalone (0.3uM) itself did not

influence the secretion, but the drug alone at a higher concentration (10uM) reduced the secretion by 55%. The inhibitory effect of alphaxalone (10uM) was not reversed by bicuculline (4uM). Cottrell et al. (1986) demonstrated that alphaxalone at 30 and 100 nM in the medium potentiated the whole cell currents evoked by GABA (100uM). At higher concentrations (1-100uM), in addition to a transient potentiating effect, alphaxalone itself evoked a dose-dependent inward current, which was blocked by bicuculline (3uM). In the rat cuneate nucleus, alphaxalone (0.1 - 1 uM) potentiated depolarizing responses to GABA and muscimol (Harrison & Simmonds, 1984). Scholfield (1980) showed that alphaxalone (0.2-50 uM) prolongs the duration of inhibitory postsynaptic conductance (i.p.s.c.) in guinea pig olfactory cortex.

Since alphaxalone at potentiating concentrations is not an agonist, it is probably not acting at the GABA binding site. The potentiating effect of alphaxalone on GABA response is probably mediated by enhancement of interaction between GABA and its receptor. This conclusion is consistent with the recent finding that alphaxalone enhanced [^{3}H]-muscimol binding to rat brain membrane (Harrison & Simmonds, 1984). However, the effect of alphaxalone alone at high concentration (10uM) might be independent of GABA receptor activation because this effect was not reversed by bicuculline. Therefore, this finding does not agree with the observation of Cottrell et al. (1986). At higher concentrations, alphaxalone might suppress the ACh-current in the cell membrane. Smaje (1976) reported that

alphaxalone (50uM) reversibly depressed the ACh-sensitivity of prepiriform neurones. Alphaxalone, at higher concentrations (3-100uM), produced a reversible blockade of ACh-evoked inward current (ED_{50} = 6uM) in cultured myoballs (Gillo & Lass, 1984). At a concentration of 10uM or more, e.p.s.p. in olfactory cortex of guinea pig was depressed by alphaxalone (Scholfield, 1980). Alphaxalone may block the ACh channel by allosteric interaction from adjacent hydrophobic sites at the receptor-channel complex or its micro-environment (Gillo & Lass, 1984).

4.6 **Conclusion**

In this investigation, nicotinic receptor activation in cultured bovine adrenal chromaffin cells stimulates catecholamine secretion, while stimulation of muscarinic receptors does not cause any release. Co-stimulation of muscarinic receptors exerts an inhibitory effect on agonist-induced secretion. GABA receptor activation decreases the agonist-stimulated CA release. Diazepam and alphaxalone decrease the secretion of catecholamines.

To conclude, though the physiological role of GABA in adrenal medullary function is yet to be evaluated, it can be speculated that GABA could be responsible for the functional alternation of chromaffin cells. Activation of chromaffin cell GABA receptors might account for the peripheral component of mechanism of action of drugs like alphaxalone.

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