# STIMULUS-SECRETION COUPLING IN GLAND CELLS

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I declare that this thesis has been composed by myself, and that the work contained in it is my own.

12/1/84

CONTENTS



NOTE: This thesis was prepared using WordStar word-processing facilities and printed on a Diablo 1620 printer. The type used has no Greek symbols, therefore the symbol u has been used throughout to represent "micro-", for example uM = micromolar; um = micrometre (micron); ug = microgram. Alpha, beta and gamma have been written out in full or the symbols inserted manually as these occur less frequently in the text.

ABSTRACT

The work described in this thesis is divided into three main sections. The first section concerns the role of calcium in the control of fluid secretion. When cockroach salivary glands are bathed in calcium-free medium the basal rate of fluid secretion increases from about 1nl/min to about 10nl/min; maintained dopamine stimulation elicits a further rise in secretory rate which gradually declines. Evidence is presented which suggests that magnesium is unable to substitute for calcium in this system. When calcium is returned to the bathing solution after a period of calcium deprivation there is a transient increase in secretory rate. The calcium ionophore A23187 also elicits an increase in secretory rate which is maintained in the presence of extracellular calcium but declines in calcium-free medium. Stimulation of the glands in certain conditions which inhibit the secretory response leads to some kind of calcium-dependent active state in the secretory cells which can outlast the interaction of the agonist with its receptors. The conclusion drawn from this section of the work is that stimulus-secretion coupling in this gland involves a calcium-dependent second messenger system.

The second section is concerned with investigating the existence and possible location of a cellular store of calcium involved in stimulus-secretion coupling. The fluorescent compound chlorotetracycline (CTC) enters the cells of the cockroach salivary gland. The acinar peripheral cells and non-secretory duct

cells become preferentially labelled by CTC. Microscopic examination of the intracellular distribution of CTC indicates that this compound labels the highly folded apical plasma membranes of the peripheral cells and the deep infolds of the basal membranes of the non-secretory duct cells. Lanthanum blocks the entry of CTC into all of the gland cells and in this condition the CTC labels the basal surfaces of the acini and ducts. The results of this investigation support the idea that CTC labels calcium ions in the vicinity of plasma membranes. A comparison was made between glands in which the calcium stores had been depleted by dopamine stimulation in calcium-free medium, prior to CTC labelling, and glands which had not been stimulated prior to labelling. No apparent reduction in the labelling of stimulated glands compared to unstimulated glands was noted.

The third section of this work is concerned with the role of cyclic AMP in the control of fluid secretion. Exposure of the glands to exogenous cyclic AMP causes an increase in fluid secretion; maximal responses to cyclic AMP virtually match the maximal responses of the same glands to dopamine. The secretory response to maintained exogenous cyclic AMP declines in calcium-free medium. The calcium-dependence of the active state which arises in the cells during stimulation in conditions preventing fluid secretion cannot be bypassed by application of exogenous cyclic AMP. Forskolin, which reputedly stimulates adenyl cyclase, does not consistently stimulate fluid secretion. The adenyl cyclase inhibitor MDL 12,330A blocks the secretory responses to dopamine, calcium readmission and A23187. Application of the phosphodiesterase inhibitor IBMX causes an increase in fluid

secretion,which declines in calcium-free medium and is blocked by MDL 12,330A. It is concluded from this section that an increase in intracellular cyclic AMP is an important step in stimulus-secretion coupling in the cockroach salivary gland. Preliminary experiments to determine intracellular cyclic AMP concentration using radio-immunoassay have been carried out.

In conclusion a model is presented of stimulus-secretion coupling in the cockroach salivary gland, in which calcium and cyclic AMP act as synarchic second messengers regulating fluid secretion.
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# CHAPTER ONE

# INTRODUCTION

The second messenger concept first proposed by Sutherland, Øye and Butcher (1965) arose from observations that when many different types of cell are activated by specific extracellular stimuli, the intracellular concentration of adenosine 3',5'-cyclic monophosphate (cyclic AMP) increases (Sutherland & Rall, 1960; Sutherland & Robison, 1966). They proposed that a specific first or extracellular messenger activates an adenylate cyclase in the plasma membrane of the target cell. The resultant increase in cyclic AMP was considered to act as a second messenger translating the extracellular message to an intracellular response, by the activation of protein kinases.

Cyclic AMP was discovered in the course of investigations into the mechanisms of the hyperglycaemic actions of adrenaline and glucagon, and was first reported by Rall, Sutherland and Berthet (1957). It was found to be formed by a particulate fraction of liver homogenate in the presence of adenosine triphosphate (ATP), magnesium ions and either adrenaline or glucagon. Subsequently these hormones were found to stimulate liver glycogenolysis by increasing the rate of formation of cyclic AMP, which in turn increases the rate of formation of active phosphorylase from inactive phosphorylase, presumably by an action on phosphorylase kinase (Riley & Haynes, 1963).

Cyclic AMP is now known or suspected to be involved not only in hepatic glycogenolysis but also in a number of other tissue functions. (Table 1:1).

Whereas many hormones appear to act by increasing the intracellular concentration of cyclic AMP, others may exert at least some of their influence by decreasing cyclic AMP levels. For

Table 1:1 Examples of systems in which cyclic AMP is believed to act as a second messenger.

| Tissue | Response | Stimulus | Reference |
|---|---|---|---|
| Cockroach salivary gland | Fluid secretion | dopamine | Grewe & Kebabian, 1982 |
| Blowfly salivary gland | Fluid secretion | 5-HT | Berridge & Patel, 1968<br>Berridge, 1980a |
| | Chloride transport | | Berridge 1980b |
| Tick salivary gland | Fluid secretion | dopamine | Kaufman, 1976<br>Needham & Sauer, 1979 |
| Parotid gland | Amylase secretion | noradren.<br>adrenaline | Babad et.al., 1967<br>Carlsöö et.al., 1982 |
| Submandibular gland | Mucin secretion | noradren.<br>isoproterenol | Quissel et.al., 1981 |
| Jejunum | Fluid transport | VIP | Beubler, 1980 |
| Gastric mucosa | Acid secretion | histamine | Rutten & Machen, 1981 |
| Kidney | Antidiuresis | vasopressin | Grantham & Burg, 1966<br>Katayama et.al., 1980 |
| Toad bladder | Antidiuresis | vasopressin | Orloff & Handler, 1962 |
| Endocrine pancreas | Insulin release | glucose | Grill, 1980<br>Flatt et.al., 1980 |
| | somatostatin release | glucose | Gerber et.al., 1981 |
| Corpora lutea | Steroidogenesis | lutenizing hormone | Ahrén et.al., 1981<br>Ling et.al., 1980 |
| Adrenal cortex | Steroidogenesis | ACTH | Haynes et.al., 1960 |

Table 1:1 continued

| Tissue | Response | Stimulus | Reference |
|---|---|---|---|
| Neurohypophysis | Vasopressin release | | Dartt et.al., 1981 |
| Cardiac muscle | Contraction | adrenergic stimulation | Epstein et.al., 1971<br>Au et.al., 1980 |
| Tracheal smooth muscle | Contraction/ relaxation | histamine | Creese et.al., 1980<br>Duncan et.al., 1980 |
| Skeletal muscle | Phosphorylase b kinase activation | | Posner et.al., 1965 |
| Liver | Glycogenolysis | adrenaline glucagon | Rall et.al., 1957 |
| Adipose tissue | Lipolysis | adrenaline | Butcher et.al., 1965<br>Arner & Ostman, 1980 |
| | Antilipolysis | insulin | Wong & Loten, 1981 |
| Frog skin | Melanin dispersal | melanocyte-stim.hormone | Bitensky & Burstein, 1965 |
| Dictyostelium (slime mould) | Aggregation/ differentiation | | Juliani et.al., 1981 |

example, the effects of insulin on carbohydrate metabolism and potassium uptake, as well as its antilipolytic effect, may be due to lowering cyclic AMP in the target cells (Exton, Jefferson, Butcher & Park, 1966).

The original hypothesis that cyclic AMP is the sole mediator of the actions of most hormones began to be seen as an oversimplification, as evidence accumulated that other substances, particularly calcium, might be acting as second messengers.

The work of Katz and Miledi on the neuromuscular junction is probably the most compelling evidence of the role of calcium as an intracellular second messenger. These authors demonstrated that increasing calcium ion concentration in the immediate vicinity of the neuromuscular junction by iontophoresis facilitates transmitter release if it occurs immediately before the depolarising pulse reaches the nerve ending, but is ineffective if given after this time (Katz & Miledi, 1967). They concluded that the utilization of calcium ions at the neuromuscular junction is restricted to a brief period which barely outlasts the depolarisation of the nerve ending, and precedes transmitter release.

The diversity of systems in which calcium mediates as a second messenger is illustrated by the examples in table 1:2.

The mechanism of action of calcium has not yet been fully understood in all of the systems listed in table 1:2, but in many it appears to depend on intracellular calcium-binding proteins. Examples of these are troponin in muscle (Ebashi & Endo, 1968; Weber & Murray, 1973) and calmodulin in many other cells (Kretsinger, 1976). For an interesting account of the discovery and function of calmodulin see Cheung (1981).

Table 1:2 Examples of systems in which calcium has been implicated as a second messenger.

| Tissue | Response | Stimulus | Reference |
|---|---|---|---|
| Cockroach salivary | Fluid secretion | dopamine | Gray & House, 1982 |
| gland | Hyperpolarisation | | Ginsborg et.al., 1980a |
| Blowfly salivary | Fluid secretion | 5-HT | Berridge & Prince, 1975 |
| gland | | | Berridge, 1979 |
| Tick salivary | Fluid secretion | cyclic AMP | Needham & Sauer, 1979 |
| | | catecholamines | |
| Exocrine pancreas | Amylase release | cholinergic | Petersen & Veda, 1976 |
| | | A23187 | Poulsen & Williams, 1977 |
| | Depolarisation | acetylcholine | Iwatsuki & Petersen 1977b |
| Parotid gland | Amylase release | adrenergic<br>cholinergic | Leslie, et.al. 1976<br>Petersen et.al., 1977 |
| Submandibular | Mucin release | noradrenaline | Quissel et.al., 1981 |
| gland | | isoproterenol | |
| Submaxilliary | Salivary | acetylcholine | Douglas & Poisner, 1963 |
| gland | secretion | noradrenaline | |
| Platelets | ATP release | collagen | Nishikawa et.al., 1980 |
| | 5-HT release | thrombin | |
| | myosin | adrenaline | |
| | -phosphorylation | | |
| Erythrocytes | (secretion) | adrenaline | Nelson & Huestis, 1980 |
| Intestinal mucosa | Ion & water | Ionophore | Bolton & Field, 1977 |
| | secretion | | Hubel & Callanan, 1980 |
| Mollusc pedal | Secretion | nerve stim. | Kater, 1977 |
| gland | | | |
| Squid giant | Transmitter | nerve stim. | Llinas et.al., 1976 |
| | | | Miledi & Parker, 1981 |

Table 1:2 continued

| Tissue | Response | Stimulus | Reference |
|---|---|---|---|
| Neuromuscular junction | Transmitter release | nerve stim. | Katz & Miledi, 1965;1967; 1969 |
| Atrium | Transmitter release | noradrenaline phenoxybenzamine | Kalsner, 1981 |
| Adrenal | Adrenaline & noradren. release | acetylcholine | Douglas & Rubin, 1963 |
| | Aldosterine release | angiotensin II ACTH, $K^+$ ouabain | Schiffrin et.al., 1981 |
| Neurohypophysis | Vasopressin release | | Dartt et.al., 1981 |
| Endocrine pancreas | Insulin release | glucose | Anjaneyulu et.al., 1980<br>Flatt et.al., 1980<br>Wollheim & Sharp, 1981 |
| Mast cells | Histamine release | | Foreman et. al., 1976 |
| Cardiac muscle | Contraction | adrenaline | Ozaki & Urakawa, 1979 |
| Smooth muscle | Contraction | catecholamines | Bülbring & Tomita, 1977<br>Van Meel et.al., 1981 |
| Skeletal muscle | Contraction | nerve stim. | Ebashi & Endo, 1968 |
| Melanocytes | Melanin dispersal | melanocyte stim.hormone | Dikstein, et.al., 1963 |

Table 1:2 continued

| Tissue | Response | Stimulus | Reference |
|---|---|---|---|
| Toad bladder | Na transport | vasopressin | Orloff & Handler, 1962 |
| | | incr.lumenal Na | Chase & Al-Awqati, 1981 |
| Photoreceptors | Decr. sodium conductance | light | Yoshikami et.al., 1980 |
| Spermatozoa | Acrosomal hyaluronidase release | initiation of capacitation | Triana et.al., 1980 |
| Oocytes | Parthenogenetic activation | temperature | Whittingham & Siracusa, 1978 |
| | | injected Ca | Fulton & Whittingham, 1978 |

It has been proposed (Kretsinger, 1977) that the sole function of calcium within the cytosol is to transmit information, which is achieved by maintaining low cytosolic calcium levels in the resting cell, and translating stimulation into an increased intracellular concentration of free calcium, which has a protein in the cytosol as its target.

In 1970 Rasmussen published one of the earliest models describing ways in which calcium and cyclic AMP may interact as second messengers within the same cell (Rasmussen, 1970). One of the best known examples of a system of this kind is the blowfly salivary gland, which has been extensively investigated by Berridge and his co-workers since 1968, when the first paper in a long and continuing series on this preparation was published. They reported that the gland secretes fluid in response to stimulation by both 5-hydroxytryptamine (5-HT) and cyclic AMP (Berridge & Patel, 1968; Berridge, 1970). However it was subsequently shown that these compounds have opposite effects on transepithelial potential (Berridge & Prince, 1971; 1972a). They then suggested that 5-HT has two effects on the blowfly salivary gland, one increasing chloride movements, and the other stimulating a potassium pump. The effect on the potassium pump was shown to be mediated by cyclic AMP (Prince, Berridge & Rasmussen, 1972) and that on chloride movement by calcium (Prince & Berridge, 1973). Berridge and Prince presented a model of hormone action in the control of fluid secretion involving calcium and cyclic AMP as second messengers (Berridge & Prince 1972b). This model has been updated (Prince & Berridge, 1973; Berridge & Lipke, 1979; Berridge & Heslop, 1981) and there are now known to be pharmacologically

separable 5-HT receptors linked to the generation of either cyclic AMP or calcium signals. Feedback mechanisms are believed to operate between the two second messengers whereby cyclic AMP may liberate calcium from intracellular stores (Berridge & Lipke, 1979), and calcium may regulate intracellular cyclic AMP levels, having the ability to activate both synthesis and degradation enzymes (Berridge, 1975; Wolff & Bronstrom, 1979; Heslop & Berridge, 1980).

In the pancreas intracellular injections of calcium mimic the electrophysiological effects of acetylcholine, causing membrane depolarisation, reduction of input resistance and uncoupling of cells. Prolonged iontophoretic application of acetylcholine also caused uncoup ling of the acinar cells (Iwatsuki & Petersen, 1977b). Uncoupling may be caused by an increase in free calcium concentration inside the cells (Rose & Loewenstein, 1975). These two observations strongly suggest that in the pancreas one of the principal actions of acetylcholine is on intracellular calcium ion concentration. Iwatsuki and Petersen (1978b) suggest that in the initial phase of acetylcholine-evoked increase in calcium concentration calcium seems to be coming from the plasma membrane, and in the second, sustained, phase calcium may be entering from the bathing medium.

The cockroach salivary gland was chosen as a preparation in which to study stimulus-secretion coupling for a number of reasons. Much of the basic information necessary for the project was already available (Ginsborg & House, 1980; House, 1980). Also the isolated cockroach salivary gland preparation developed by Smith & House (1977) is one where the relative ease and reliability with which a response can be elicited and measured,

combined with the accessibility of the tissue and its suitability for many different experimental approaches, permit practically unlimited manipulations of experimental conditions in order to gather information about the mechanisms involved in stimulus-secretion coupling.

When this work was started, in 1980, it was believed that calcium might be a second messenger in fluid secretion from the cockroach salivary gland (Ginsborg, House & Mitchell, 1980a;b; Mitchell & Martin, 1980) but that cyclic AMP was probably not involved (House, 1980). However, since then evidence has begun to accumulate which suggests that cyclic AMP may in fact also be a second messenger in this system, indicating that the mechanisms of fluid secretion from the blowfly and cockroach salivary glands may have some features in common.

# CHAPTER 2

# THE ROLE OF CALCIUM IN FLUID SECRETION FROM THE COCKROACH SALIVARY GLAND

# INTRODUCTION

Both calcium ions and cyclic AMP have been proposed as 'second messengers' in secretory tissues as diverse as the blowfly salivary gland (Berridge, 1979) and mammalian parotid gland (Leslie, Putney & Sherman, 1976). How these second messengers control the rate of secretion of enzymes, ions and water is not thoroughly understood. However there is evidence from pancreatic acinar cells (Petersen & Iwatsuki, 1978) and nerve cells (Meech, 1972) that calcium mediates changes in the permeability of cell membranes to ions. In the blowfly salivary gland calcium probably controls the chloride permeability of the secretory cells and the rate of active potassium transport into the saliva (Prince et.al., 1972). A calcium dependent rise in the potassium permeability of cockroach salivary gland acinar cells has also been reported (Ginsborg, et.al. 1980a,b).

Observations of declining responses to stimulation in calcium-free media have been made in a number of tissues, e.g. cockroach salivary gland (Ginsborg et al., 1980a), blowfly salivary gland (Prince & Berridge, 1973), rat parotid gland (Petersen & Pedersen, 1974; Rudich & Butcher, 1976; Petersen, Ueda, Hall & Gray, 1977) and exocrine pancreas (Argent, Case & Scratcherd, 1973; Petersen & Ueda, 1976; Kanno & Yamamoto, 1977; Iwatsuki & Petersen, 1977a and Laugier, 1979). These have led to the proposal of intracellular calcium-dependent stores of some substance (possibly calcium itself) essential for the response to stimulation.

Certain characteristics of this calcium-dependent store in the cells of the cockroach salivary gland have been examined by

recording the secretory response of the isolated perfused preparation developed by Smith & House (1977; 1979), and the results are presented in this chapter.

The experiments described in this chapter also investigate whether dopamine stimulation under conditions preventing fluid secretion can produce some kind of active state in the secretory cells which outlives the interaction of the agonist with its receptors. The calcium-dependence of this state was also investigated. The conclusion of this chapter is that stimulus-secretion coupling in this gland involves a second messenger system which is calcium-dependent.

## METHODS

### Preparation

The paired salivary apparatus was dissected from adult cockroaches and mounted in a channel in a Perspex slab, through which varying solutions were perfused (Smith & House, 1977). One of the salivary ducts was ligated near its end, passed through a small hole in the side of the channel into a pool of liquid paraffin, and anchored by the ligature. A puncture was made in the duct posterior to the ligature to allow the secreted fluid to escape.Figure 2:1 shows the preparation set up in this way, and photographed at regular intervals after addition of dopamine to the bathing solution.

### Volume of secretion

The volume of secretion was measured by taking up the droplet of saliva from the duct with a micropipette and transferring it to another pool of liquid paraffin, where it assumed a spherical shape. The diameter could then be measured and the volume calculated.

### Composition of bathing fluids

The control solution contained (mM); NaCl, 160; KCl, 10; HCl, 4; $CaCl_2$, 5; Tris(hydroxymethyl)aminomethane, 5 and glucose, 20. For the calcium-free EGTA solution, $CaCl_2$ was replaced by 1mM EGTA. The free calcium concentration of such a solution has been estimated to be less than $10^{-9}$M (Hubbard, Jones & Landau, 1968). For brevity this will be referred to as calcium-free solution

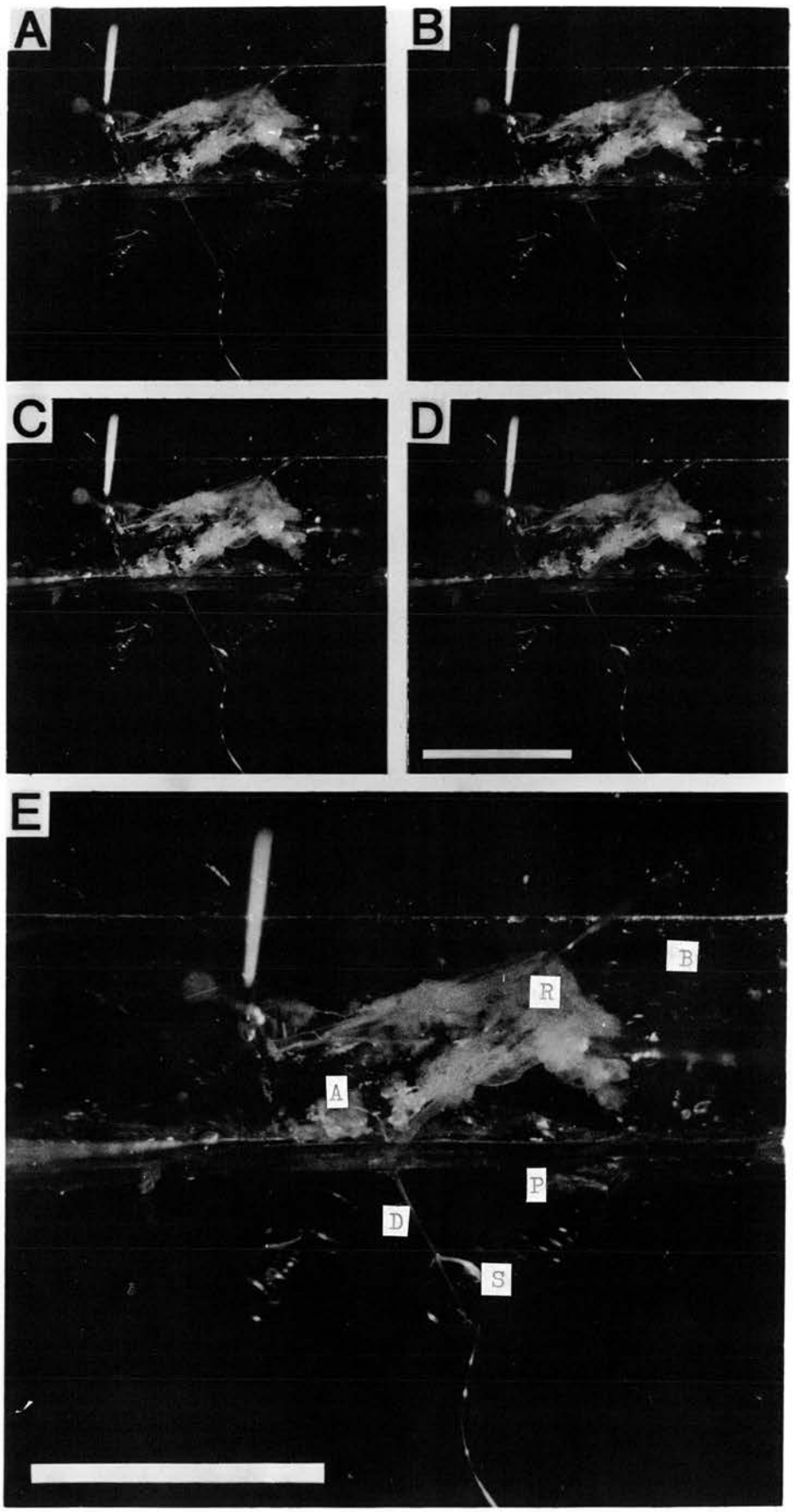
Figure 2:1

The preparation, mounted as described in 'Methods', and photographed at regular intervals after the addition of dopamine (1uM) to the bathing solution.

A = 2 min, B = 4 min, C = 6 min, D = 8 min, E = 10 min.

B = bathing solution
A = acini
R = reservoir
P = liquid paraffin
D = duct
S = saliva

The scale bars in D and E both represent 5mm


A
B
C
D
E
B
R
A
P
D
S

throughout.

For the calcium-free magnesium solution, $CaCl_2$ was replaced by 5mM MgCl.

For the sodium-free solution, NaCl was replaced by 160mM Choline chloride (repurified before use).

The composition of the chloride-free solution was (mM): sodium methylsulphate, 160; $K_2SO_4$, 5; $CaSO_4$, 1; Tris., 5; $H_2SO_4$, 2; glucose, 20, with 1mM EGTA replacing the $Ca.SO_4$ in the case of Ca-,Cl-free solution.

The pH of all solutions used was in the range 7.3 - 7.7.

The calcium ionophore A23187 was obtained from Calbiochem-Behring Corporation. A stock solution of 1mg/ml methanol was prepared, and then diluted to give the required final concentration (5-10ug/ml) in the bathing solution, as needed.

Protocol

At both the beginning and end of each experiment the gland was exposed to a test dose of 1uM dopamine in control solution. If the final response was more than 10-15% smaller than the first it was assumed that the preparation had in some way been damaged during the experiment and the results were discarded. Variations in the protocol according to the aims of each experiment are detailed in the Results.

Except where stated otherwise, all experiments were repeated at least three times. If some variation in the responses was noted this number was increased, to five or seven.

# RESULTS

## Calcium store

The effects of calcium-free medium on the secretory response of the isolated cockroach salivary gland are shown in figure 2:2. The important feature of the tissue's response to the withdrawal of extracellular calcium is that the secretory response to maintained dopamine stimulation gradually declines (see section headed "Decline"). It was also noted that the basal or unstimulated secretory rate increases slightly (see "Basal Rate") in the absence of extracellular calcium ions. When calcium is returned to the bathing fluid there is a transient secretory response in the absence of dopamine stimulation (see "Readmission Response").

After a short recovery period in control solution the gland is capable of giving a response as large as that obtained in control solution prior to calcium-free treatment; therefore the effect of calcium-deficient medium is reversible and cannot be attributed to permanent damage to the tissue (Figure 2:2).

## Decline

Figure 2:3 shows that a declining response occurs when the preparation is stimulated after exposure to calcium-free solution has started, and that the decline occurs whether the stimulation is prolonged or given in ten-minute pulses. Other experiments of this type have been done and have shown that the order of presentation of pulsed and prolonged stimulation is not important. The declining response to dopamine is consistent with the idea

Figure 2:2

Effect of calcium-free medium on the secretory response to dopamine. The periods during which calcium (5mM) and dopamine (1uM) were present in the bathing medium are indicated by horizontal bars labelled Ca and DA respectively.

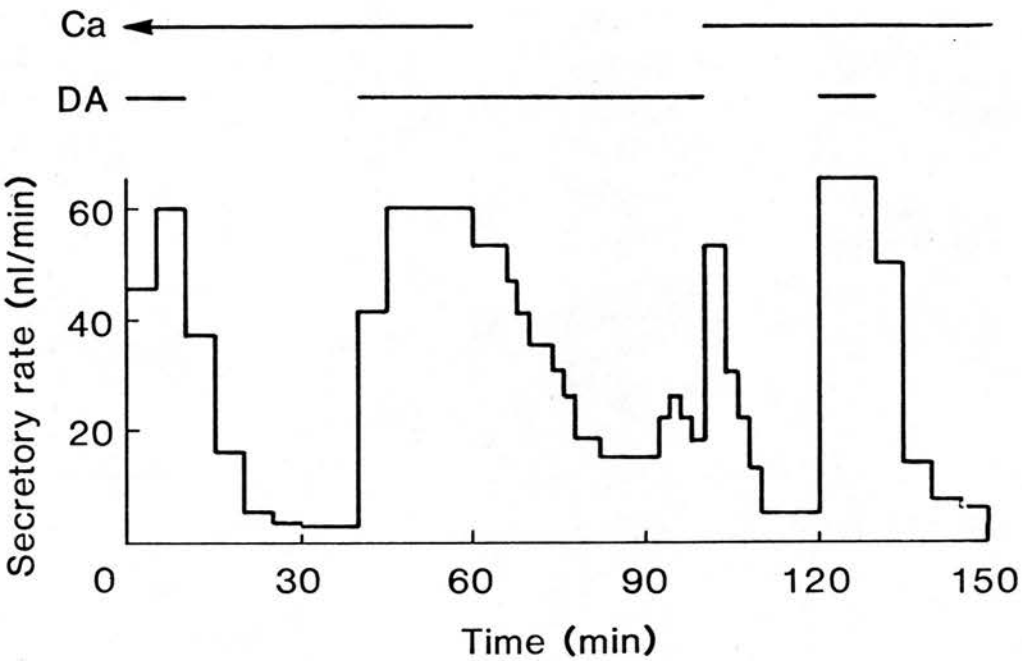
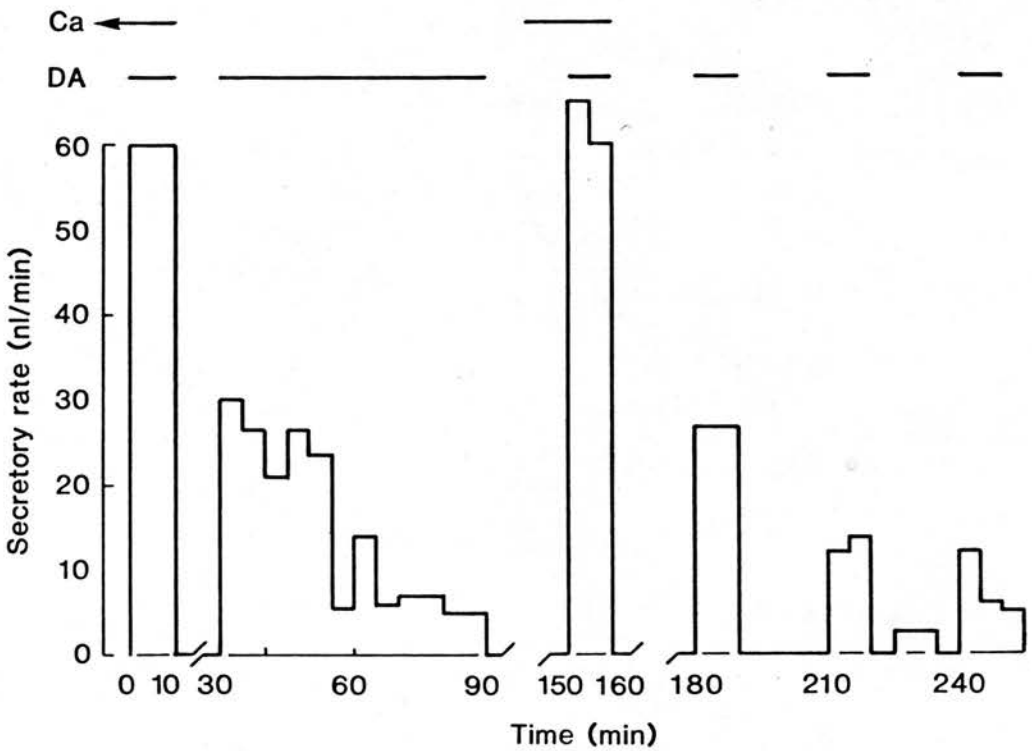

Ca
DA
Secretory rate (nl/min)
60
40
20
0
30
60
90
120
150
Time (min)

Figure 2:3

Effect of calcium-free medium on secretory response to dopamine. The periods during which calcium (5mM) amd dopamine (1uM) were present in the bathing medum are indicated by horizontal bars labelled Ca and DA respectively.


Ca
DA
60
50
40
30
20
10
0
Secretory rate (nl/min)
0 10 30 60 90 150 160 180 210 240
Time (min)

that stimulation in calcium-free conditions causes depletion of an intracellular store of some substance necessary for secretion. The decreasing responses to repeated pulses of stimulation in calcium-free conditions indicate that no significant replenishment of the store occurs between periods of stimulation.

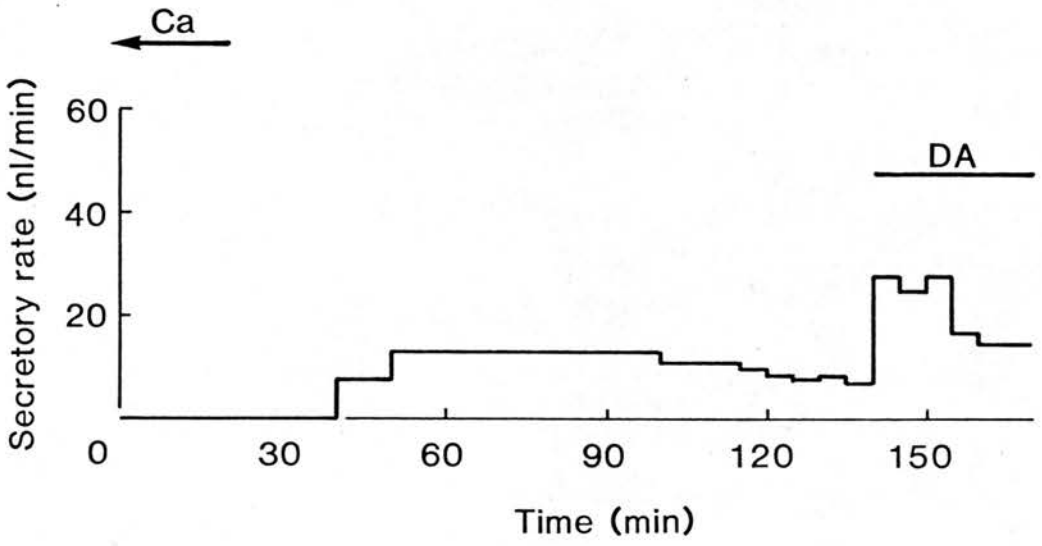
Basal Rate

It was observed that after exposing the glands to calcium-free medium, the basal rate of secretion began to rise without any stimulation. Further experiments were carried out to study this effect more closely and to see whether this increased rate of secretion would have any effect on the subsequent response to stimulation. Figure 2:4 shows the gradual increase in basal rate which begins after a short incubation in calcium-free medium (typically 20 minutes) and eventually reaches a plateau. Figure 2:4 also shows that it is still possible to obtain an increase in secretory rate in response to stimulation after 120 minutes exposure to calcium-free medium but that the rate is significantly reduced. This preparation gave a satisfactory response after a recovery period in control solution (see Methods: Protocol) indicating that no permanent damage had occurred.

A range of pre-stimulation times was tried on 25 glands and there appears to be a trend towards a reduction in response with longer prestimulation periods, indicating that the increased basal rate secretion possibly may be connected with the reduction in the response to dopamine. Six attempts were made to extend the range of pre-stimulation periods to include 180 minutes, and in none of these experiments was any secretion collected in response to

Figure 2:4

Effect of calcium-free medium on basal secretory rate. The periods during which calcium (5mM) and dopamine (1uM) were present in the bathing medium are indicated by horizontal bars labelled Ca and DA respectively.


Ca
DA
Secretory rate (nl/min)
60
40
20
0
30
60
90
120
150
Time (min)

dopamine. In all cases the basal secretory rate dropped to zero during the pre-stimulation period, and recovery responses after the return of calcium were very poor.

Magnesium

Magnesium is believed to displace calcium from membranes (e.g. Petersen & Ueda, 1976) or compete with calcium for membrane binding sites (e.g. Smith & House, 1979). Experiments were carried out to determine whether 5mM magnesium could (i) substitute for 5mM calcium, or (ii) displace calcium from an intracellular store. As shown in Figure 2:5 the response to stimulation in calcium-free magnesium medium is small, indeed smaller than might be expected in calcium-free solution without magnesium. This indicates that not only is magnesium unable to substitute for calcium but that there may be a slight inhibitory effect (Smith & House, 1979), since the secretory rate rises slightly, even before replacement of calcium, when magnesium is withdrawn. When the gland is stimulated in calcium-free medium after incubation in calcium-free magnesium medium (Figure 2:6) the response obtained is within the range expected for that prestimulation exposure time in calcium-free solution without magnesium; thus magnesium has not had a lasting effect on the calcium-dependent store. An increase in basal rate during exposure to calcium-free magnesium solution was observed less frequently and was smaller than the rise during incubation in calcium-free solution.

5HT

5-Hydroxytryptamine, an agonist evidently stimulating the

Figure 2:5

Effect of calcium-free medium containing 5mM-magnesium on the subsequent secretory response to dopamine. The periods during which calcium (5mM), magnesium (5mM) and dopamine (1uM) were present in the bathing medium are indicated by horizontal bars labelled Ca, Mg and DA respectively.

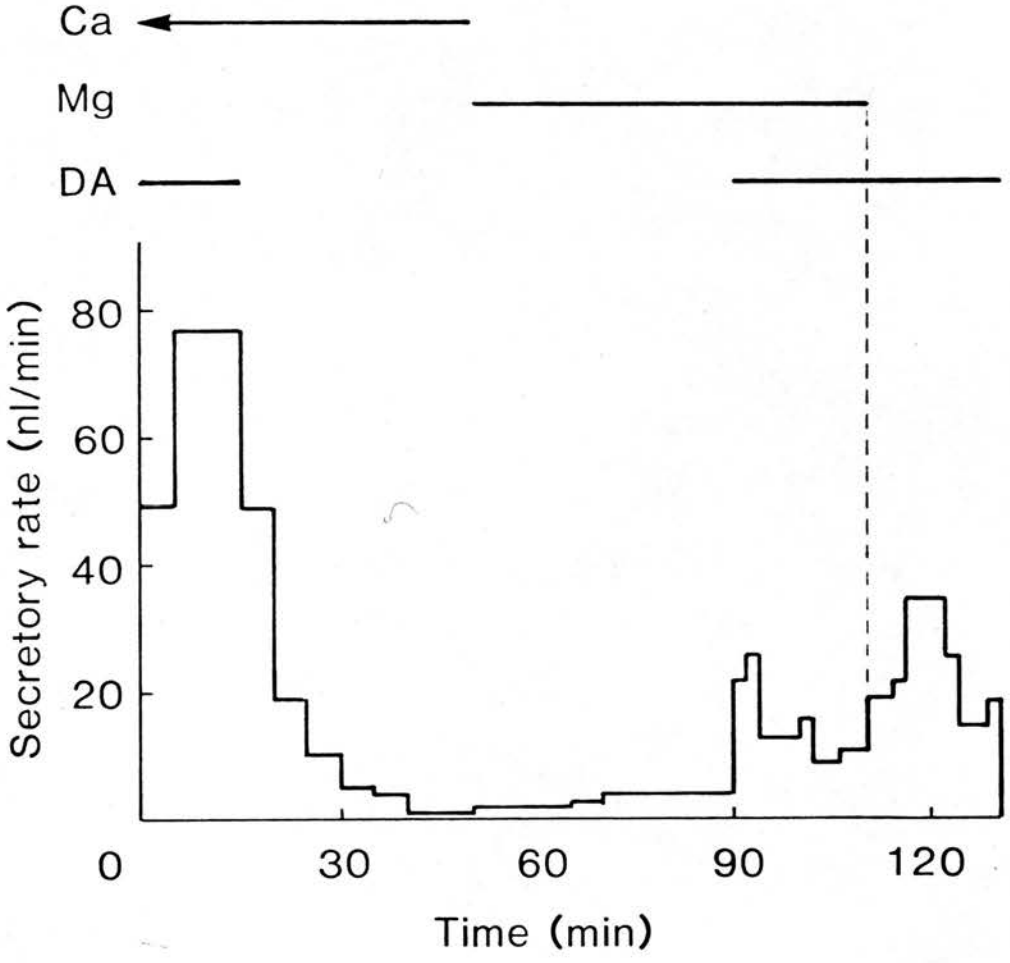
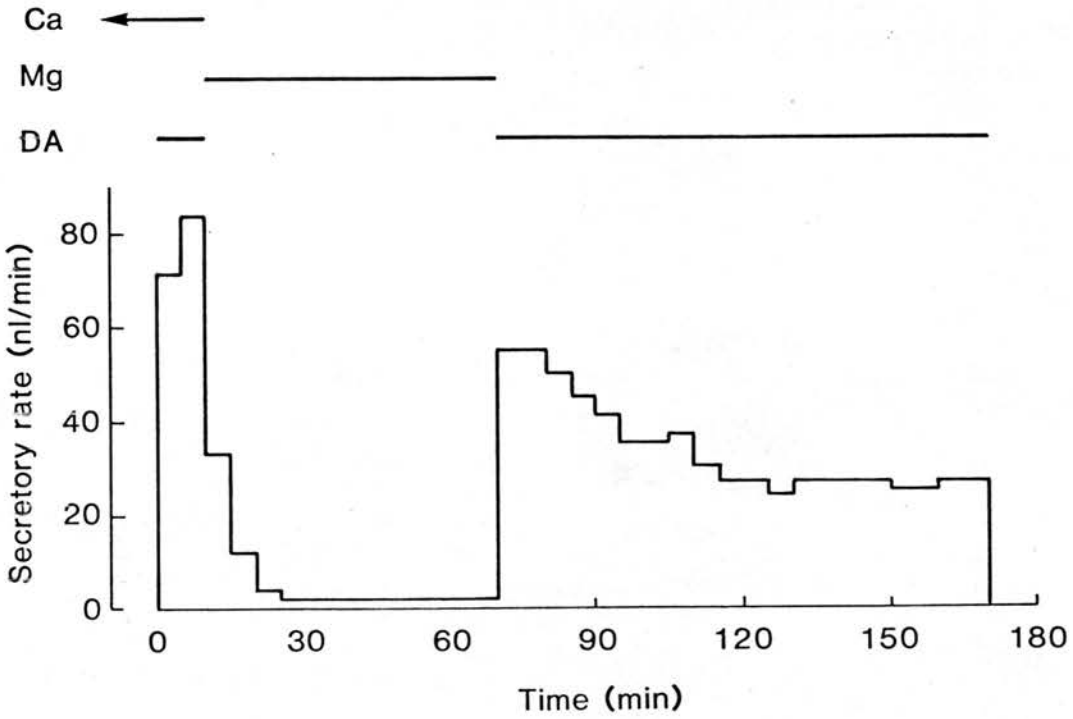

Ca
Mg
DA
Secretory rate (nl/min)
80
60
40
20
0
30
60
90
120
Time (min)

Figure 2:6

Effect of pre-incubation in calcium-free medium containing 5mM-magnesium on the subsequent secretory response to dopamine in calcium-free medium. The periods during which calcium (5mM), magnesium (5mM) and dopamine (1uM) were present in the bathing medium are indicated by horizontal bars labelled Ca, Mg and DA respectively.


Ca
Mg
DA
Secretory rate (nl/min)
80
60
40
20
0
0
30
60
90
120
150
180
Time (min)

gland via receptors other than those activated by dopamine (House, Ginsborg & Silinsky, 1973; Bowser-Riley, House & Smith, 1978) was used to test whether more secretion could be elicited from a preparation 'exhausted' by dopamine stimulation in calcium-free conditions. As Figure 2:7 shows, no increase in secretion was caused by the application of 1uM 5HT to an 'exhausted' preparation.

## SECOND MESSENGER

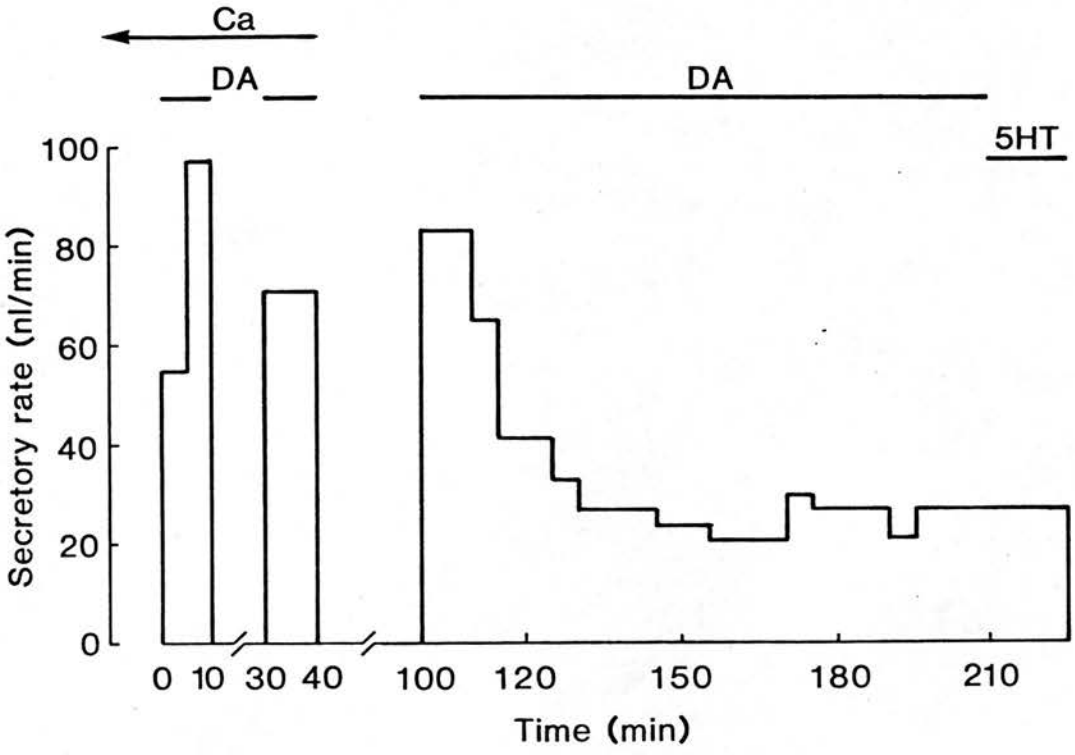
The second part of the work reported here was an investigation of the possible calcium-dependence of a second messenger in stimulus-secretion coupling in the cockroach salivary gland. The object of the experiments was to look for the effect of a build-up of second messenger in the cells when they were stimulated in the absence of an essential ion for salivary secretion. It was anticipated that such a build-up might be expressed, when the missing ion was returned, as a transient secretory response larger than the normal readmission response to that ion.

The experiments described below were prompted by our finding that the replacement of sodium or chloride ions to the bathing solution after a period of dopamine stimulation caused a transient secretion of fluid lasting up to 20 minutes. It is unlikely that residual dopamine in the medium produced this response because the time to change solutions within the bath is less than 40 seconds; moreover the response ocurred even when the missing ion was replaced up to 10 minutes after the end of dopamine stimulation.

The protocol of these experiments was designed with a control

Figure 2:7

Effect of 5-hydroxytryptamine stimulation on fluid secretion from a gland previously stimulated by dopamine in calcium-free medium. The periods during which calcium (5mM), dopamine (1uM) and 5-HT (1uM) were present in the bathing medium are indicated by horizontal bars labelled Ca, DA and 5-HT respectively.


Ca
DA
DA
5HT
100
80
60
40
20
0
Secretory rate (nl/min)
0 10 30 40 100 120 150 180 210
Time (min)

part built into each experiment to enable direct comparison of experimental and control results to be made for each gland. Following the initial test stimulation, the 'switching-off' phase was measured (i.e. the secretion during the 20 minutes immediately following withdrawal of dopamine - phase I in Figures 2:8, 2:10 & 2:11). The control solution was then changed for one of the test solutions (chloride-free; chloride- and calcium-free; sodium-free; and sodium- and calcium-free) and the preparation incubated in this solution for 60 minutes. The control solution was then returned and the readmission response measured, again for 20 minutes (phase II in the figures). That completed the control part. While the preparation was perfused with control solution another test response was obtained to check that the condition of the preparation was similar to that at the start of the experiment. Then the ion deficient test solution was returned and after 20 minutes the preparation was stimulated in test solution for 40 - 60 minutes. A prolonged period of dopamine stimulation was chosen so that the cytosolic concentration of the suspected second messenger might reach an optimum (see Heslop & Berridge 1980); in separate experiments it was shown that the `switching off` phases after brief and prolonged stimulation periods were similar.At the end of this stimulation period the missing ion was returned and the response over the next 20 minutes was recorded as the experimental response (phase III in the figures). Lastly, a test response to 1uM dopamine was obtained after a recovery period in control solution, as a final check on the condition of the preparation. This protocol was repeated on three preparations for each of the test solutions specified above. When calcium was withdrawn as well as either sodium or chloride, calcium was

returned some time after the other ion, to separate the responses. In order to ensure that no contribution to the crucial part of the results could be made either by dopamine remaining in the bath, or by release of transmitter from the nerve endings, 100uM phentolamine, an effective antagonist of dopamine's action (House & Smith, 1978) was included in the medium during phases I,II and III.

Figure 2:8 shows one of the experiments with chloride as the missing ion and with calcium present throughout. There is a small readmission response (II) in the control part, and a much larger response to the return of chloride following stimulation in the experimental part (III).

Since the fluid secretion during both the "switching-off" after stimulation (phase I), and the readmission of the missing ion (phase II) might contribute to the experimental response (phase III) the experiments were analysed by subtracting the sum of phases I and II from phase III. Figure 2:9 shows the results of this analysis plotted for two of the chloride-free experiments. Figure 2:9A is the result of the analysis for the experiment shown in figure 2:8. The positive difference between III and the sum of I and II indicates that a part of the experimental response must be due to the preceding period of stimulation.

When the experiment was repeated with calcium withheld as well as chloride there was no large response on the return of chloride following stimulation in the test solution (Figure 2:10) and phase III was not greater than the sum of phases I and II. These experiments were also repeated using sodium as the missing ion, with or without calcium (figures 2:11A & 2:11B). However in

Figure 2:8

Effect of chloride-free solution containing calcium on the secretory response to dopamine. The periods during which calcium (5mM), chloride (184mM), phentolamine (100uM) and dopamine (1uM) were present in the bathing medium are indicated by horizontal bars labelled Ca, Cl, Pnent. and DA respectively.

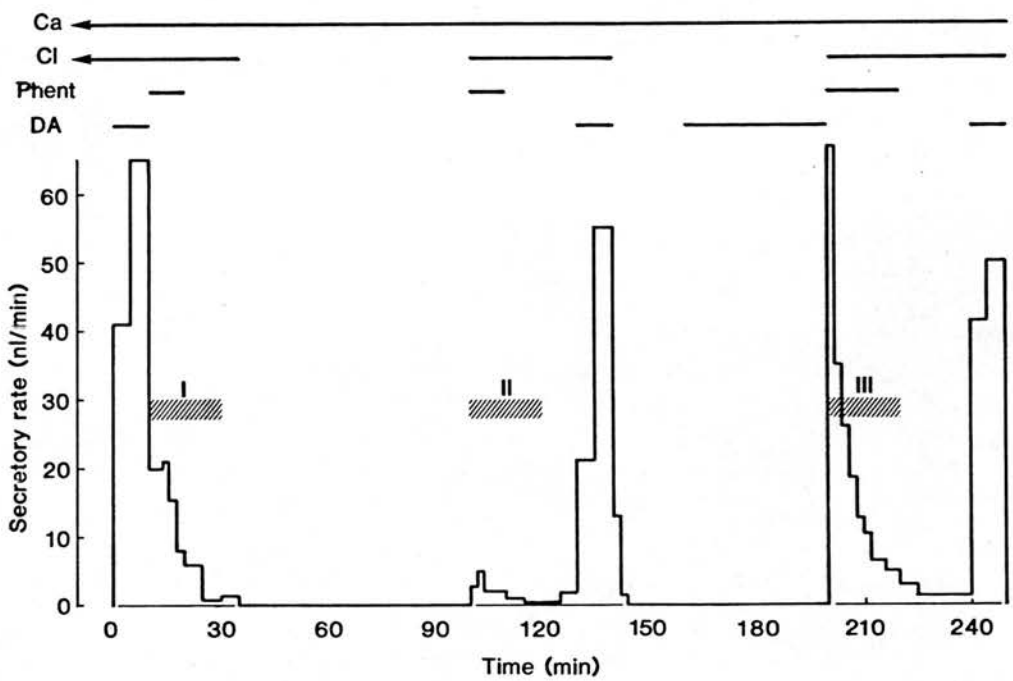

Ca
Cl
Phent
DA
Secretory rate (nl/min)
0
10
20
30
40
50
60
I
II
III
0
30
60
90
120
150
180
210
240
Time (min)

Figure 2:9

Analysis of the results shown in Figure 2:8. The difference between phase III and the sum of phases I and II has been plotted as a function of time in A. The results of the analysis of a similar experiment are shown in B.

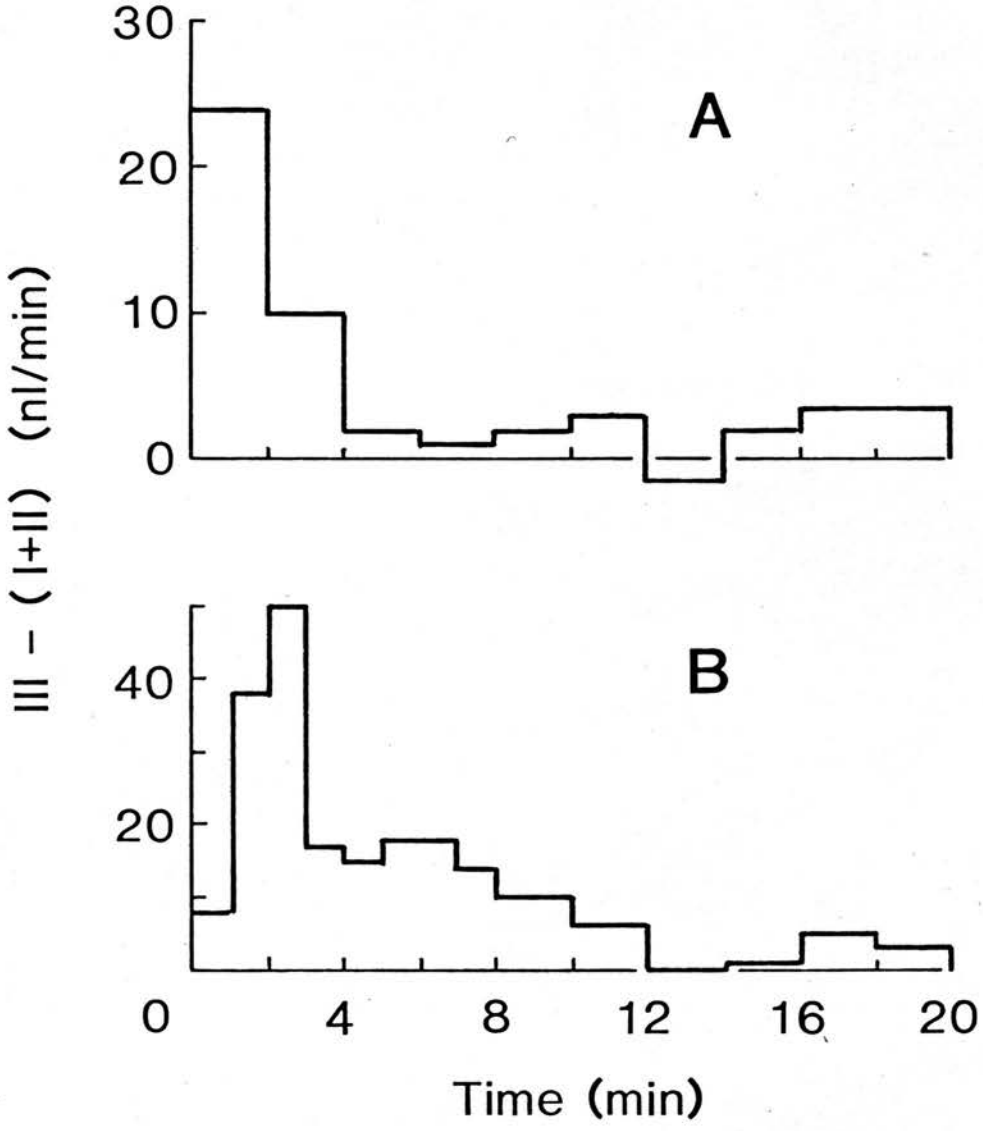

30
20
10
0
A
40
20
B
0
4
8
12
16
20
Time (min)
III - (I+II) (nl/min)

Figure 2:10

Effect of chloride-free, calcium-free solution on the secretory response to dopamine. The periods during which calcium (5mM), chloride (134mM), phentolamine (100uM) and dopamine (1uM) were present in the bathing medium are indicated by horizontal bars labelled Ca, CL, Phent. and DA respectively.

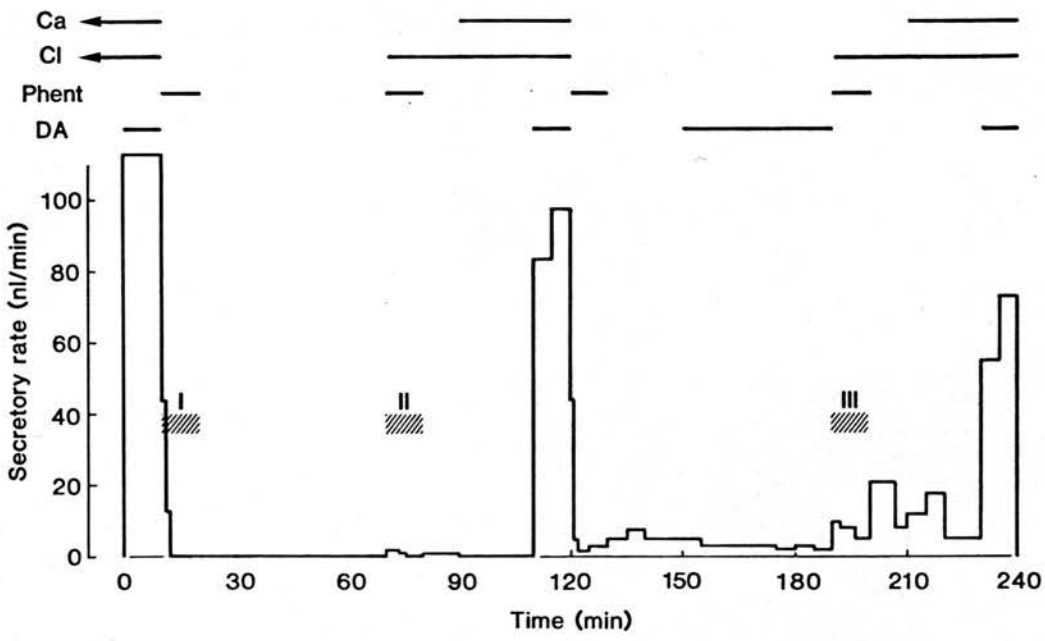
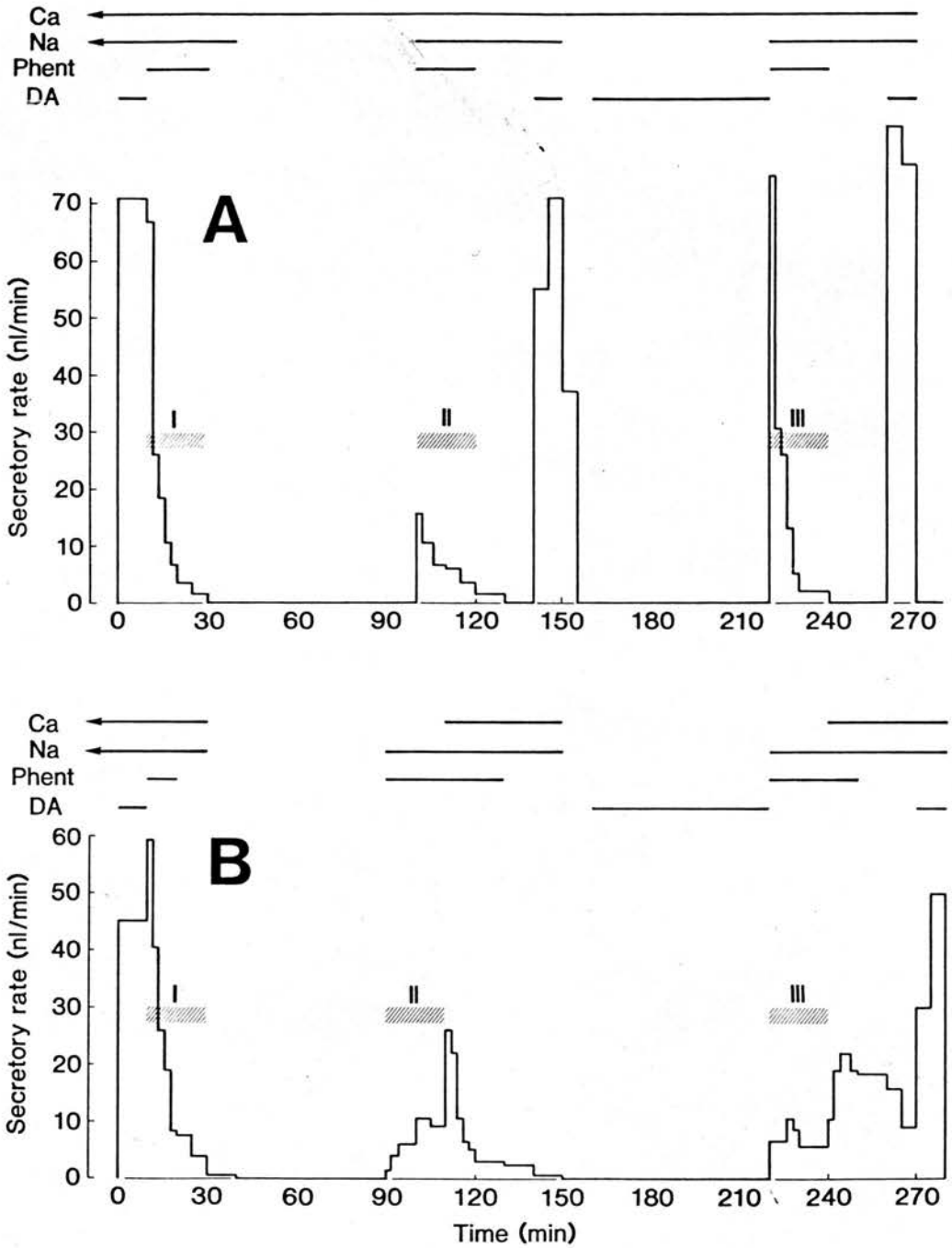

Ca
Cl
Phent
DA
Secretory rate (nl/min)
100
80
60
40
20
0
I
II
III
0
30
60
90
120
150
180
210
240
Time (min)

Figure 2:11

Effect of sodium-free solution, in the presence (A) or absence (B) of calcium, on the secretory response to dopamine. The periods during which calcium (5mM), sodium (160mM), phentolamine (100uM) and dopamine (1uM) were present in the bathing medium are indicated by horizontal bars labelled Ca, Na, Phent. and DA respectively.


Ca
Na
Phent
DA
A
Secretory rate (nl/min)
70
60
50
40
30
20
10
0
I
II
III
0
30
60
90
120
150
180
210
240
270
Ca
Na
Phent
DA
B
Secretory rate (nl/min)
60
50
40
30
20
10
0
I
II
III
0
30
60
90
120
150
180
210
240
270
Time (min)

this case phase III was approximately equal to the sum of phases I and II (in the presence of calcium) because in all of the experiments the sodium readmission response (phase II) was so large that it defeated the purpose of the comparison.

Readmission Responses

As can be seen in Figure 2:12A, when calcium is returned to the medium after a period of calcium deprivation, there is a transient secretory response. This has also been observed when other ions, for example chloride or sodium, have been withheld and then replaced. Figure 2:12 also shows examples of readmission responses to chloride and sodium ions.

Ionophore

When the gland is exposed to 5-10ug/ml of the ionophore A23187 in a normal, calcium-containing medium, there is a marked increase in secretory rate. The maximum secretory response to A23187 was equal to 84% of the maximum response to dopamine (mean of 5 experiments) and in most cases was reached within 6-10 minutes of exposure to A23187.

In calcium-free solution the response to A23187 is reduced to about 28% of the maximum response to dopamine and declines rapidly (2 experiments). Figure 2:13 shows typical responses to A23187 with and without extracellular calcium.

Figure 2:12

Examples of secretory responses to the replacement of calcium (A), chloride (B) or sodium ions (C) in the bathing medium. The periods during which calcium (5mM), chloride (184mM) and sodium (160mM) were present in the bathing medium are indicated by horizontal bars labelled Ca, Cl and Na respectively.

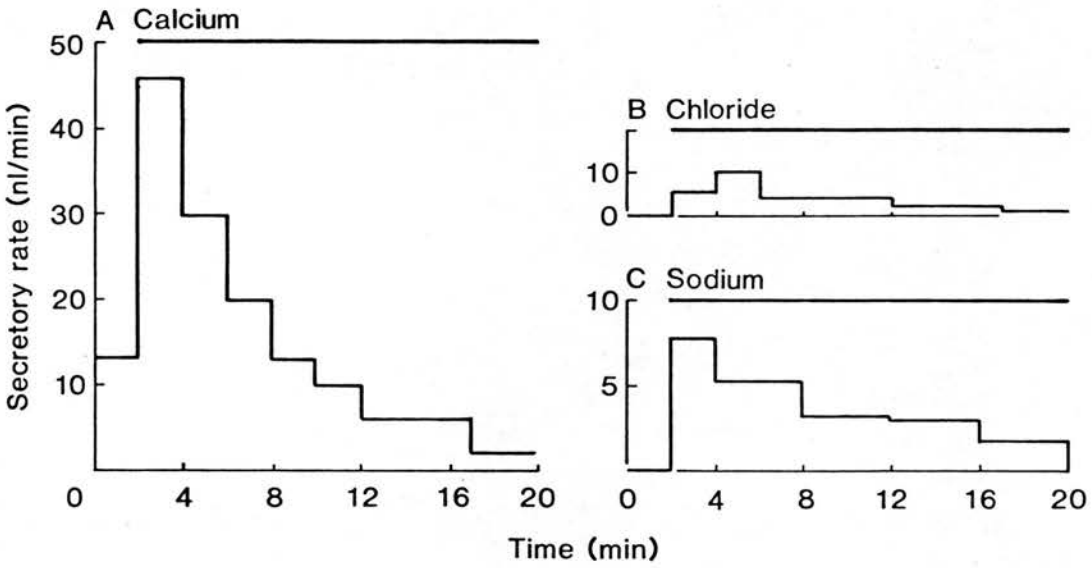
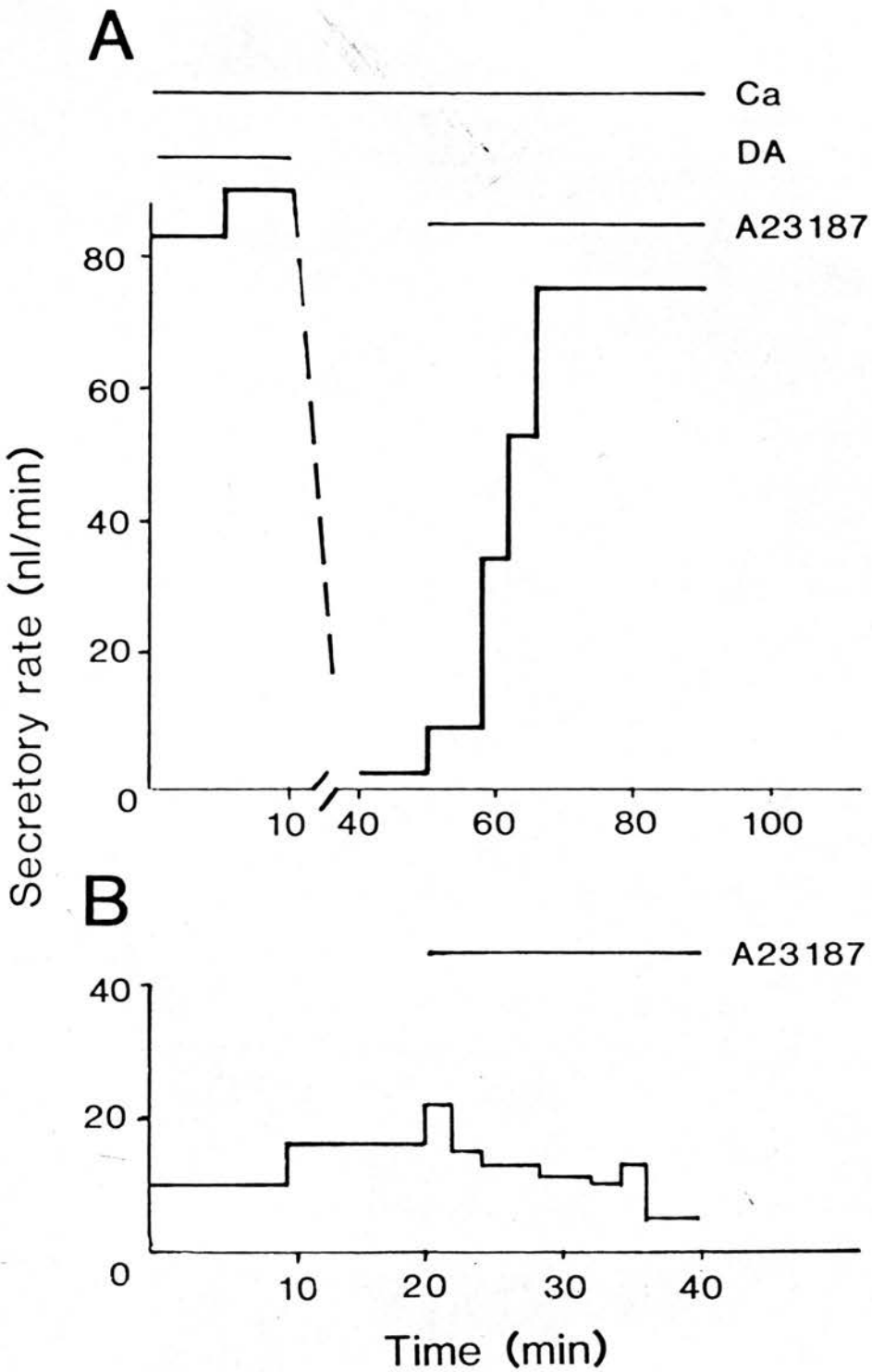

A Calcium
B Chloride
C Sodium
Secretory rate (nl/min)
50
40
30
20
10
0
10
0
10
5
0
4
8
12
16
20
Time (min)

Figure 2:13

Typical responses to A23187 in the presence (A) and absence (B) of extracellular calcium ions. The periods during which A23187 was present in the bathing medium are indicated by horizontal bars labelled A23187.


A
Ca
DA
A23187
80
60
40
20
0
10
40
60
80
100
Secretory rate (nl/min)
B
A23187
40
20
0
10
20
30
40
Time (min)

# DISCUSSION

## CALCIUM STORE

The decline of the response to maintained stimulation in calcium-free solution can be interpreted as further evidence of the role played by calcium in stimulus-secretion coupling in the cockroach salivary gland, and for a calcium-dependent store of a necessary substance in the gland cells. It is possible that the necessary substance is calcium itself and that the calcium store is associated with the plasma membrane of the acinar cells. Such a store could give up calcium ions to the cytosol during stimulation, and be replenished from the exterior (Ginsborg et al., 1980a).The experiments described in chapter 3, using chlorotetracycline, a fluorescent probe (Caswell, 1979), have demonstrated the presence of calcium associated with the plasma membranes of the peripheral cells (Bland & House, 1971) which probably are solely responsible for ion and water secretion into the acinar lumen (Maxwell, 1981).

Cellular calcium stores have previously been proposed on the basis of several different types of evidence (see Table 2:1). Many of the observations of declining responses to stimulation in calcium-free media have been interpreted as the result of stimulation causing release of a limited amount of calcium from a store in the cell, which brings about a transient increase in cytosolic free calcium and secretory rate, both declining when the calcium from the store is used up.

Table 2:1 Observations of declining responses to stimulation in calcium-free conditions.

| Tissue | Response | Stimulus | Reference |
|---|---|---|---|
| Cockroach salivary gland | Hyperpolarization of acinar cells | Ionophoretic dopamine | Ginsborg et.al., 1980a |
| " | " | Ionophore A23187 | Mitchell & Martin, 1980 |
| Blowfly salivary gland | Fluid secretion | 5-HT | Prince & Berridge, 1973 Prince et. al., 1972 |
| " | Transepithelial potential | " | Prince & Berridge, 1973 Berridge & Prince, 1971; 1972a |
| " | Calcium efflux | " | Prince et. al. 1972 |
| Rat parotid | Amylase release | ACh & $\alpha$-adrenergic | Petersen et. al., 1977 |
| " | $K^+$ efflux Amylase release | Substance P Eledoisin Cyclic nucleotides | Rudich & Butcher, 1976 |
| Mouse parotid | Hyperpolarisation of acinar cells | Adrenaline | Petersen & Pedersen, 1974 |
| Mouse/rat pancreas | Amylase release | ACh | Petersen & Ueda, 1976 |
| " | Depolarisation of acinar cells | ACh | " Laugier, 1979 Iwatsuki & Petersen, 1977a |
| Rat pancreas | " | CCK-PZ* | Petersen & Matthews, 1972 Yamamoto, 1977 |
| " | Fluid secretion | " | Kanno & Yamamoto, 1977 |
| Cat pancreas | " | " | Argent et .al.1973 |

*CCK-PZ = Cholecystokinin-pancreozymin

Basal rate in calcium-free medium

Prince and Berridge (1973) reported a significant rise in secretion from blowfly salivary glands exposed to a calcium-free solution containing 5mM EGTA. This increase in the 'unstimulated' secretory rate, although of greater amplitude, follows the same time-course as that reported here for cockroach glands bathed in calcium-free solution containing 1mM EGTA. Also, as is the case with the cockroach gland, the response of the blowfly gland to the agonist was reduced after this period of enhanced secretion.

Rose and Loewenstein (1975) found an increase in cytosolic calcium concentration in Chironomus salivary gland cells exposed to calcium-free medium. This increase in cytosolic free calcium is presumably supplied by intracellular calcium stores - for example the mitochondria may release calcium (Carafoli, Tiozzo, Lugli, Crovetti & Kratzing, 1974).

It is also possible that EGTA itself may have a stimulatory effect by causing some redistribution of intracellular calcium. Huddart and Saad have found that the rate of efflux of radioactive calcium from the cells of rat ileum placed in calcium-free medium is significantly increased when EGTA is added to the medium (H.Huddart, personal communication). This rise in efflux has a time-course similar to that of the increase in basal secretory rate observed in cockroach salivary glands exposed to calcium-free EGTA solution, reaching a peak about 20 minutes after addition of EGTA. The fact that EGTA induced an increase in calcium efflux from rat ileum might indicate that EGTA is bringing about the release of bound calcium. If a similar phenomenon occurs in the cockroach gland it could produce a rise in basal secretion if

calcium efflux is linked to sodium influx.

Magnesium

The observation reported here, that the response to stimulation in calcium-free magnesium solution is less than that observed in control medium, indicates that magnesium cannot simply replace calcium in this secretory system. The same conclusion was reached for fluid secretion from the blowfly salivary gland (Prince & Berridge, 1973), and for catecholamine release from the chromaffin cell, based on the observation that while returning calcium to a calcium-deprived preparation stimulated release, adding magnesium instead of calcium to such a preparation had no effect (Douglas & Rubin, 1963), and for the potassium permeability response of the rat parotid gland (Putney, 1978).

Magnesium is believed to have the ability to displace calcium from membranes -when magnesium is added to pancreatic cells incubated in calcium-free medium there is a small increase in amylase output (Petersen & Ueda, 1976). This has been interpreted as an indication of magnesium displacing bound calcium into the cytosol, rather than of magnesium entry, as the intracellular requirement for calcium appears to be very specific (see for example Miledi, 1973, and Kanno, Cochrane & Douglas, 1973). Although there is some evidence that a small hyperpolarization of cockroach salivary gland acinar cells occurs by displacement of calcium by magnesium (Mitchell & Martin, 1980) it seems unlikely that such a process contributes greatly to fluid secretion, since the basal rate is very low in calcium-free magnesium solution, moreover magnesium apparently is unable to displace calcium from

the cellular store which can be exhausted by dopamine stimulation (Figure 2:6).

The increase in secretory rate on removal of magnesium from the medium, in the continued absence of calcium, is indicative of a degree of inhibition by magnesium. Smith and House (1979) reported that in the absence of calcium, magnesium has an inhibitory effect on this preparation, but much less in the presence of calcium,and that this inhibition is rapidly reversed by the return of calcium. Indeed there is evidence of inhibition by magnesium, in the presence of calcium, of catecholamine release from the cat adrenal medulla (Douglas & Rubin, 1963). Fluid secretion from the cat submaxilliary gland was depressed similarly by magnesium (Douglas & Poisner 1963).

Magnesium appears to be transported across some cell membranes by the same mechanism as calcium, and to compete with calcium for those transport systems (Hodgkin & Keynes, 1957).The effect of magnesium in reducing the frequency and degree of increase in basal secretory rate in calcium-free medium may be linked to the `protective effect` of magnesium in calcium-free solution observed by Douglas and Rubin (1963). There is evidence that magnesium can maintain the normal permeability of cell membranes in the absence of calcium from a variety of tissues, for example, squid axon (Frankenheuser & Hodgkin, 1957) and erythrocytes (Bolingbroke & Maizels, 1959). This might be due to magnesium occupying some site in the transport system which would otherwise be unoccupied in the absence of calcium.

5-Hydroxytryptamine

The inability of 5HT to elicit an increase in secretory rate

from a gland previously 'exhausted' by dopamine stimulation in calcium-free solution indicates that, although the two agonists evidently stimulate the gland through different receptors (Bowser-Riley et al., 1978) they operate a common post-receptor mechanism, and there is no store of calcium accessible to 5HT which is not accessible to dopamine. Parod & Putney (1978) also suggested a common calcium-requiring post-receptor mechanism operated by both alpha-adrenergic and muscarinic receptors mediating the control of potassium permeability in the rat lacrimal gland.

## SECOND MESSENGER

The results presented in the second part of this chapter are evidence that when cockroach salivary glands are stimulated in conditions which inhibit fluid secretion, a change takes place within the glands which persists, and which can be expressed as an increase in secretory rate after the removal of both agonist and inhibition. This change is probably the build-up of a second messenger within the cell. The observation that, in the absence of calcium in the bathing fluid, this change does not occur indicates that calcium is necessary for the build-up of second messenger or may itself be the second messenger. The amount of calcium that can be released from the cellular stores by stimulation in calcium-free solution is apparently not sufficient to permit the concentration of second messenger in the cells to increase sufficiently to cause secretion when the inhibition is removed.

Berridge (1979) has proposed a scheme for excitation-

secretion coupling for the blowfly salivary gland which involves both calcium and cyclic AMP as second messengers with rather complex feedback control. The normal biphasic cyclic AMP response to 5HT stimulation is curtailed in the absence of calcium, and the conclusions drawn from this are that the initial increase in cyclic AMP is due to mobilisation of internally stored calcium, and that calcium may play an important part in the differential activation of adenylate cyclase and phosphodiesterase (Heslop & Berridge 1980). They have also presented evidence for two separate 5HT receptors, one acting through calcium and one through cyclic AMP as second messengers (Berridge & Heslop 1981).

Readmisson responses

Transient electrical and secretory responses to the return of calcium after a period of calcium deprivation have been reported in a number of preparations, namely the adrenal chromaffin cell (Douglas & Rubin, 1961), exocrine pancreas (Petersen & Ueda, 1976, Kanno & Yamamoto, 1977) and salivary gland (Mitchell, Ginsborg & House 1980; Ginsborg et al., 1980a,b). These may be further evidence for the role of calcium as, or in the regulation of, a second messenger. In the cockroach salivary gland there is always a marked increase in secretion in response to the return of calcium after a period of calcium deprivation (Figure 2:12). The electrical response to calcium readmission is a large transient hyperpolarization (Ginsborg et al., 1980a, 1980b) which reflects an increase in potassium permeability (Ginsborg et al., 1980b).

The electrical response to dopamine stimulation is also a hyperpolarisation due to increased potassium permeability

(Ginsborg, House & Silinsky, 1974) and it seems likely, therefore, that receptor activation causes an increase in the concentration of cytosolic calcium, which opens potassium channels in the plasma membrane of acinar cells as found in other cells, for example, Aplysia nerve cells (Meech & Strumwasser, 1970), and pancreatic acinar cells (Petersen & Iwatsuki, 1978).

Treatments which increase the cytosolic free calcium concentration without activating receptors on the cell membrane are capable of mimicking the stimulation of secretion. In the cockroach salivary gland the ionophore A23187, which is known to transport calcium ions (Reed & Lardy, 1972) produces a hyperpolarization due to increase in membrane potassium permeability (Mitchell & Martin, 1980) similar to that produced by stimulation of the gland by dopamine (House, 1973, Ginsborg,et.al. 1974). As shown in Fig. 2:13, there is also a marked increase in the rate of fluid secretion on exposure of the gland to the ionophore in the presence of extracellular calcium. The smaller, transient secretory response to A23187 observed in calcium-free medium is in agreement with the finding that the electrical response to the ionophore is smaller and of shorter duration in calcium-free than in calcium-containing medium (Mitchell & Martin, 1980). This supports the concept of a membrane-associated store of calcium ions in the acinar cells, which is normally maintained by extracellular calcium, but which can be drawn upon and depleted in the absence of extracellular calcium ions.

# CHAPTER 3

# CHLOROTETRACYCLINE FLUORESCENCE ASSOCIATED WITH PLASMA MEMBRANES OF ACINAR CELLS

Measurement of intracellular concentrations of second messenger substances is an important aspect of the study of stimulus-response coupling. Preliminary experiments to measure intracellular cyclic AMP concentration by radio immuno-assay were undertaken and are discussed in the appendix to Chapter 4. Although no attempt to measure intracellular calcium concentration has been made in the course of the present work, the main methods (see Blinks, Weir, Hess and Prendergast, 1982) currently available are discussed below.

Total cell calcium is divided between three 'sources'; cytoplasmic free calcium, calcium bound to membrane surfaces and calcium sequestered inside subcellular organelles. Most of the techniques available to measure intracellular calcium are designed to measure free cytoplasmic calcium. They can be grouped into four main categories; bioluminescent proteins, metallochromic dyes, fluorescent probes and calcium-sensitive microelectrodes.

Of the bioluminescent proteins, which are mostly extracted from marine invertebrates, aequorin is the most widely used. The first reported use of aequorin to measure intracellular calcium was in giant barnacle muscle (Ridgway & Ashley, 1967). It is activated by calcium to emit blue light at a rate that is highly sensitive to the free calcium ion concentration. Aequorin luminescence can be stimulated by a number of other di- and trivalent cations (Blinks, Allen, Prendergast & Harrer, 1978) but calcium is the only one likely to be found in biological systems. Magnesium ions do not stimulate aequorin luminescence but do

antagonise the effect of calcium. This appears to be due to a major conformational change which is distinctly different from the change caused by calcium (Rao, Kemple & Prendergast, 1980). Aequorin is capable of detecting concentrations of calcium as low as $10^{-7}$M, with a time resolution of around 10 ms (see Allen, Blinks & Prendergast for a review of the properties of aequorin).

Bioluminescent proteins can be used to study the localisation of calcium and calcium transients within the cell, by means of microscopic image intensification (Rose & Loewenstein, 1975; 1976).

Two major problems arise with the use of aequorin and other photoproteins. Firstly, the reaction between the aequorin and calcium is irreversible and gives an inactive product. This means that stringent measures must be taken to protect the indicator from calcium contamination prior to its introduction into the cell of interest. Also, it means that once inside the cell the photoprotein is consumed in the course of its light-giving reaction. However, because the calcium concentration in resting cells is very low (around $10^{-7}$M) it has been suggested that the half-life of aequorin in a cell would be about three weeks (Blinks et.al., 1982). When an increase in intracellular $[Ca^{2+}]$ occurs photoprotein consumption becomes appreciable. It has been estimated that during a twitch one part in $10^5$ of the aequorin in a frog muscle fibre is consumed (Blinks, Rudel & Taylor, 1978). The second major disadvantage of aequorin is that the relation between the signal and $[Ca^{2+}]$ is not linear. Over part of the range of sensitivity the signal is proportional to $[Ca^{2+}]^{2.5}$ (Allen, et.al.,1977). Calcium is non-uniformly distributed in many cells, particularly during excitation, and in these circumstances

the signal will be dominated by contributions from those parts of the cell where $[Ca^{2+}]$ is highest, leading to overestimation of the average $[Ca^{2+}]$.

Aequorin does not pass readily through biological membranes, and methods which have been used to introduce it into the cytoplasm include microinjection, temporary membrane disruption by osmotic shock, ultrasound or incubation with EGTA, and liposome fusion. Once inside the cell aequorin remains there and does not accumulate in subcellular organelles.

Aequorin has been used to study intracellular calcium in more than 40 cell types (see Blinks et.al., 1982). Two examples of particular interest here are the squid giant synapse (Llinas & Nicholson, 1975, Miledi & Stinnakre, 1977) and Chironomus larva salivary gland cells (Rose & Loewenstein, 1975; 1976).

Metallochromic dyes are low molecular weight substances which change colour, and therefore absorbance spectrum upon binding metal ions. The five metallochromic dyes which have been used in the study of intracellular calcium are arsenazo III, antipyrylazo III, murexide, tetramethylmurexide and dichlorophosphonazo III. Of these the first two are most commomly used.

The use of arsenazo III in calcium determinations was first described in detail by Michaylova and Ilkova in 1971. It has a high affinity for calcium ions which permits measurement of the low physiological levels of $[Ca^{2+}]$. Murexide and tetramethylmurexide have apparently greater selectivity for calcium over magnesium, but lower affinity for calcium. Antipyrylazo III has an affinity for calcium intermediate between those of arsenazo III and murexide. $Mg^{2+}$ complexes with

antipyrylazo III and reduces the concentration of the dye available for calcium complexation, but its selectivity for $Ca^{2+}$ over $Mg^{2+}$ is good (Blinks et.al., 1982).

The linear relationship between the signal and $[Ca^{2+}]$ is maintained as long as the ratio of bound calcium to free $Ca^{2+}$ remains constant. Because of the existence of intracellular calcium gradients the spatial distribution of the dye in the cell is important in determining the linearity between average $[Ca^{2+}]$ and absorbance change.

The ions most likely to interfere with $Ca^{2+}$ measurement by metallochromic dyes are $Mg^{2+}$ and $H^{+}$. The absorbance spectrum is strongly influenced by pH -near pH 7.4 the absorbance change at 654nm (a wavelength often used for detecting $Ca^{2+}$) is linearly related to pH and is thirty times as great for a change in $[H^{+}]$ as for an equimolar change in $[Ca^{2+}]$ (Ogan & Simons, 1979). In order to quantify intracellular $[Ca^{2+}]$ from absorbance change measurements it is essential to perform calibrations <u>in vitro</u> in conditions which resemble as far as possible those in the cells of interest, particularly with regard to concentrations of dye, $Ca^{2+}$, $Mg^{2+}$ and $H^{+}$.

As is the case with the photoproteins, metallochromic dyes do not pass readily through membranes. Methods of introduction which have been used include iontophoresis, pressure injection (Ahmed & Connor, 1980), internal dialysis (DiPolo, Requena, Brinley, Mullins, Scarpa & Tiffert, 1976) and diffusion through cut surfaces (Palade & Vergara, 1982).

The major advantages of metallochromic dyes over bioluminescent proteins are that they are more readily available,

easier to inject into cells, absorbance changes are linear in relation to $[Ca^{2+}]$, the reaction time is shorter (time constant about 1 ms.) and changes can be measured with high signal to noise ratio. The main disadvantages are that signals can be contaminated by movement artifacts, which are particularly serious in studies on contractile cells, and that they are extremely pH-sensitive.

Most intracellular applications of metallochromic dyes have been in frog skeletal muscle (see Blinks et.al., 1982). Non-contractile cells in which metallochromic dyes have been successfully employed include pacemaker neurones (Gorman & Thomas, 1980), photoreceptors (Brown & Rydqvist, 1981) and squid giant axon (DiPolo et.al., 1976; Brinley, Tiffert & Scarpa, 1978).

Fluorescent probes are of two types, fluorescent chelating molecules such as chlorotetracycline, and fluorescently-tagged calcium-binding proteins. The first sucessful use of a fluorescent indicator for the detection of $Ca^{2+}$ in biological systems was reported by Caswell and Hutchison (1971) who used chlorotetracycline (CTC) -still the most widely used fluorescent probe.

Interaction of the probe with $Ca^{2+}$ or other metal ions has three possible consequences; a change in fluorescence intensity, a shift in the emission spectrum or a shift in the excitation spectrum. In practice, rapid changes are usually recorded as changes in emission intensity at a particular wavelength. For work on bulk preparations such as cell suspensions the sensitivity of the CTC technique is sufficient to allow physiological transients to be readily detected. When the subcellular localisation of calcium is to be observed a fluorescence microscope is used, and if necessary may be coupled to an image intensification system.

The linearity of the fluorescence response to metal ion concentration is influenced by the same factors as that of the metallochromic dyes, with the addition of the so-called inner filter effect. This occurs when the absorbtion spectrum of the indicator partly overlaps its fluorescence emission spectrum, so that part of the light emitted is reabsorbed. The effect is increased when high concentrations of the indicator are present.

Although CTC binds both $Ca^{2+}$ and $Mg^{2+}$, in the presence of cell membranes the fluorescence of the Ca chelate is 65% greater than that of the Mg chelate (Hallett, Schneider & Carbone, 1972) and the emission spectra of the two are sufficiently different to allow distinction between them.

One of the major advantages of CTC is that it is able to cross cell membranes, so no complicated technique is required for its introduction into the cell. The altered charge of the divalent cation chelate may cause it to associate with membranes but it is widely accepted that it does not cross them. One complication which arises in the interpretation of data given by this technique is that a change in fluorescence intensity could result from either a change in the number of probe-ion complexes, or from migration of the complex into a more, or less, polar environment, which could alter the quantum yield of each fluorescing molecule. Another disadvantage of CTC as a calcium probe is that it may be altering the very thing that it is supposed to measure, and by chelating calcium may act as a calcium sink. It might also affect cell physiology by means unrelated to calcium chelation. Effects of millimolar concentrations of CTC on cell function have been reported (Le Breton, Sandler & Feinberg, 1976; Behn, Lübbemeier &

Weskamp, 1977). No harmfull effects have been reported with concentrations of 100uM or less (Caswell, 1979). Bathing media with low divalent cation concentrations must be used during CTC uptake, to prevent binding of extracelullar calcium to the probe and subsequent loss of uptake. However it is possible to return the tissues to normal media after CTC accumulation, as release of CTC is relatively slow.

CTC has been used to observe calcium transients in squid axon action potentials (Hallett et.al., 1972), and calcium movements in endocrine and exocrine pancreatic cells (Täljedal, 1978;1979 and Chandler & Williams 1978a;b, respectively). It has also been used to study the role of calcium in platelet aggregation (Le Breton, Dinerstein, Roth & Feinberg, 1976)and in many other biological systems (Blinks et.al., 1982).

Calcein is another fluorescent probe which can be used to monitor calcium. Howeveer at physiological pH levels the cation-induced changes in fluorescence are relatively slight, so for a long time little use was made of this indicator. Recently it has been suggested (Chiu & Haynes, 1977) that provided proper attention was given to its properties at neutral pH, calcein could be of use in biological systems. Both absorbtion and excitation spectra change when the indicator binds with either $Ca^{2+}$ or $Mg^{2+}$, resulting in a decrease of up to 40% in fluorescence intensity. However calcein appears to be approximately equally sensitive to $Ca^{2+}$ and $Mg^{2+}$ and so is unsatisfactory for measurement of $Ca^{2+}$ in living cells.

Fluorescently labelled calcium-binding proteins are a recent development. Troponin C has been proposed for use in intracellular calcium measurement (Johnson, Collins & Potter, 1978). The

fluorescence of the labelled protein doubles when it interacts with $Ca^{2+}$. This effect appears to be specific to calcium, and it is sensitive to calcium concentrations as low as $10^{-7}$M *in vitro*. Magnesium slightly decreases the fluorescence of the labelled troponin C, and changes in pH can alter the response to $Ca^{2+}$ but of themselves do not significantly alter the fluorescence. The response to $Ca^{2+}$ is very rapid and takes place in less than 1 ms (Johnson, Charlton & Potter, 1979).

Unpublished observations cited by Blinks et.al.(1982) indicate that calmodulin may also be fluorescently labelled for possible use as an intracellular calcium probe, and appears to retain its normal functions. Parvalbumin has also been tried but is not likely to be satisfactory as the fluorescence change is also evoked by $Mg^{2+}$.

Quin2 is a fluorescent compound recently developed by Tsien and colleagues (Tsien, 1980) for use as an intracellular calcium indicator. It binds calcium with a selectivity over magnesium of more than $2x10^4$. Fluorescence signals can be calibrated at the end of the experiment by lysing the cells in the measuring cuvette and recording signals from the dye that they contain in the presence of very low and saturating calcium concentrations.

The most important advantage of the quin2 technique is that the indicator can be introduced with ease into even very small cells, by incubating the cells with the acetoxymethyl ester of quin2. The ester penetrates the cell membranes, and once inside is hydrolysed, and releases the tetra-anionic form of the indicator (Tsien, 1981). It does not appear to accumulate inside mitochondria or other subcellular organelles (Pozzan, Rink &

Tsien, 1981).

The most serious disadvantage of quin2 is that it has to be used at high concentrations because its fluorescence spectrum is in the same range as the natural cell constituents. Because of this it may significantly buffer intracellular $Ca^{2+}$ and damp any rapid $Ca^{2+}$ transients. Also the dye becomes virtually saturated within the physiological range of intracellular $Ca^{2+}$, and the signal to $[Ca^{2+}]$ relationship will not be linear over any range of calcium concentration likely to be found in living cells.

Quin2 has been used for measurement of intracellular calcium in lymphocytes (Pozzan et.al., 1981) and macrophages (Kesteven, 1982).

Calcium-sensitive microelectrodes work on the principle that the activity of a particular ion species in a solution of unknown composition can be deduced from the electrical potential difference measured across an ion-selective membrane separating the solution to be analysed from a reference solution inside the electrode. There are two types of cation-selective membranes, one type containing a charged ion exchanger and the other a neutral carrier. When the sensor components are incorporated into polyvinylchloride membranes the useful range of the electrodes may be extended to below $10^{-7}$M-$Ca^{2+}$ (Tsien & Rink, 1981). These authors reported that $H^{+}$ and $Mg^{2+}$ had virtually no effect on the measured potentials, but that $Na^{+}$ increased the potentials by amounts that increased with increasing $[Ca^{2+}]$. None of the $Ca^{2+}$ sensors developed so far is completely specific, so quantitative interpretation of signals obtained using these electrodes depends to a certain extent on assumptions made about intracellular concentrations of interfering ions.

The most serious disadvantage of $Ca^{2+}$-sensitive microelectrodes is their slow response to changes in calcium. Ion exchanger microelectrodes are reported to have time constants of about 10 seconds (Christofferson & Simonsen, 1979), and that of neutral carrier microelectrodes is longer, especially at low $[Ca^{2+}]$ (Berridge, 1980c; Tsien & Rink, 1980). Blinks et.al. (1982) give a list of resting intracellular calcium concentrations ranging from $5.5 \times 10^{-8}$M in frog skeletal muscle (Coray, Fry, Hess, McGuigan & Weingart, 1980) to $1.2 \times 10^{-6}$ in Helix neurones (Christofferson & Simonsen, 1979), measured in a wide variety of cell types, with calcium-sensitive microelectrodes.

Chlorotetracycline was chosen as a tool for a preliminary investigation of intracellular calcium in the cockroach salivary gland. The results of the investigation are presented in this chapter.

## METHODS

The method of dissection used was the same as that described in Chapter 2. The composition of the bathing solutions was also the same, except that in those solutions containing calcium, the calcium concentration was 1mM not 5mM. In some experiments $MgCl_2$ was added to the control (1mM calcium) solution to give a final magnesium concentration of 5mM. Chlorotetracycline (Sigma Chemical Co.) and lanthanum nitrate (Taab Laboratories, Reading) were added to solutions to give the final concentrations cited in the text. In some experiments, where measurements of salivary secretion were made, the glands were stimulated by adding dopamine to the bathing solutions.

### Secretory measurements

Secretory measurements were made as described in Chapter 2.

### Microscopy

Glands were examined by transmitted visible or ultraviolet light on a Carl Zeiss Universal microscope fitted with fluorescence illuminator and filters.

Pilot experiments showed that solutions containing 100uM chlorotetracycline (CTC) gave optimal staining within a convenient experimental period. The results of each treatment described in RESULTS were obtained on at least six glands. Representative light

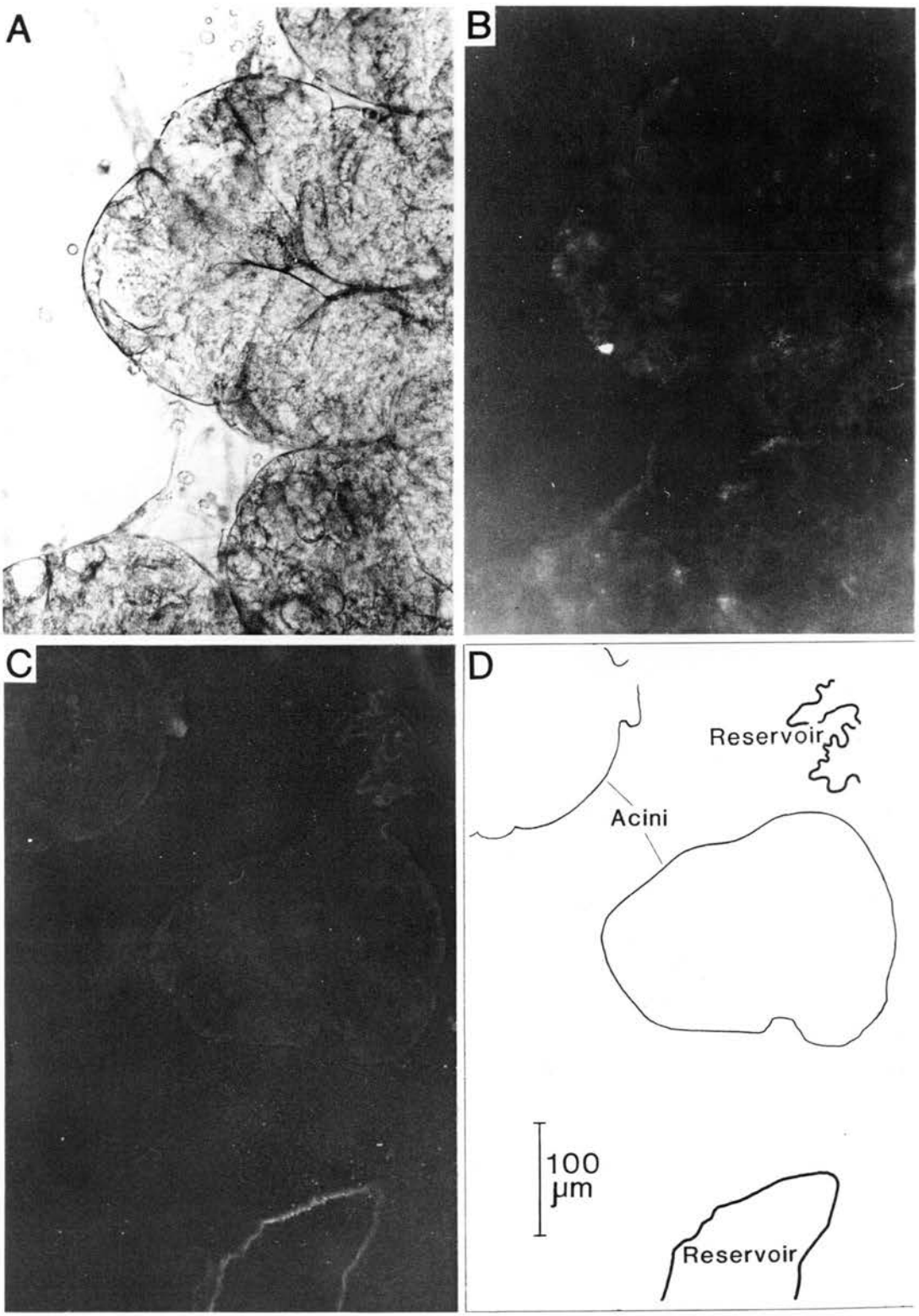
and fluorescence pictures of CTC stained glands and tissue sections (see Histology) are shown because the results obtained under the different conditions were consistent and no other reliable way of giving the results is available. All fluorescence pictures, except those in Figure 3:5 were obtained under the same photographic conditions and have been reproduced by an identical procedure for the sake of comparison. Fluorescence pictures of control glands and tissue sections incubated in normal solution are shown in Figure 3:1; these pictures were obtained under the conditions of photography and printing which were used for the experimental groups in the study. The corresponding control pictures obtained for other experimental solutions have not been reproduced because, like those shown in Figure 3:1, these glands had insufficient autofluorescence to make them visible under these conditions.

Histology

Glands bathed in various solutions containing 100uM CTC were fixed in 10% formalin. After wax embedding, 15uM sections were prepared for examination by fluorescence microscopy. In most experiments photographs were made of wax sections because the process of de-waxing reduced the fluorescent staining of the sections. In some experiments where high power light and fluorescence pictures were desired glands were exposed to 500uM CTC, fixed and the sections were de-waxed and mounted in Gurr`s UV inert mountant. After fluorescence pictures of these sections were obtained the cover slips were floated off the slides by prolonged immersion in xylene and the tissue sections stained with Erlich`s Haemotoxylin and Eosin and finally mounted in DPX (Raymond A.

Figure 3:1

Light and fluorescence pictures of salivary gland acini incubated in normal solution. A and B are respectively light and fluorescence pictures of living acini. C is a fluorescence picture of a wax section of acini from another control gland. D is a line drawing showing the boundaries of the acini and reservoirs in C.


A
B
C
D
Reservoir
Acini
100
µm
Reservoir

Lamb) for examination by light microscopy.

Electron microscopy

Glands from control and experimental groups were fixed at 4°C in 4% glutaraldehyde in 0.05M sodium cacodylate buffer with added sucrose (28g/l). the glands were then processed conventionally for electron microscopy and stained with uranyl acetate and lead citrate. Ultra-thin sections (gold) were cut from araldite blocks and examined through a Philips 400 electron microscope.

## RESULTS

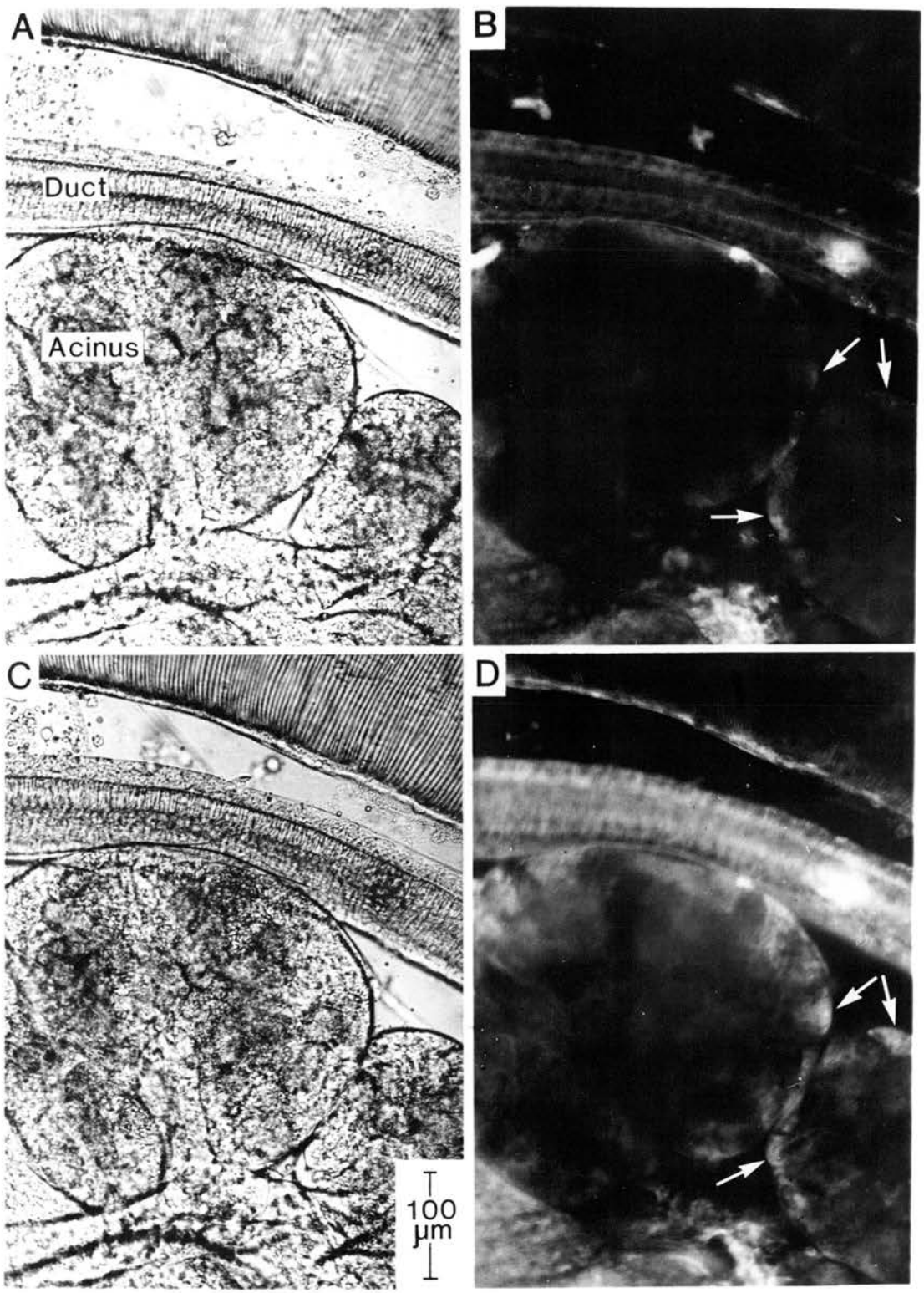
The uptake of CTC into cockroach salivary gland cells is relatively slow, as observed in other cells (Chandler & Williams, 1978b; Naccache, Showell, Becker & Sha`afi, 1979; House & Bland, 1982). Figure 3:2 shows light and fluorescence pictures of an acinus and duct bathed in normal solution containing 100uM CTC. Within about 5 minutes of exposing the gland to CTC the acinus and duct were weakly fluorescent. In the acinus the fluorescent label occurred at particular places (arrows, Fig.3:2B). When the same field was examined 10 min. later the intensity of fluorescence in the acinus and duct had increased. Whereas the labelling of the duct did not vary along its length the labelling of the acinus was clearly concentrated in triangular shaped regions (Fig.3:2D). The area of these fluorescent regions (arrows) suggested that CTC preferentially had labelled the acinar peripheral cells (Bland & House, 1971) which have a characteristic pyramidal shape. The intensity of fluorescence continued to increase during further incubation in CTC solution, the gland cells being optimally labelled after 60-90 min. Examples of maximal labelling are shown

It was important to establish whether CTC interfered with the anatomy or physiology of gland cells. An electron microscopical study was made and the results are discussed below (see Ultrastructure of CTC stained cells).

A check on the physiological function of the CTC stained gland was made as follows. The effect of CTC on the power of the gland to secrete fluid in response to stimulation by dopamine was

Figure 3:2

Light and fluorescence pictures of living gland cells during uptake of CTC. A and B show corresponding light and fluorescence pictures of an acinus and duct about 5 min after immersion in normal solution containing 100uM-CTC. C and D show the same acinus and duct about 10 min later. The fluorescent CTC is taken up into duct cells and preferentially into acinar cells with a pyramidal shape (arrows).


A
Duct
Acinus
B
C
100
µm
D

investigated,and the results of a representative experiment are shown in Fig.3:3. The gland was stimulated by passing a solution containing 1uM dopamine through the chamber holding the gland. The rate of fluid secretion rose from a basal rate of about 1nl/min (not shown) to about 60nl/min during dopamine application. After recovery from dopamine stimulation a solution containing 100uM CTC was passed through the chamber. At this concentration CTC itself evoked secretion and did not depress the ability of the gland to secrete fluid in response to subsequent dopamine stimulation.

Sites of CTC staining

The fluorescent staining in glands incubated in normal solution containing 100uM CTC for periods of 60-90 min was confined to certain acinar cells and duct cells. Fig.3:4 shows a representative pattern of acinar labelling by CTC where the fluorescence originates from pyramidal-shaped cells (arrows, Fig.3:4B). To establish the site of CTC deposition with greater precision CTC labelled glands were fixed in 10% formalin and the tissue was then processed for the preparation of wax sections. Evidently these procedures do not remove a substantial proportion of CTC from the cells because the tissue sections remained fluorescent. Fig.3:4C shows a section from a gland incubated for 30 min in 100uM CTC; fluorescent regions can be seen in acinar cells (small arrows) and duct cells (large arrows). Fig.3:4D shows a section from a gland incubated for 60 min in normal solution containing 100uM CTC; the fluorescence of acinar cells is more intense than in Fig.3:4C but the subcellular distribution is similar. In an effort to determine the site of CTC staining within acinar cells sections of stained cells were examined at higher

Figure 3:3

Effect of CTC on the rate of fluid secretion by an isolated salivary gland. The periods during which dopamine and CTC were present in the bathing medium are indicated by horizontal bars labelled Ca and CTC respectively.

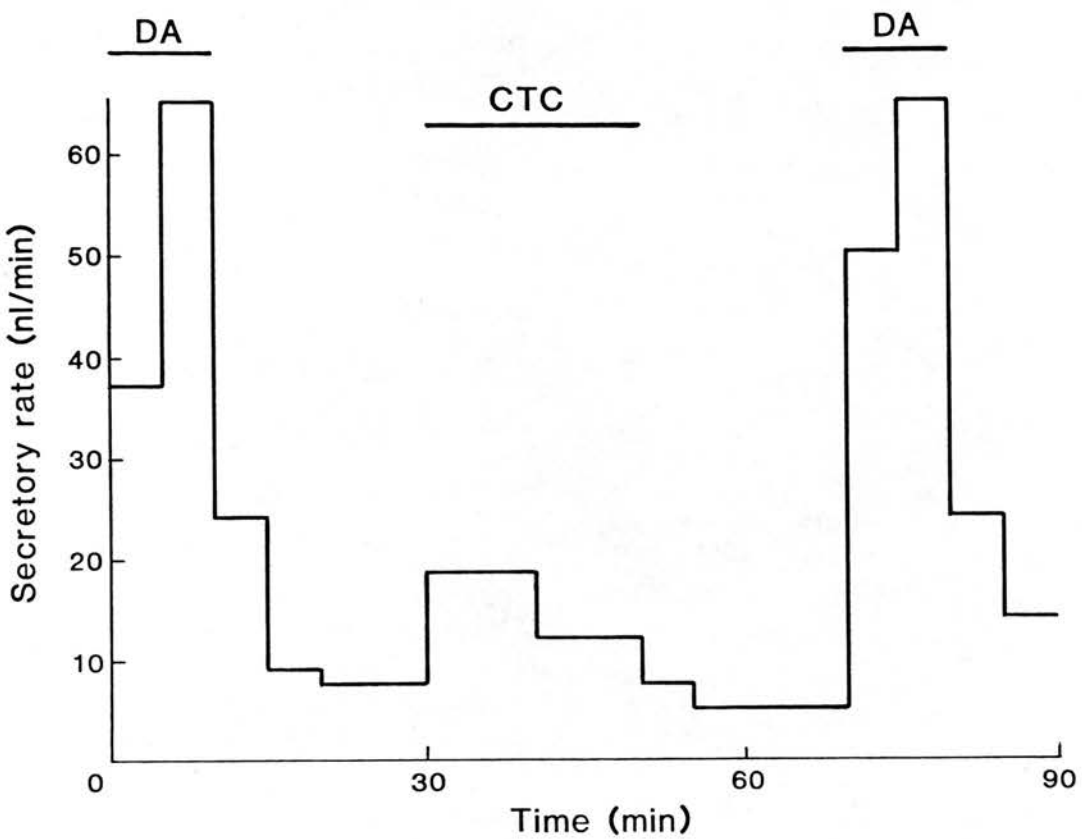
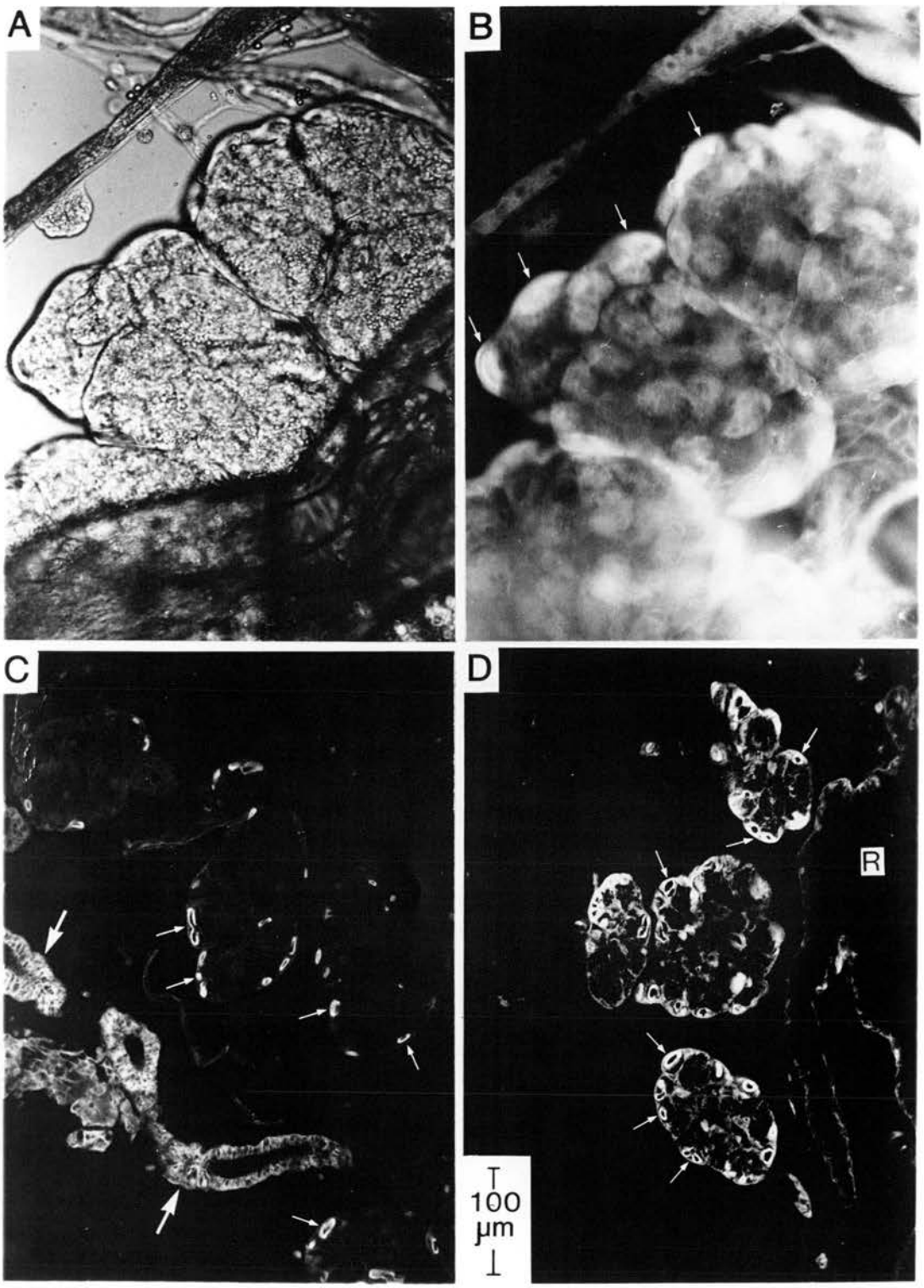

DA
CTC
DA
Secretory rate (nl/min)
60
50
40
30
20
10
0
30
60
90
Time (min)

Figure 3:4

Light and fluorescence pictures of glands labelled by CTC. A and B show pictures of living acini incubated for about 40 min in normal solution containing 100uM-CTC. C is a fluorescence picture of a section of acini and ducts (large arrows) incubated for 30 min in normal solution containing 100uM-CTC; the CTC is non-uniformly distributed within some acinar cells (small arrows). D is a fluorescence picture of a section of acini and reservoir (R) incubated for 60 min in a normal solution containing 100uM-CTC; as in C the CTC is non-uniformly distributed (small arrows).


A
B
C
D
R
100
µm

magnification after incubation in normal solution containing 500uM CTC for 2 hours. Fig.3:5 shows examples of acinar cells where the CTC staining occurred in a ring-like structure within the cells. These cells were confirmed to be peripheral cells by subsequently staining the sections with haemotoxylin and eosin and examining them by light microscopy. Thus the ring structure, which is an origin of intense staining, is the microvillar border formed by the prominent folding of the apical plasma membrane. The other type of acinar cell is the central cell, which has numerous granules and endoplasmic reticulum (Bland & House, 1971); evidently CTC does not stain granules in this kind if cell but the adjacent regions containing endoplasmic reticulum are fluorescent.

The idea that the acinar sites of CTC staining are associated with regions of prominent foldings of plasma membrane is compatible with the observation that certain duct cells are also intensely stained, since these have prominent basal membrane infolds (Bland & House, 1971).

Magnesium solution

The enhanced fluorescence of CTC depends on the presence of divalent cations. Our normal solution contained calcium but not magnesium. Was the pattern of normal CTC labelling modified by the addition of magnesium to the normal solution? Figures 3:6A & B show respectively the CTC staining in glands incubated in normal solution and normal solution containing 5mM $MgCl_2$. It is clear that magnesium ions partially reduce the CTC staining of gland cells and it seems unlikely that trace amounts of magnesium are the source of the relatively intense staining of glands bathed in

Figure 3:5

Fluorescence pictures of sections of gland cells incubated in normal solution containing 500uM-CTC. A, B and C show examples of acinar cells each containing intense CTC labelling of a ring-like structure. Subsequent histochemical examination revealed that the labelled cells were peripheral cells. D is a line drawing of the acinar cells in C. The scale bar in each part of the Figure indicates 10uM.

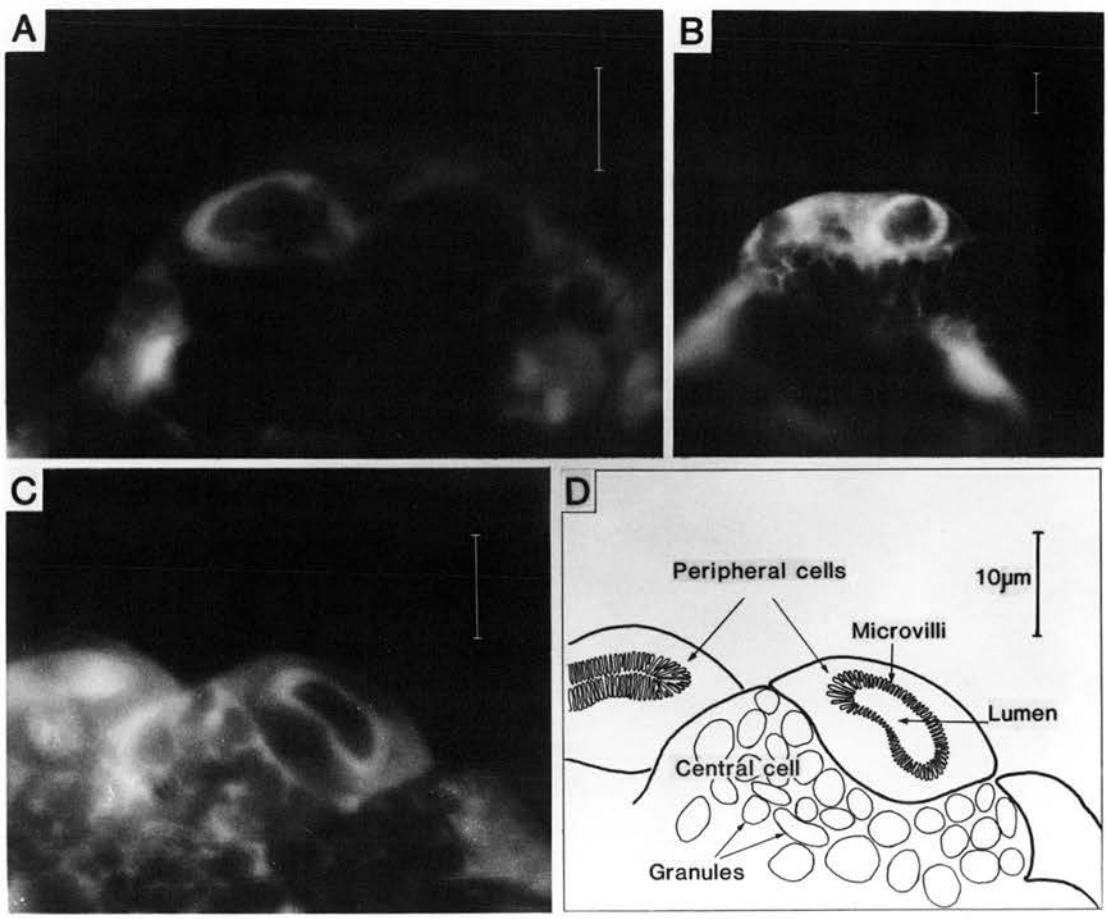
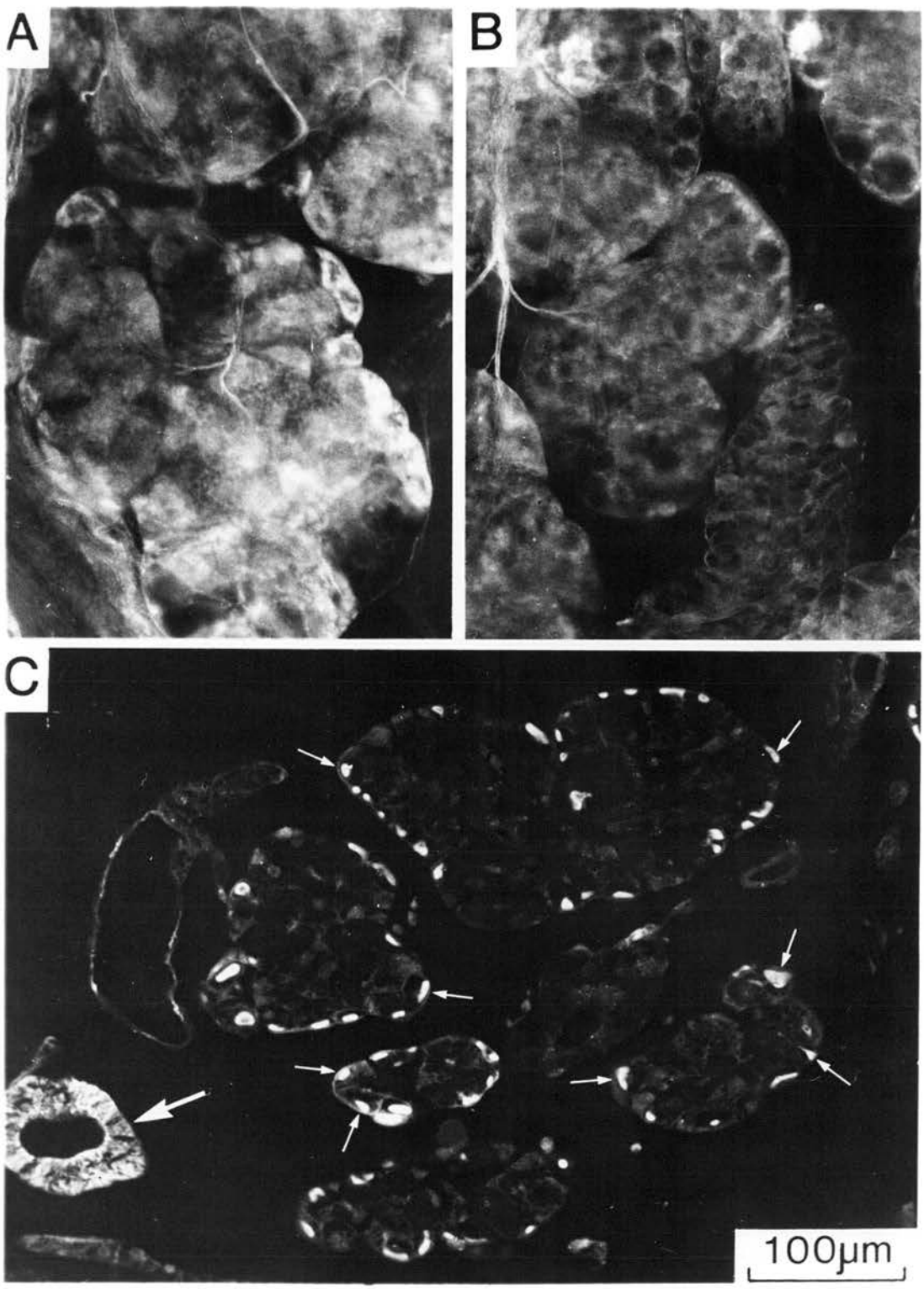

A
B
C
D
Peripheral cells
10μm
Microvilli
Lumen
Central cell
Granules

Figure 3:6

Fluorescent pictures of glands labelled by CTC. A andB are pictures of living acini incubated for 60 min respectively in normal solution containing 100uM-CTC and in normal solution containing 5mM-Mg and 100uM-CTC. C shows a picture of a section of acini and duct (large arrow) in a gland incubated for 60 min in normal solution containing 5mM-Mg and 100uM-CTC.


A
B
C
100μm

normal solution. Nevertheless, the CTC staining present in glands incubated in solutions containing magnesium is still associated with the same structures in cells (Fig.3:6C) as those seen in glands in normal solution (Fig.3:4D).

Calcium-deficient solution

CTC labelling persists in glands incubated in calcium-deficient solution for several hours (Fig.3:7) although it is less intense than in glands bathed in normal solution. The staining pattern in both whole glands and tissue sections strongly suggests that CTC labels the peripheral cells in the acini (white arrows, Fig.3:7B &C). The labelling is relatively intense in the foldings of the apical membranes of these cells (black arrows, Fig.3:7B).

Solutions preventing fluid secretion

Previous work (Smith & House, 1979) has shown that cockroach salivary glands bathed in sodium-free or chloride-free solutions do not secrete fluid in response to dopamine. Another extracellular ion which is necessary for maintained, but not transient, fluid secretion is calcium (see Chapter 2, also Gray & House, 1982). One way of reducing calcium movement across the plasma membrane is to add lanthanum ions to the bathing solution (e.g. Herchuelz & Malaisse, 1978). In the blowfly salivary gland, for example, lanthanum ions appear to block both the influx of calcium ions and the fluid secretion normally produced by 5-HT stimulation (Prince & Berridge, 1973; Hansen Bay, 1978). Lanthanum ions also block dopamine-stimulated (or CTC-stimulated) fluid secretion by isolated cockroach salivary glands (Fig.3:8).

Glands bathed in sodium-free, chloride free or normal

Figure 3:7

Light and fluorescence pictures of glands labelled by CTC. A and B show corresponding light and fluorescence pictures of an acinus in a gland incubated for 60 min in calcium-free solution containing 100uM-CTC: the fluorescence is located at the surface (white arrows) and intracellular structures of some acinar cells in B. C is a fluorescence picture of a section of acini from a gland incubated for 60 min in calcium-free solution containing 100uM-CTC. D is a line drawing showing the boundaries of the acini and reservoir in C.

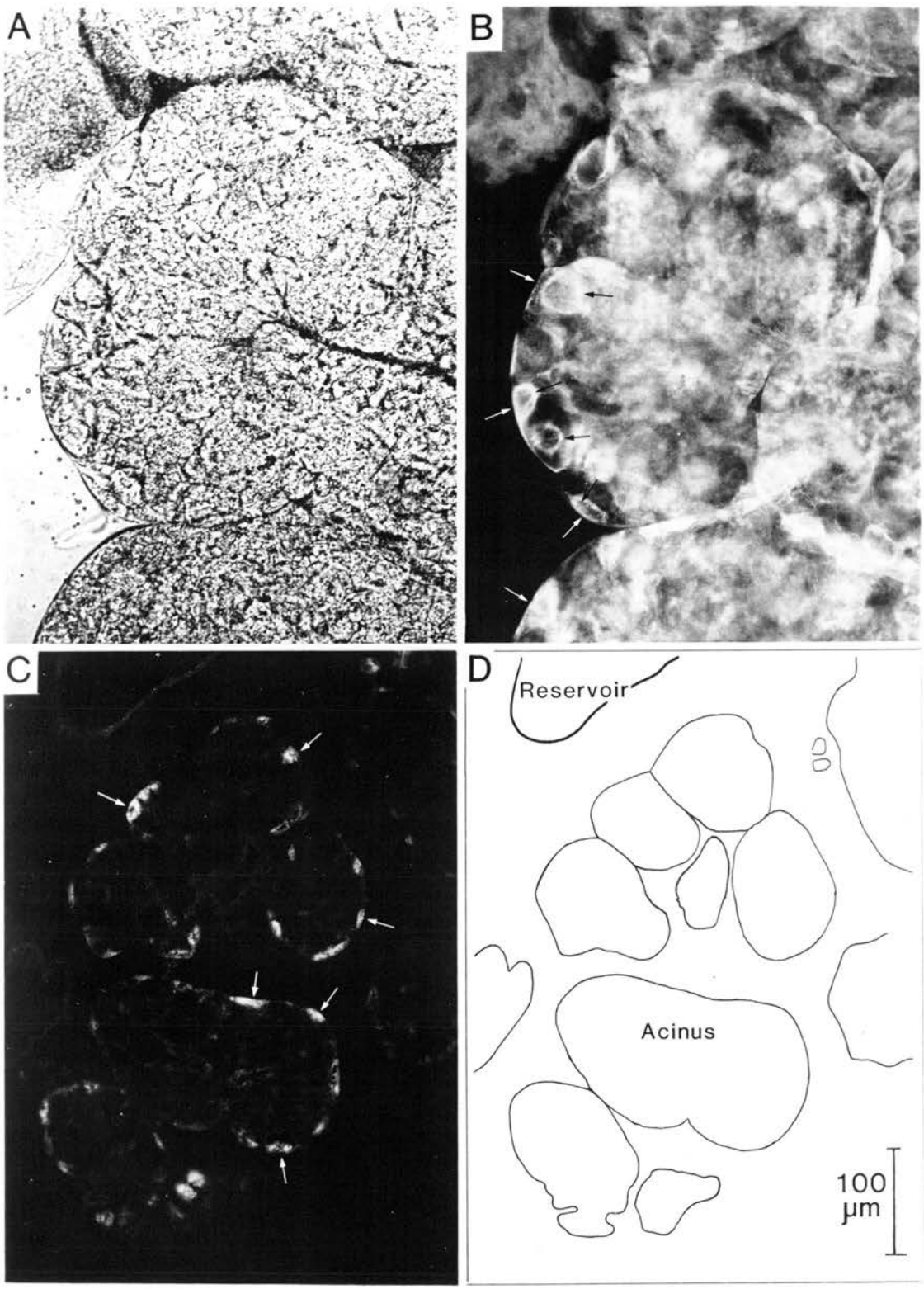
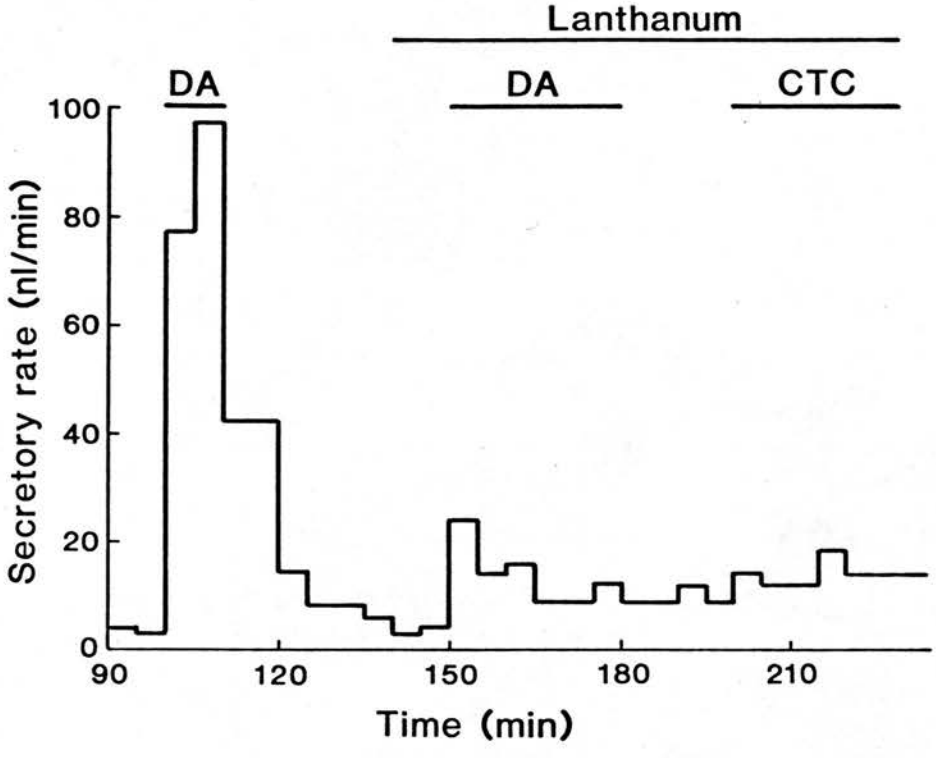

A
B
C
D
Reservoir
Acinus
100
µm

Figure 3:8

Effect of lanthanum on the rate of fluid secretion by an isolated salivary gland. The periods during which dopamine (1uM), CTC (100uM) and lanthanum (2mM) were present in the bathing medium are indicated by horizontal bars labelled DA, CTC and Lanthanum


Lanthanum
DA
DA
CTC
100
80
60
40
20
0
Secretory rate (nl/min)
90
120
150
180
210
Time (min)

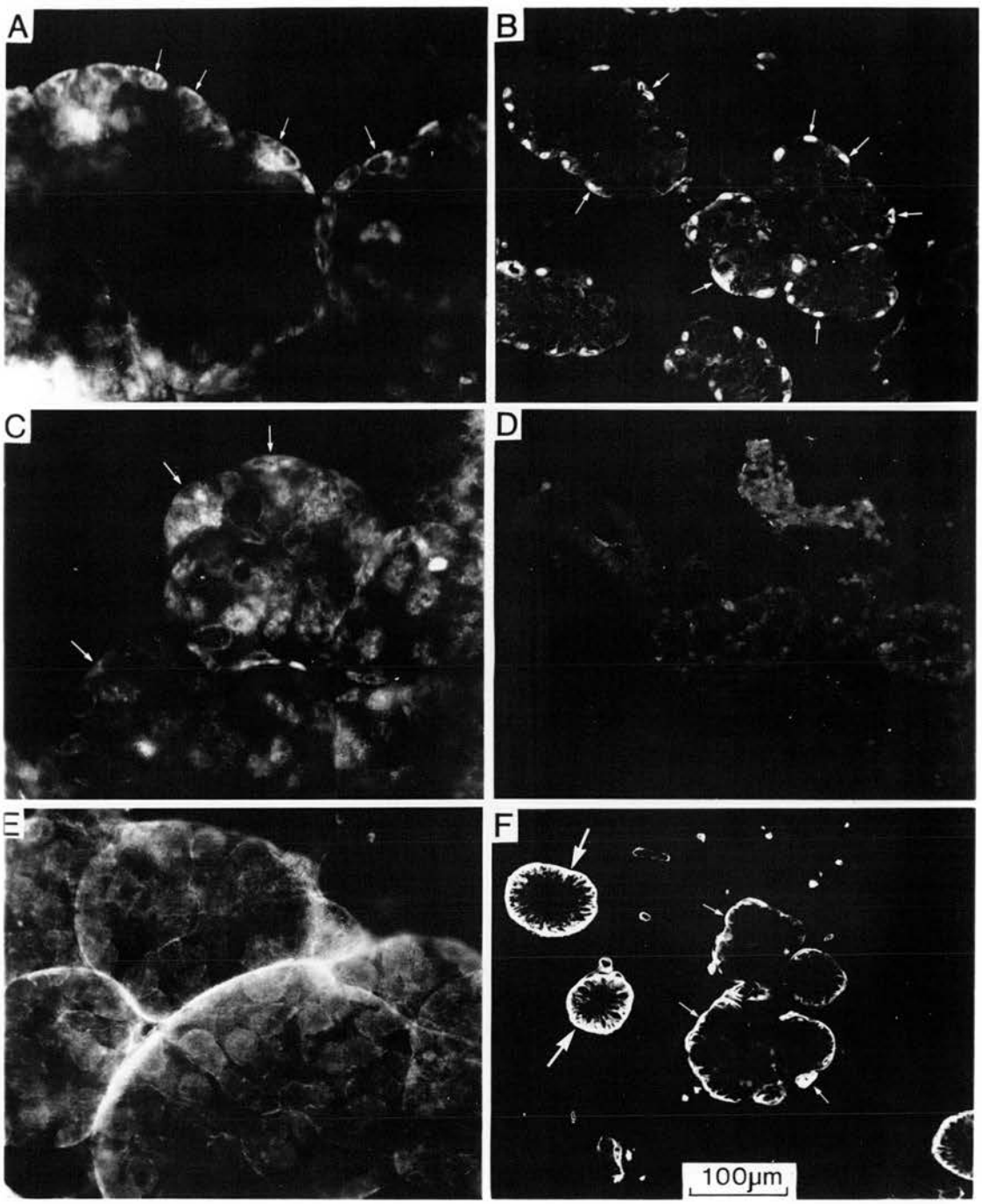
solution containing lanthanum were examined. Each solution contained 100uM CTC and all glands were exposed to CTC for 60 min. Figure 3:9 shows corresponding whole glands and tissue sections for chloride-free (A &B), sodium-free (C & D) and lanthanum solutions (E & F). The CTC labelling of glands incubated in chloride-free solution is similar to that observed in calcium-deficient solution. In sodium-free solution, however, CTC labelling is relatively weak; although there appears to be labelling of peripheral cells (white arrows, Fig.3:9C). In glands incubated in normal solution containing 2mM lanthanum nitrate the pattern of CTC labelling was substantially different from that found in all other solutions used in this study. Figure 3:9C shows that CTC was confined to the surface and extracellular space in the acini (small arrows) and ducts (large arrows). This surface labelling was non-uniform and examination of whole acini (Fig.3:9E) revealed patches of fluorescence which occurred in pairs.

Effects of dopamine stimulation on CTC staining

An important aim of the present study was to establish whether the plasma membranes of cockroach salivary gland cells had an associated store of calcium ions. Another aim of great interest was to discover whether such a store might be depleted by dopamine stimulation. Isolated glands were bathed in calcium-free solution containing 1uM dopamine for 2 hours to deplete the calcium store (see Chapter 2, also Gray & House, 1982). Another group of glands was bathed in calcium-free solution for the same period. Stimulated and unstimulated glands were then bathed in calcium-free solution containing 100uM CTC for 1 hour and subsequently

Figure 3:9

Fluorescence pictures of glands labelled by CTC in solutions preventing fluid secretion. A,C and E show pictures of living acini, and B,D and F show pictures of sections of glands incubated under the same conditions. A and B show CTC labelled acini incubated for 60 min in chloride-free medium containing 100uM-CTC; selective uptake occurred (arrows). C and D show CTC-labelled acini incubated for 60 min in sodium-free solution containing 100uM-CTC; selective uptake of CTC occurred (arrows). E and F show pictures of acini incubated for 60 min in normal solution containing 2mM lanthanum and 100uM-CTC. In F the CTC labeling is located evidently at the surfaces of ducts (large arrows) and acini (small arrows).


A
B
C
D
E
F
100μm

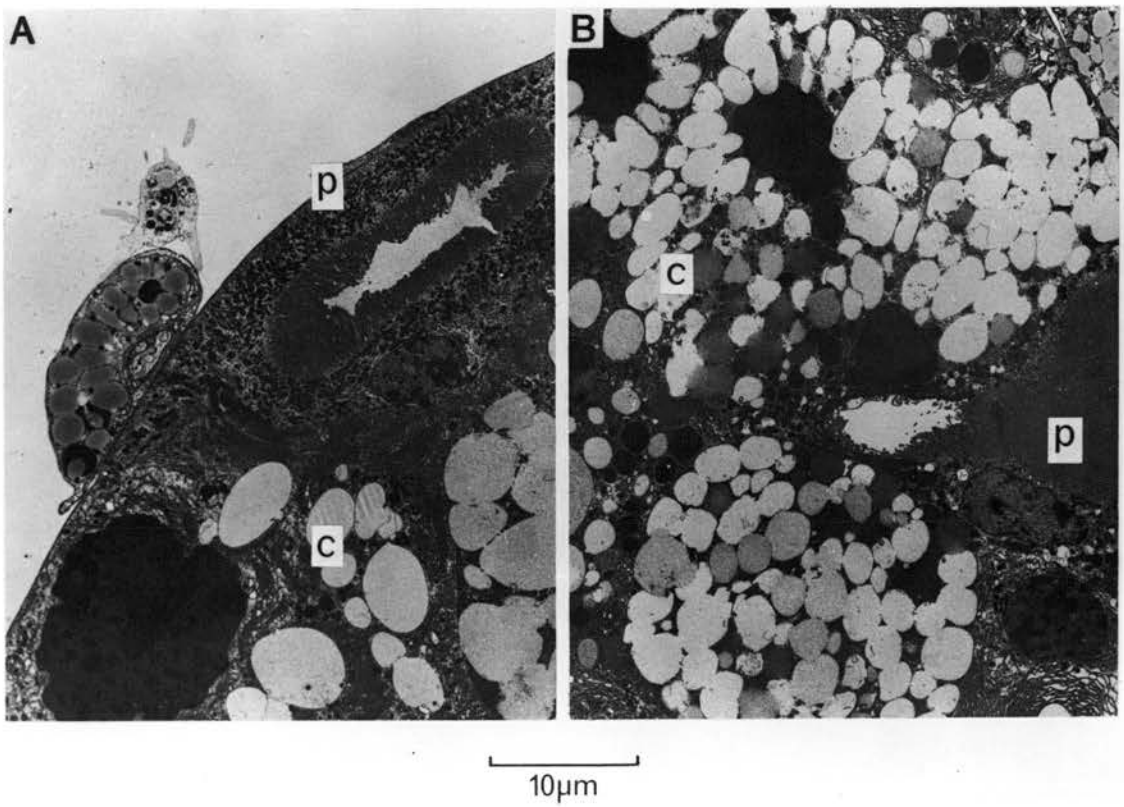
examined by fluorescence microscopy. No significant reduction in the staining of stimulated compared with unstimulated glands was noted.

Ultrastructure of CTC stained cells

Light and fluorescence pictures of CTC stained glands shown above (Figs 3:2,4-7,9) have no signs of gross cellular damage. Moreover there is no evidence that CTC causes ultrastructural damage in mammalian pancreatic acinar cells (Chandler & Williams, 1978b) and eggs (House & Bland, 1982). Nevertheless, it seemed prudent to compare the ultrastructure of CTC stained and control cells of cockroach glands. No significant differences in the ultrastructure of control and stained cells were noted. Figure 3:10 shows representative areas from electron microscopical sections taken from acini bathed for 60 min in normal solution containing 50 (A) or 100 (B) uM CTC. Both electron micrographs show regions of peripheral (p) and central (c) cells which are indistinguishable from pictures of normal p and c cells already published (Bland & House, 1971).

Figure 3:10

Electron micrographs of acinar cells incubated for 60 min in normal solution containing CTC prior to fixation. A shows peripheral (p) and central (c) cells in a gland exposed to 50uM-CTC. B shows similar acinar cells in a gland exposed to 100uM-CTC.


A
p
c
B
c
p
10μm

## DISCUSSION

### Distribution of CTC within cells

The pattern of CTC labelling of the acinar and duct cells of the cockroach salivary gland closely resembles the distribution of folded plasma membranes of the gland cells (Kessel & Beams, 1963; Bland & House, 1971; Whitehead, 1971).

In acinar peripheral cells the basal plasma membrane has shallow infolds of about 1 um whereas the apical plasma membrane forms an elaborate surface of microvilli extending about 5um into the lumen (Fig.3:10). The peripheral cells contain numerous mitochondria. The finding that CTC staining of peripheral cells is particularly intense, especially in the region of the microvillar surface (Fig.3:5), indicates that CTC labels plasma membranes and probably also mitochondrial membranes. Evidence for plasma membrane labelling by CTC has also been obtained in mammalian erythrocytes (Chandler & Williams, 1978b), pancreatic islet cells (Taljedal, 1978) and eggs (House & Bland, 1982). Mitochondrial labelling by CTC is also probably the origin of the isolated fluorescent spots in isolated liver cells (DuBuy & Showacre, 1961; Caswell, 1972; Chandler & Williams, 1978b).

In contrast to peripheral cells, the central cells do not have prominently folded plasma membranes and most of their volume is taken up by granules destined for secretion. The central cells are not well stained by CTC except in sparse cytoplasmic regions where there is endoplasmic reticulum between granules (Fig.3:5).



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Indirectly, the poor staining of central cells supports the idea that CTC fluorescence is associated with membranes. In these cells the site of staining is probably endoplasmic reticulum, in accord with the finding that in pancreatic acinar cells the regions containing rough endoplasmic reticulum (or Golgi vesicles) are labelled by CTC (Chandler & Williams , 1978b).

The secretory ducts adjacent to the acini are comprised of cells which apparently make, store and secrete a mucoid substance (Bland & House, 1971). The ultrastructural features of these cells are similar to those of acinar central cells and make them unlikely candidates for intense labelling of plasma membranes.

Another type of duct cell is found at some distance from the acini in this gland. It contains numerous mitochondria interleaved between long infolds of the basal plasma membrane. The apical plasma membrane of this cell has short infolds. Apparently the intense, but non-uniform, labelling by CTC of this type of duct cell is consistent with a pattern of plasma membrane labelling expected from observations on the acinar peripheral cells.

Thus it appears that the pronounced foldings of the plasma membranes of particular cells in the cockroach salivary gland are the origin of strong CTC fluorescence. Compartments in rod photoreceptors containing folded disc membranes are equally intense sources of CTC fluorescence (Chandler & Williams, 1978b). Plasma membrane in an unfolded state is not such an effective source of fluorescence, although in the early stages of the labelling of mammalian pancreatic islet cells (Taljedal, 1978) and eggs (House & Bland, 1982) discrete patches of fluorescence have been observed at the surface of these cells. That this surface labelling originates in the plasma membrane has not been

established beyond doubt. However, when both cell types are incubated for 60 minutes in solutions containing CTC and lanthanum the surface labelling becomes relatively intense. It seems that lanthanum ions interfere with the entry of CTC into these cells and thereby enhance the relative intensity of labelling at the cell surface as has been found in the present study (Fig. 3:9E & F).

Influence of calcium on CTC staining

When CTC binds to calcium or other divalent cations in aqueous solution the complex formed has an enhanced fluorescence. The enhancement is further increased if the binding takes place in a less polar medium such as membrane lipid. Thus CTC may act as an indicator of divalent cations stored in the vicinity of cell membranes.

These results suggest that calcium, rather than magnesium, ions increase the fluorescence of CTC associated with the plasma membranes of cockroach salivary gland cells. The intensity of labelling is reduced in glands incubated in calcium-free solution or normal solution containing magnesium. Thus magnesium cannot substitute for calcium at the sites where CTC fluorescence originates in the cells. Nor can magnesium substitute for calcium as an agent influencing the control of fluid secretion by this gland (chapter 2, also Gray & House, 1982).

Possibly CTC is a calcium ionophore as suggested by Taljedal (1978) for pancreatic islet cells. It produces an increase in insulin release from pancreatic islet cells (Taljedal, 1978) and a rise in the calcium influx into these cells (Taljedal, 1979). A similar phenomenon might also occur in the cockroach salivary

gland since CTC evokes fluid secretion (Fig.3:3). Lanthanum ions block fluid secretion evoked by dopamine or CTC (Fig.3:8) and also reduce the entry of CTC into gland cells (Fig.3:9E & F). Poor cytoplasmic labelling by CTC in the presence of lanthanum has been observed also in mammalian pancreatic islet cells (Taljedal, 1978) and eggs (House & Bland, 1982). Perhaps lanthanum blocks a co-entry process mediating the influx of calcium and CTC.

The prevention of intracellular staining by CTC in the presence of lanthanum is an interesting observation. The enhanced fluorescence around the acinar surfaces indicates some CTC chelation of lanthanum. The La-CTC complex is apparently not able to enter the cell. In addition lanthanum may block the entry of uncomplexed CTC -the labelling observed inside cells incubated with lanthanum appears considerably less than that from inside cells incubated in calcium-free medium, in which conditions a proportion of CTC entering the cell is probably uncomplexed. It is likely that in control solution some of the CTC which enters the cell is complexed with calcium, and some is free CTC.

In this connection it is interesting to note that the acinar surface labelling in normal solution containing lanthanum seems to occur in patches similar in size and distribution to the basal surfaces of peripheral cells.

These results suggest that the plasma membranes of cockroach gland cells have a store of calcium ions. Is this the calcium store affected by dopamine stimulation? The experiments on this point were inconclusive and perhaps a quantitative method of monitoring the CTC fluorescence from areas of plasma membrane would yield definative results. Fluorimetric measurements of CTC fluorescence from whole cells, including pancreatic acinar cells

(Chandler & Williams, 1978a), islet cells (Taljedal, 1978) and neutrophils (Naccache et al. 1979) indicate that stimulation by various agonists causes calcium release from intracellular stores. The subcellular location of these stores, however, remains to be identified.

# CHAPTER 4

# THE ROLE OF CYCLIC AMP IN FLUID SECRETION FROM THE COCKROACH SALIVARY GLAND

# INTRODUCTION

Since the discovery of adenosine 3',5'-cyclic monophosphate a quarter of a century ago (Rall, et. al.1957), much research has been carried out into the role of this nucleotide in stimulus-response coupling. Cyclic AMP has been shown to mediate the actions of many hormones and neurotransmitters, examples of which are listed in table 1:1, with key references. Indeed, it was in the light of the early discoveries about the role of cyclic AMP in hormone action that the second-messenger concept was first proposed (Sutherland, et. al.1965).

Interest in cyclic AMP in relation to stimulus-secretion coupling in the cockroach salivary gland was stimulated by the work of Berridge and co-workers on the blowfly salivary gland, in which cyclic AMP and calcium act as second messengers (Berridge & Prince, 1972b), being activated by the interaction of 5-HT with two pharmacologically distinct types of receptor (Berridge, 1980b; Berridge & Heslop, 1981). Cyclic AMP appears to stimulate a potassium pump (Prince, et. al. 1972),while calcium influences chloride movement in these cells (Prince & Berridge, 1973). The two second messengers may also operate subtle feedback mechanisms by which each is capable of influencing the other (Berridge, 1975).

In the cockroach salivary gland, C.R. House,M.R. Mitchell & R.K. Smith (unpublished) found that neither cyclic AMP or dibutyryl cyclic AMP, applied exogenously at concentrations of up to 1mM, evoked any electrical or secretory responses. This seems

to indicate that cyclic AMP is not involved in fluid secretion from this tissue. Research into stimulus-secretion coupling in the cockroach salivary gland was therefore concentrated on the role of calcium as a second messenger. The influence of calcium on the electrical responses of acinar cells has been described by Ginsborg et.al.(1980a; 1980b), Mitchell & Martin (1980) and Mitchell, et.al., (1980). An account of the investigation into the role of calcium in the secretory response is to be found in Chapter 2, and has been published (Gray & House, 1982).

At the same time as the work described in chapters 2 and 3 was being completed, it was discovered that the cyclic AMP content of cockroach salivary gland acinar cells increased under certain conditions of stimulation (Grewe & Kebabian, 1982). This finding immediately re-awakened interest in the role of cyclic AMP in the secretory response of the gland, and the resultant research is described in this chapter.

Two basic approaches to the problem have been used. Firstly the effects of direct bath application of cyclic AMP on fluid secretion were examined. The second approach which has been used in this part of the work is the manipulation of intracellular cyclic AMP concentration by the use of pharmacological agents which selectively stimulate or inhibit either synthesis or degradation of cyclic AMP, as it is the balance between these two continuous processes which determines the concentration of cyclic AMP in the cell at any time.

It was hoped that it would be possible to use a third approach to investigate the role of cyclic AMP in the secretory response, by measuring intracellular cyclic AMP concentration, and comparing stimulated and unstimulated tissues. The cyclic AMP

content of glands during stimulation in conditions preventing fluid secretion (for example, in chloride-free medium) would have been of particular interest. Attempts were made to measure intracellular cyclic AMP in cockroach acini, using radio-immunoassay. The preliminary experiments yielded promising results but there were a number of technical difficulties, and it was not possible to continue the radio-immunoassay experiments due to lack of time. For this reason the results of radio-immunoassay experiments have been omitted from the main part of this chapter and are described in the appendix.

## METHODS

The methods described in Chapter 2 were used in the experiments described in this chapter.

For the cobalt-containing solution, cobalt chloride was added to calcium-free solution to give a concentration of 5mM.

Cyclic AMP, in both the free acid and sodium salt forms, was obtained from Sigma Chemical Company. Both were dissolved directly in the bathing solution at the required concentration.

Forskolin (7B-Acetoxy-8,13-epoxy-1a,6B,9a-trihydroxy-LABD-14-en-11-one) was obtained from Calbiochem-Behring Corporation. A 15mM stock solution was made up in 95% ethanol and then diluted to the required concentration with bathing solution, as needed (Seamon, Padgett & Daly, 1981).

MDL 12,330A (N-(cis-2-phenylcyclopentyl)azacyclotridecan-2-imine hydrochloride, formerly known as RMI 12,330A) was obtained from Merrell Dow Pharmaceuticals Incorporated. The amount needed to give the required final concentration was dissolved in 2ml polyethylene glycol 400 (Sigma) at slightly above room temperature, before being made up to 100ml with bathing solution.

Theophylline (Sigma) was dissolved directly in the bathing solution to give a concentration of 10mM.

IBMX (3-isobutyl-1-methylxanthine) was obtained from Aldrich Chemical Company. The amount needed to give the required final concentration was dissolved in 0.5ml deoxymethyl sulphate (Sigma) before being made up to 100ml with bathing solution.

A23187 was obtained and used as described in chapter 2 (Methods).

# RESULTS

When isolated salivary glands are bathed in a solution containing cyclic AMP (sodium salt) there is a dose-dependent increase in secretory rate. Figure 4:1 shows a typical secretory response to 100uM cyclic AMP, also shown is the maximal response of the same gland to dopamine stimulation.

The dose-response relationship of the secretory response to cyclic AMP is illustrated in Fig.4:2. There is some variability in the responsiveness of individual glands to exogenous cyclic AMP, as there is in the sensitivity to dopamine. Maximal responses to cyclic AMP were evoked by concentrations of 100 (8 preparations), 200 (1 preparation) or 500 (2 preparations) uM cyclic AMP. In half of the glands tested the maximal responses to cyclic AMP virtually matched the maximal responses of the same glands to dopamine.

In contrast to the responses described above, evoked by the sodium salt of cyclic AMP, no response at all was obtained to a variety of concentrations of the free acid form of cyclic AMP tried on ten preparations. In some experiments both forms of cyclic AMP were applied, separately, to the same gland, the sodium form being active and the free acid form being ineffective. One possible explanation for this apparent anomaly is that the sodium salt might contain some contaminant not present in the free acid, and that the secretory response might be due to the contaminant rather than to the sodium salt of cyclic AMP. Conversely an inhibitory contaminant might be present in the free acid, and not in the sodium salt. Several batches of each form of cyclic AMP

Figure 4:1

Effect of exogenously applied cyclic AMP. Also shown is the maximal response of the same gland to dopamine. The periods during which dopamine (1uM) and cyclic AMP (100uM) were present in the bathing medium are indicated by horizontal bars labelled DA and cAMP respectively.

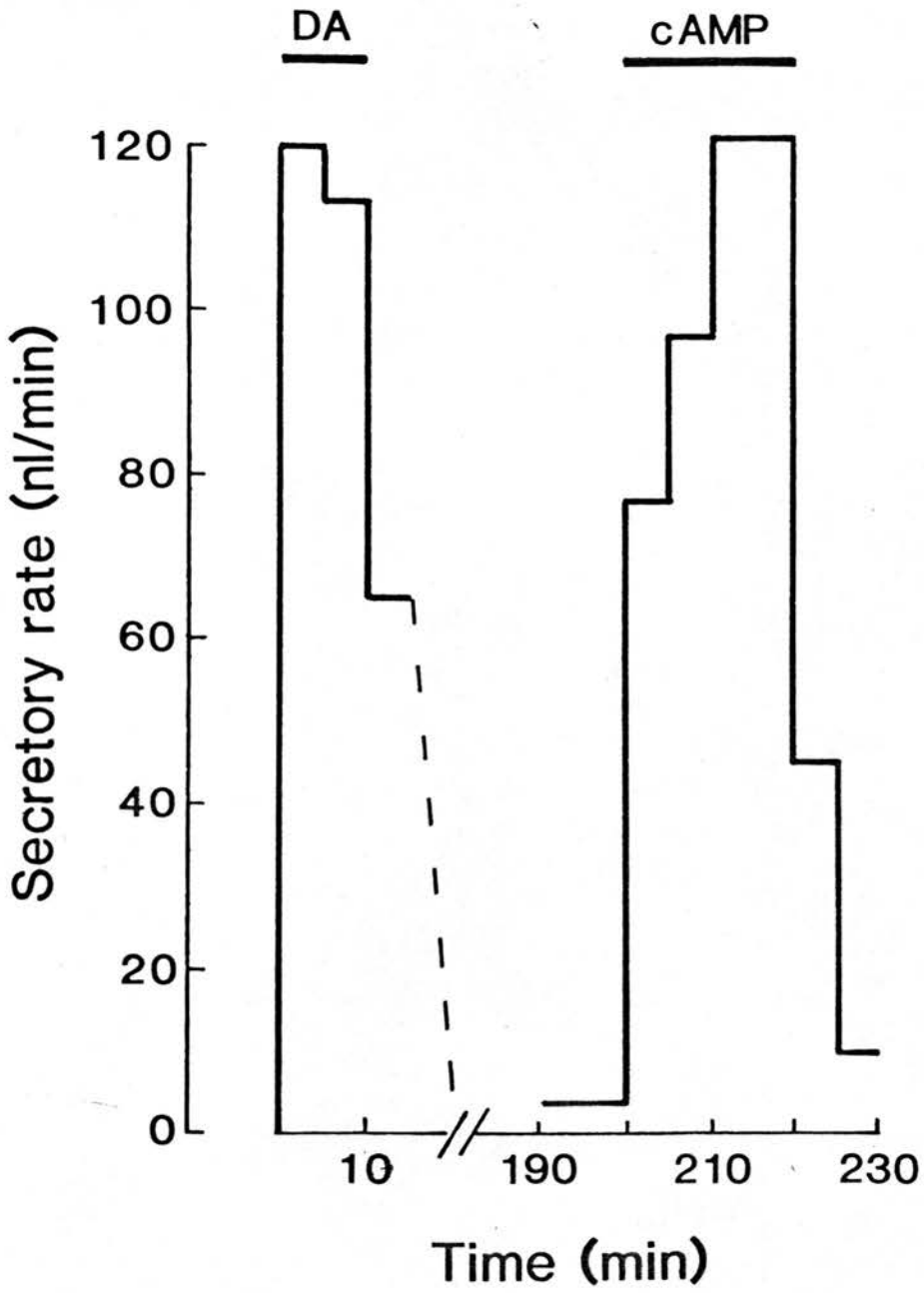
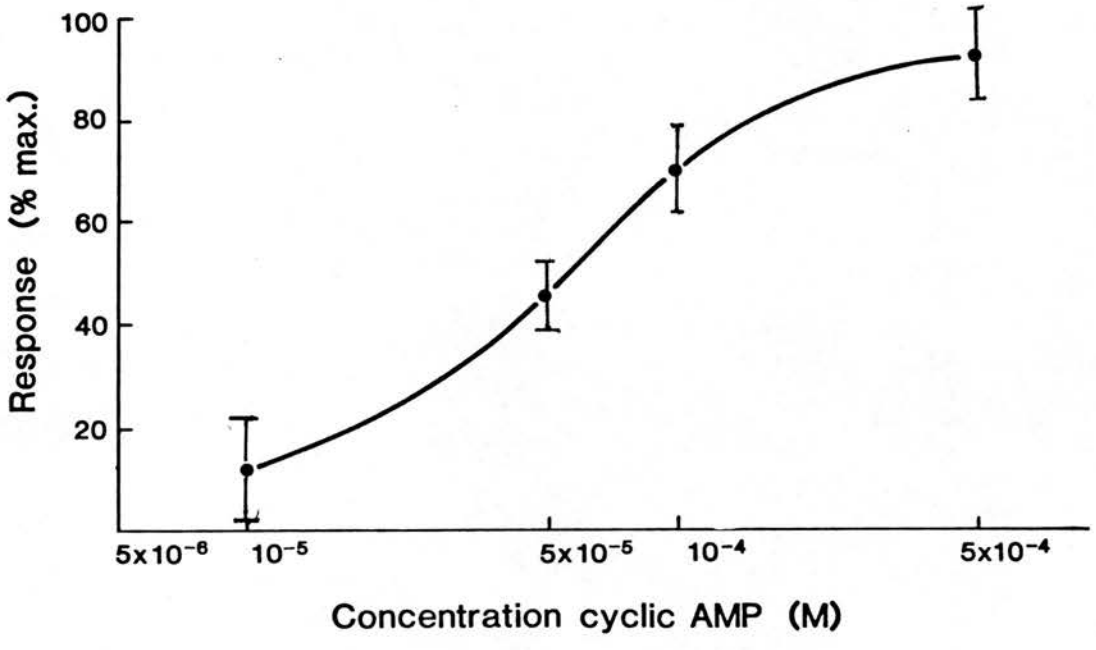

DA
cAMP
120
100
80
60
40
20
0
Secretory rate (nl/min)
10
190
210
230
Time (min)

Figure 4:2

Dose-response relationship for fluid secretion elicited by cyclic AMP. 12 glands were exposed to some or all of a range of concentrations of cyclic AMP from $10^{-5}$M to $5x10^{-4}$M. 22 values were used to calculate the points shown. Responses are shown as percentages of the maximal response of the same gland to dopamine. Standard deviations about the means are indicated by vertical bars.


Response (% max.)
100
80
60
40
20
5x10⁻⁶
10⁻⁵
5x10⁻⁵
10⁻⁴
5x10⁻⁴
Concentration cyclic AMP (M)

were obtained from the manufacturers at different times, and tested, but the results were always the same.

Vanadium is thought to be a contaminant of the sodium salt of cyclic AMP but not of the free acid form. Two pairs of glands were used in control experiments for a vanadium effect; exposure to 100uM sodium metavanadate (added to the control solution) slightly depressed the secretory response of the tissue to dopamine, and had no effect on the basal secretory rate. Thus it seems unlikely that vanadium contamination of the sodium salt of cyclic AMP could account for the observed differences in response to the two forms of cyclic AMP. It seems likely that the free acid form of cyclic AMP contains an unidentified inhibitory contaminant.

Unless otherwise specified, all further references to applied cyclic AMP signify the sodium salt.

Cyclic AMP and phentolamine

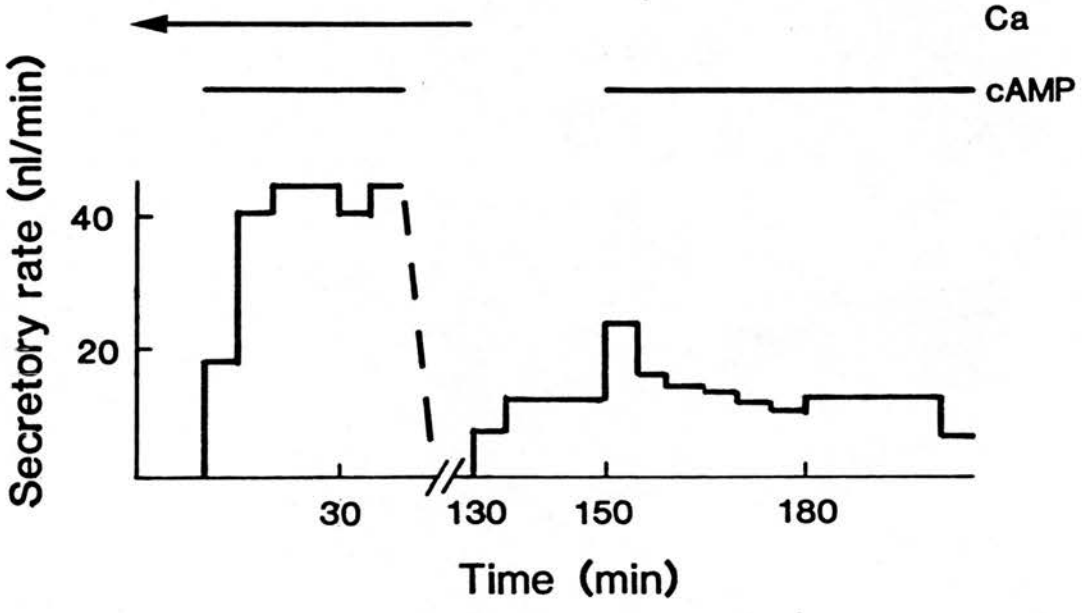
The stimulatory effect of exogenously applied cyclic AMP can be reversibly blocked by phentolamine, at a concentration of phentolamine which blocks the response of the tissue to dopamine stimulation (Bowser-Riley et.al., 1978). This is in accord with the observation that, in rat adipose tissue, alpha-adrenergic blocking agents (including phentolamine) act non-specifically to interfere with the responses to all lipolytic agents, including dibutyryl cyclic AMP (Aulich, Stock & Westermann, 1967).

Cyclic AMP and calcium-free media

Responses to continued exposure to cyclic AMP in normal, calcium-containing solution are well maintained over periods of 30-60 minutes. In calcium-free solution the initial response to

Figure 4:3

Effect of calcium-free solution on the secretory response to cyclic AMP. The periods during which calcium (5mM) and cyclic AMP (100uM) were present in the bathing medium are indicated by horizontal bars labelled Ca and cAMP respectively.


Ca
cAMP
Secretory rate (nl/min)
40
20
30
130
150
180
Time (min)

cyclic AMP is reduced, and declines with continued exposure to cyclic AMP (Fig.4:3). (3 experiments)

When a gland which has been "exhausted" by exposure to cyclic AMP in calcium-free solution is then exposed to dopamine, still in calcium-free solution, there is a small, transient increase in secretory rate (Fig.4:4A). (3 experiments) However, subsequent exposure to cyclic AMP does not elicit any increase in secretory rate from a gland previously "exhausted" by exposure to dopamine in calcium-free solution (Fig.4:4B) (2 experiments)

Cyclic AMP and the "build-up" phenomenon

The experiment, described in chapter 2, designed to look for a build up of second messenger within the cells during stimulation in conditions preventing fluid secretion was repeated using cyclic AMP to stimulate the glands in place of dopamine. The result of a typical experiment is shown in Fig.4:5. (2 experiments). Using the analysis described in chapter 2 the difference between phase III and the sum of phases I and II was positive, that is, on the return of chloride after stimulation of the tissue by cyclic AMP in chloride-free solution the secretory transient was greater than the chloride readmission response and the secretion during the "switching-off" period following cyclic AMP stimulation. This suggests a build up of second messenger during the stimulation period which cannot be expressed as secretion due to the absence of chloride, which outlasts the stimulation and is expressed when chloride is returned to the medium.

When this experiment was repeated with calcium absent from the bathing medium as well as chloride there was no significant difference between the secretory rates following the return of

Figure 4:4

A: Effect of dopamine on a gland previosly exposed to cyclic AMP in calcium-free medium. In B the treatments were reversed. The periods during which calcium (5mM), cyclic AMP (100uM) and dopamine (1uM) were present in the bathing medium are indicated by horizontal bars labelled Ca, cAMP and DA respectively.

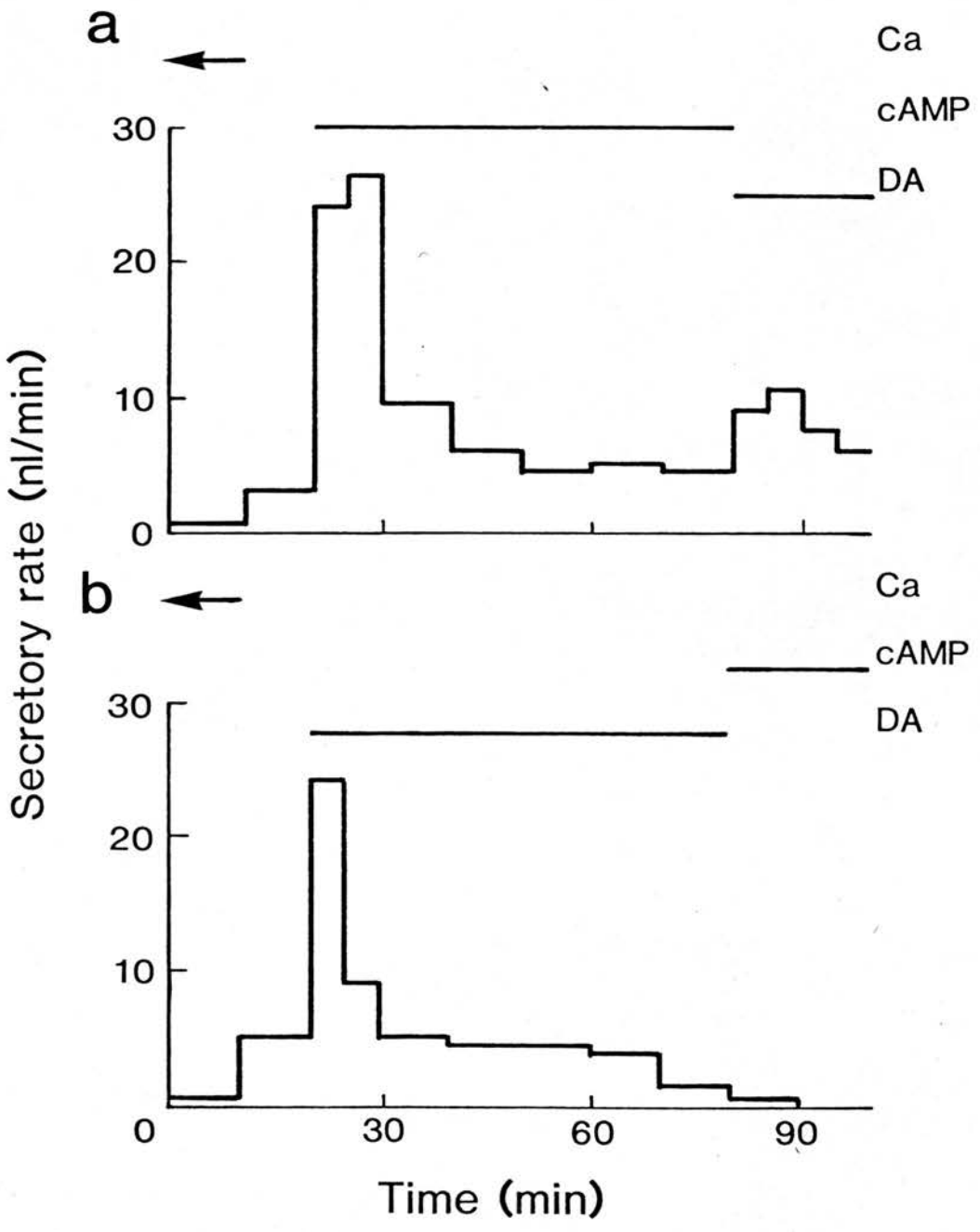
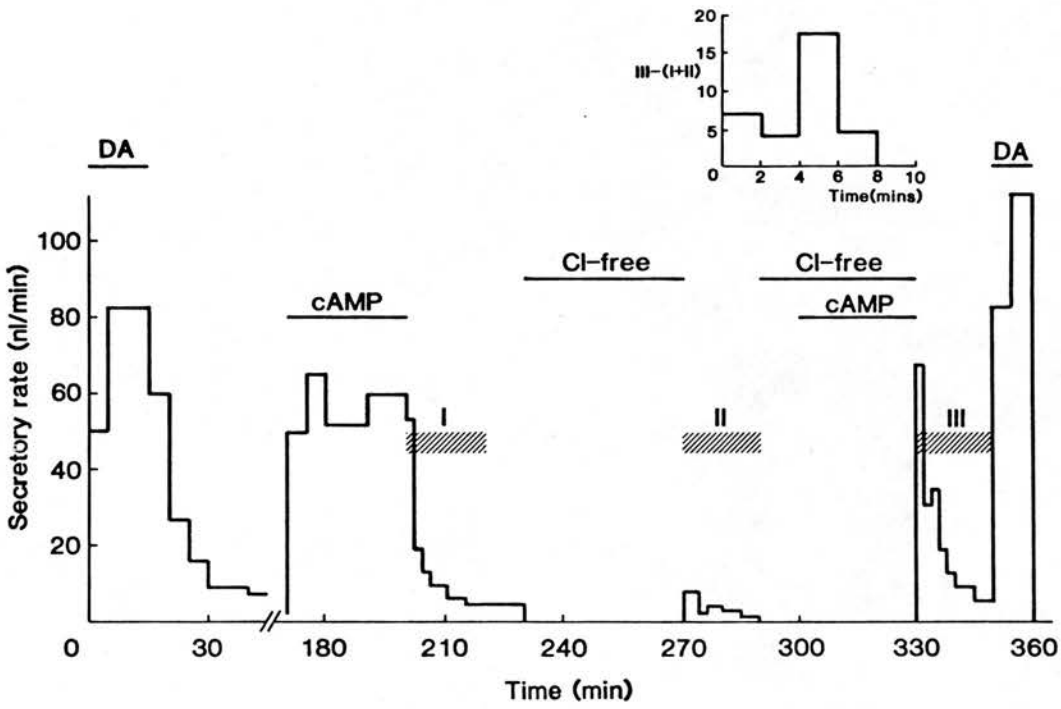

a
Ca
cAMP
DA
30
20
10
0
b
Ca
cAMP
DA
30
20
10
0
30
60
90
Time (min)
Secretory rate (nl/min)

Figure 4:5

Effect of chloride-free solution containing calcium on the secretory response to cyclic AMP. The periods during which chloride-free solution, dopamine (1uM) and cyclic AMP (100uM) were present are indicated by horizontal bars labelled Cl-free, DA and cAMP respectively. The inset shows the analysis of these results. The difference between phase III and the sum of phases I and II has been plotted as a function of time.


DA
cAMP
Cl-free
Cl-free
cAMP
DA
I
II
III
Secretory rate (nl/min)
100
80
60
40
20
0
30
180
210
240
270
300
330
360
Time (min)
III-(I+II)
20
15
10
5
0
2
4
6
8
10
Time(mins)

chloride (and calcium) after a period of absence, or after a period of exposure to cyclic AMP in the absence of chloride and calcium (Fig.4:6). This suggests that a build-up of second messenger in conditions preventing fluid secretion is dependent on the presence of extracellular calcium ions even when the agonist is cyclic AMP.

Further experiments to investigate the role of cyclic AMP in fluid secretion in the cockroach salivary gland were carried out using pharmacological agents to alter the synthesis and degradation of cyclic AMP within the cells.

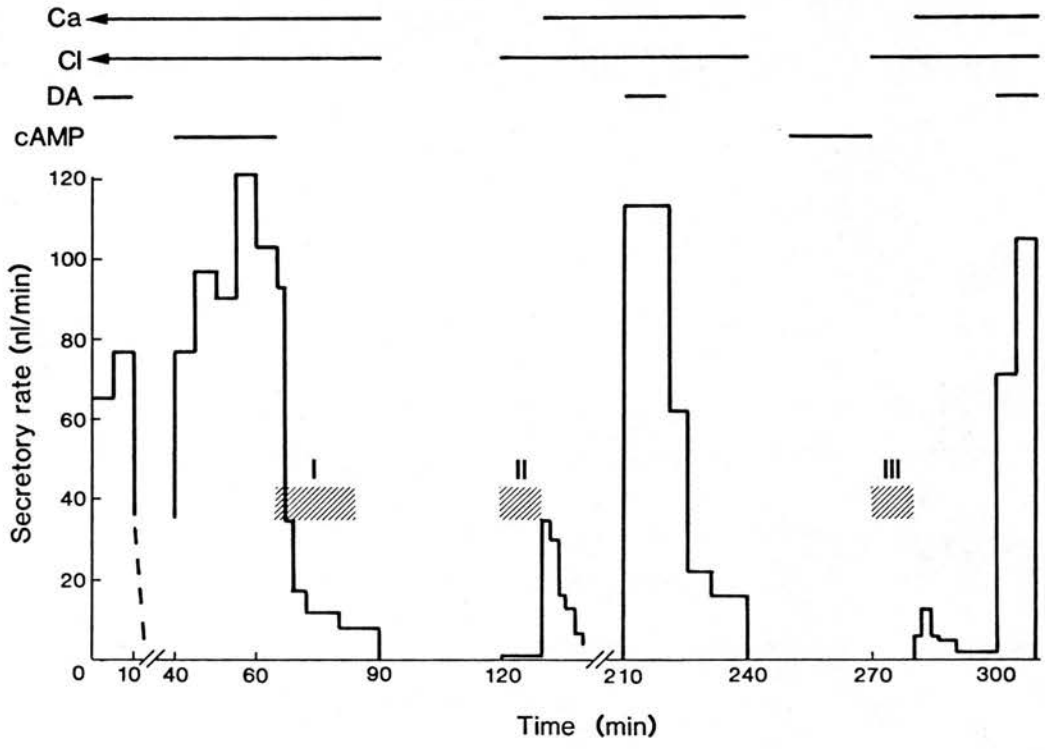
Forskolin

The diterpene forskolin reputedly acts by stimulating adenyl cyclase (Seamon et. al., 1981), the enzyme which synthesises cyclic AMP. Exogenously applied forskolin has a slight stimulatory effect on the cockroach salivary gland. However it is not very satisfactory as a secretagogue as some glands did not respond at all, and in those that did, the maximal response to forskolin was only 30% of the maximal response of the same gland to dopamine. Moreover the sensitivity of individual glands to forskolin varied a great deal,and there did not appear to be a clear dose-response relationship either for individual glands exposed to a range of doses from 0.5uM to 100uM forskolin, or over the six glands tested.

In two control experiments the concentration of ethanol (used to dissolve the forskolin, see Methods) present with the higher doses of forskolin were found to have no effect on basal secretory rate, but had a slight depressant effect on the response of glands

Figure 4:6

Effect of chloride-free, calcium-free solution on the secretory response to cyclic AMP. The periods during which calcium (5mM), chloride (184mM), dopamine (1uM) and cyclic AMP (100uM) were present in the bathing medium are indicated by horizontal bars labelled Ca, Cl, DA and cAMP respectively.


Ca
Cl
DA
cAMP
120
100
80
60
40
20
0
Secretory rate (nl/min)
I
II
III
10
40
60
90
120
210
240
270
300
Time (min)

to dopamine. This could partially mask any stimulatory effect of forskolin.

MDL 12,330A

The compound MDL 12,330A is an inhibitor of adenyl cyclase (Siegel & Wiech, 1976; Guellaen, Mahu, Mavier, Berthelot & Hanoune, 1977; Hunt & Evans, 1980). It reversibly blocks the secretory response to dopamine at a concentration of 100uM (Fig.4:7). At higher concentrations the reversibility is lost. The blocking effect of a lower concentration can be partially overcome by increasing the concentration of dopamine (Fig.4:8).

MDL 12,330A also inhibits secretion caused by the calcium readmission response (Fig.4:9A) (see chapter 2) which is presumably brought about by an influx of calcium ions, and is not related to receptor activation. The calcium readmission responses of cells preconditioned by the inclusion of cobalt in the calcium-free solution (Mitchell et.al.1980) were similarly inhibited. The secretory response to the ionophore A23187 (see chapter 2) is also inhibited by MDL12,330A (Fig. 4:9B).This suggests that in the normal course of stimulus-secretion coupling in this tissue the activation of adenyl cyclase is necessary for secretion, and increasing cytosolic calcium concentration is not sufficient to bring about a secretory response.

The maximum concentration of PEG 400 (used to dissolve the MDL 12,330A) present in the bathing solution was 2%. Tested alone, this concentration of PEG 400 had no effect on the basal secretory rate or the secretory response of the two control glands to dopamine.

Figure 4:7

Effect of MDL 12,330A on the secretory response to dopamine. The periods when dopamine (1uM) and MDL 12,330A (100uM) were present in the bathing medium are indicated by horizontal bars labelled DA and MDL respectively.

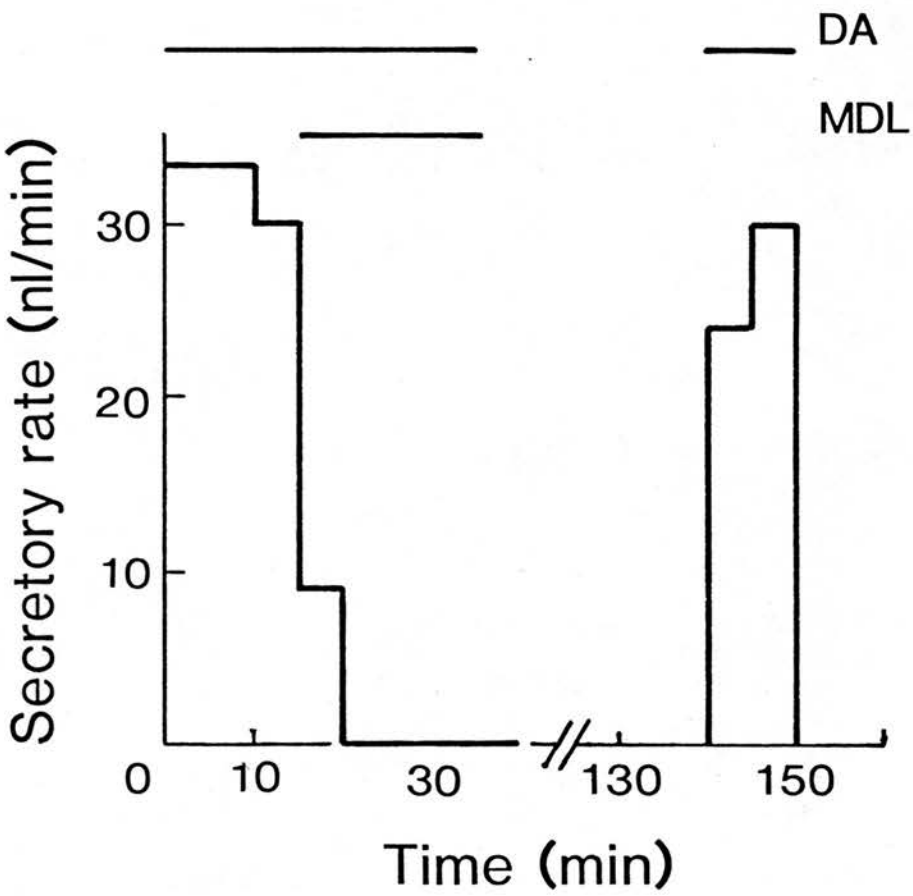

DA
MDL
Secretory rate (nl/min)
30
20
10
0
10
30
130
150
Time (min)

Figure 4:8

Effect of increasing dopamine concentration on the inhibitory effect of a lower concentration of MDL 12,330A. The periods during which MDL 12,330A (50uM) and dopamine were present in the bathing medium are indicated by horizontal bars labelled MDL and DA respectively. The two dopamine concentrations are indicated above the horizontal bars.

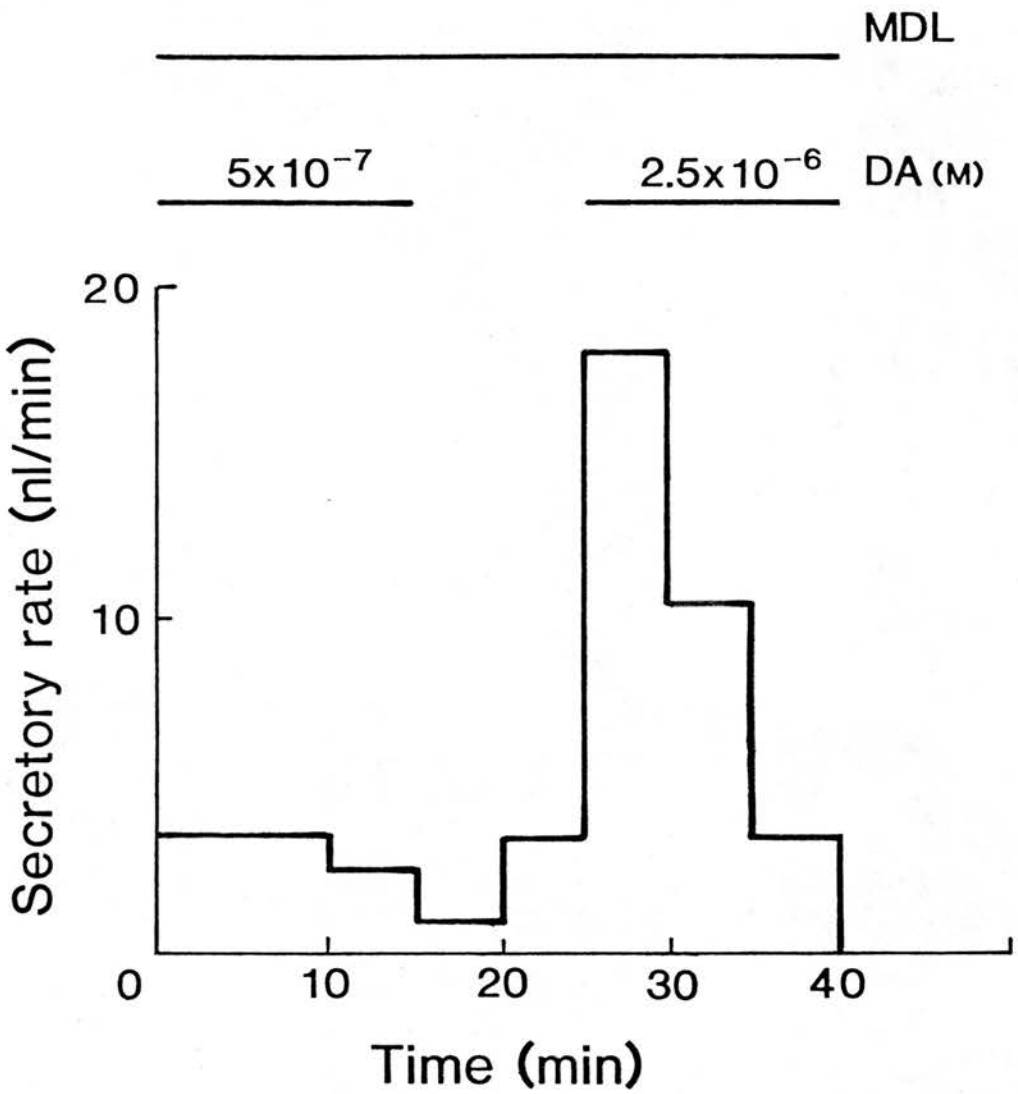
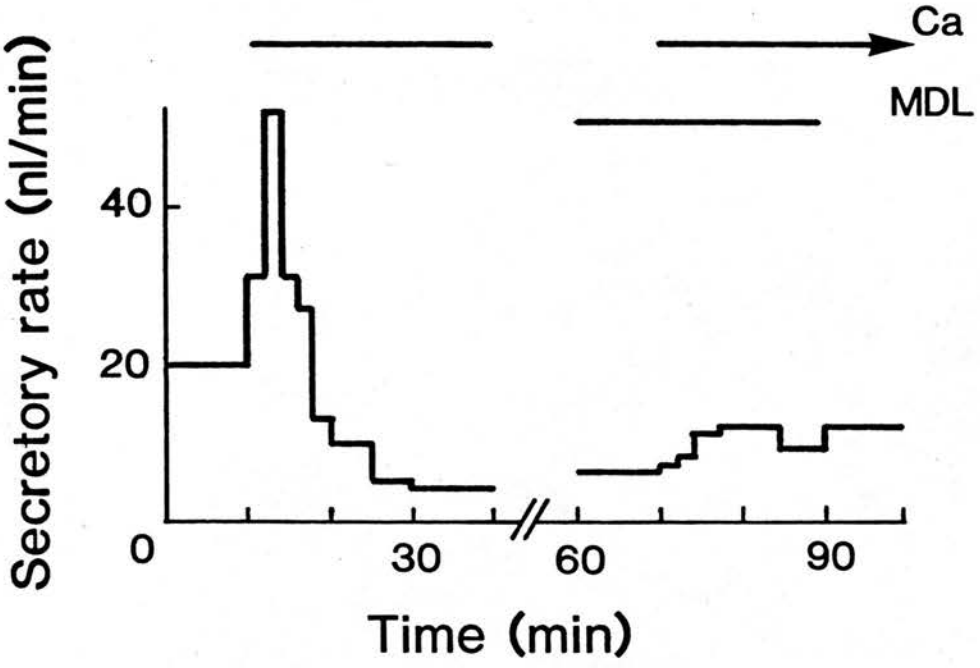

MDL
5x10⁻⁷
2.5x10⁻⁶
DA (M)
20
10
0
10
20
30
40
Secretory rate (nl/min)
Time (min)

Figure 4:9

Effect of MDL 12,330A on the response to calcium readmission. The periods during which calcium (5mM) and MDL 12,330A (100uM) were present in the bathing solution are indicated by horizontal bars labelled Ca and MDL respectively.


Ca
MDL
Secretory rate (nl/min)
40
20
0
30
60
90
Time (min)

Phosphodiesterase inhibitors

As previously observed (C.R.House & R.K.Smith, unpublished) the phosphodiesterase inhibitor theophylline had no effect on the secretory response of the cockroach salivary gland, although it is effective on the blowfly salivary gland (Berridge, 1970). Theophylline at a concentration of 10 mM had no effect on basal secretory rate, secretory response to dopamine stimulation or the build-up phenomenon observed after stimulation in chloride-free medium.

However, another phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) was found to increase the secretory rate in a dose-dependent manner. Five out of seven glands exposed to 100uM IBMX produced a secretory response equal to 75-100% of that of the same gland to maximal dopamine stimulation (Fig.4:10). The dose-response relationship of the secretory response to IBMX is illustrated in Fig.4:11. This was calculated from the responses of 12 glands exposed to some or all of a range of doses of IBMX from 5uM to 1mM.

The secretory response to IBMX is reduced by phentolamine, as shown in Fig.4:12A. This reduction can be briefly and partially overcome by increasing the concentration of IBMX. Increasing dopamine concentration is more effective at overcoming phentolamine blockade of dopamine stimulation (Fig.4:12B).

It was anticipated that a phosphodiesterase inhibitor might enhance the response of the gland to submaximal dopamine stimulation. However when IBMX was used to test this idea the responses to low doses of dopamine were in fact depressed. Figure 4:13 shows an example of one of the 4 experiments of this type, in which the dose of IBMX was chosen to have little intrinsic

Figure 4:10

Effect of IBMX on fluid secretion. Also shown is the maximal response of the same gland to dopamine. The periods when IBMX (100uM) and dopamine (1uM) were present in the bathing medium are indicated by horizontal bars labelled IBMX and DA respectively.

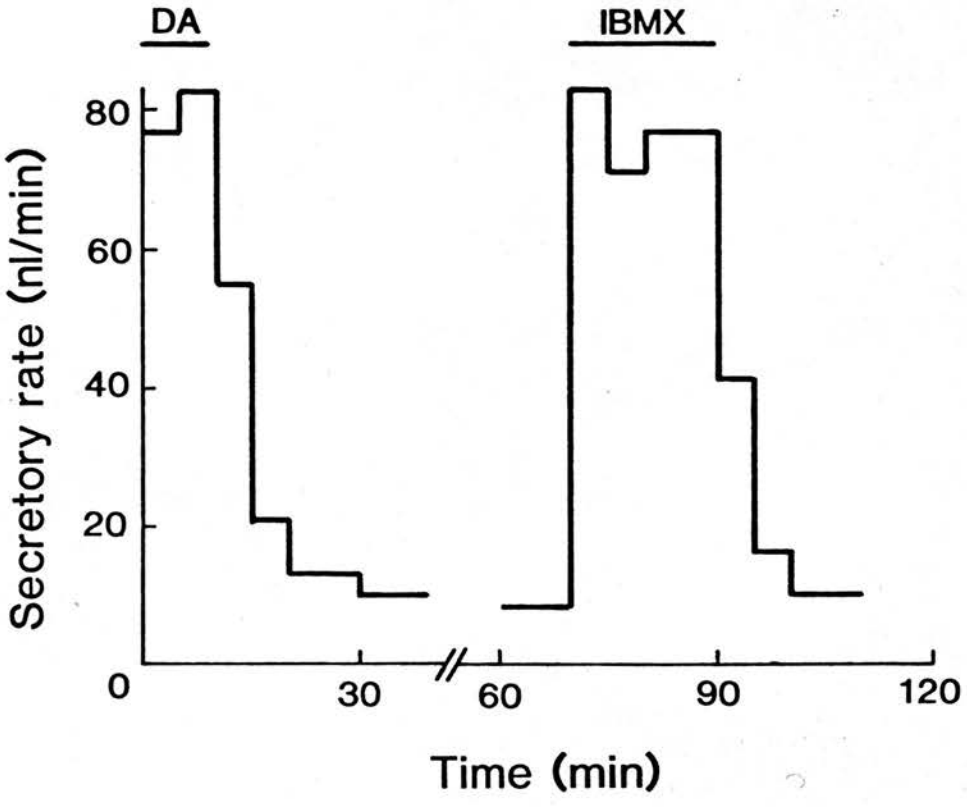

DA
IBMX
80
60
40
20
0
30
60
90
120
Secretory rate (nl/min)
Time (min)

Figure 4:11

Dose-response relationship for fluid secretion elicited by IBMX. 12 glands were exposed to some or all of a range of concentrations of IBMX from $5 \times 10^{-6}$M to $10^{-3}$M. 36 values, expressed as percentage of the maximal response of the same gland to dopamine, were used to calculate the points shown, each of which is the mean of the values obtained for that concentration of IBMX. Standard deviations about the means are indicated by vertical bars.

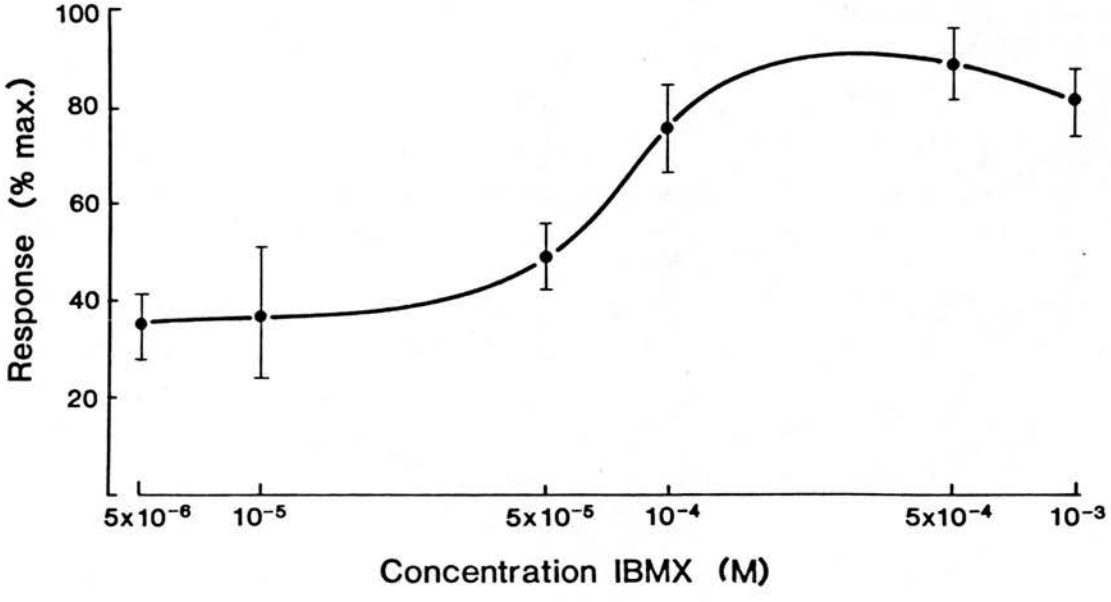

100
80
60
40
20
Response (% max.)
5x10⁻⁶
10⁻⁵
5x10⁻⁵
10⁻⁴
5x10⁻⁴
10⁻³
Concentration IBMX (M)

Figure 4:12

Effect of phentolamine on the secretory responses to (A) IBMX and (B) dopamine. The effects of increasing the concentration of IBMX and dopamine are also shown. The periods during which IBMX, dopamine and phentolamine (100uM) were present in the bathing medium are indicated by horizontal bars labelled IBMX, DA and PHENT respectively. Concentrations of IBMX and dopamine are indicated above the horizontal bars.

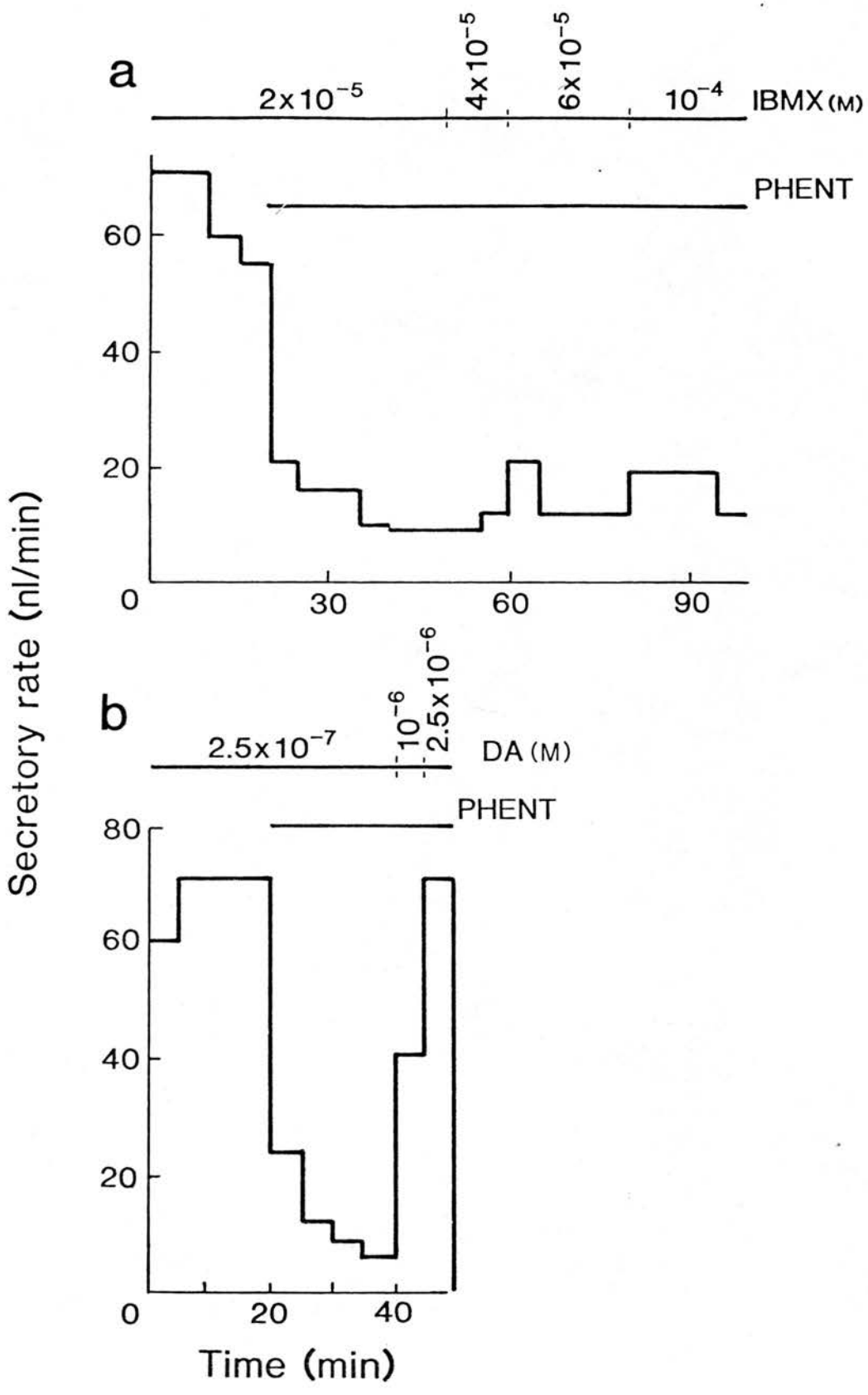
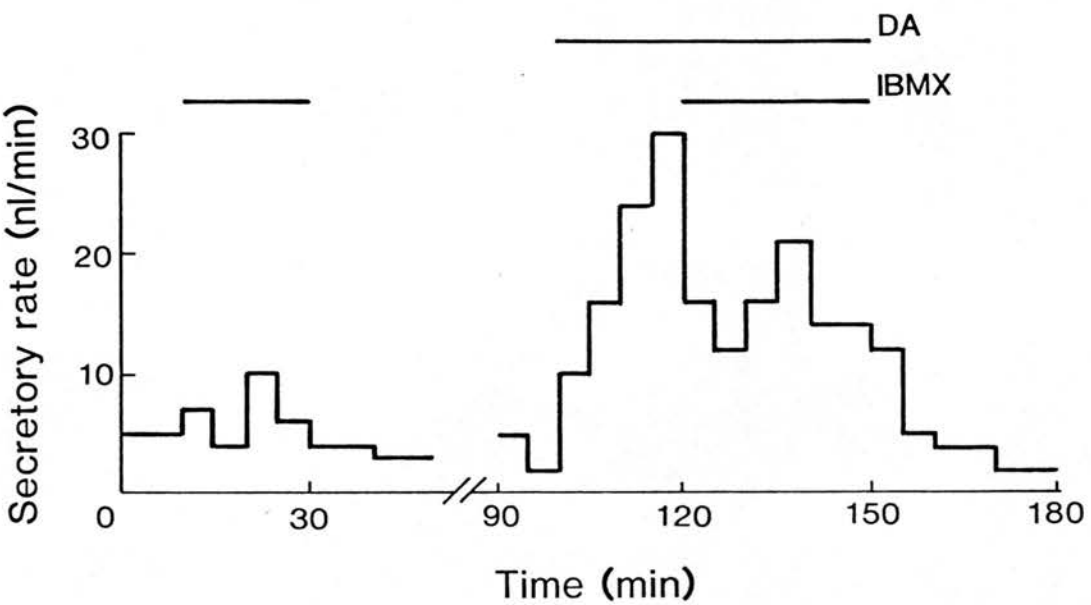

a
2x10⁻⁵
4x10⁻⁵
6x10⁻⁵
10⁻⁴
IBMX (M)
PHENT
60
40
20
0
30
60
90
b
2.5x10⁻⁷
10⁻⁶
2.5x10⁻⁶
DA (M)
PHENT
80
60
40
20
0
20
40
Time (min)
Secretory rate (nl/min)

Figure 4:13

Effect of a low concentration of IBMX on a submaximal secretory response to dopamine. The periods during which dopamine (0.01uM) and IBMX (5uM) were present in the bathing medium are indicated by horizontal bars labelled DA and IBMX respectively.


DA
IBMX
Secretory rate (nl/min)
30
20
10
0
30
90
120
150
180
Time (min)

stimulatory effect on the secretory rate. This suggests that IBMX may have part of its stimulatory effect by acting as a partial agonist, and that it may reduce the accessibilty of receptors to dopamine.

The effect of calcium-free solution on the response to IBMX is essentially similar to that on the response to exogenously applied cyclic AMP. The initial response to IBMX is lower in calcium-free solution than in control solution, and the response to maintained stimulation with IBMX declines in calcium-free solution in 3 out of 4 experiments (Fig.4:14). Stimulation of the gland with dopamine in calcium-free solution after "exhaustion" of the response by stimulation with IBMX in calcium-free solution produces a small transient increase in secretory rate. This is shown in Fig.4:15A which is a typical example of the 3 experiments of this type. Conversely, addition of IBMX to the calcium-free solution bathing a gland previously "exhausted" by dopamine stimulation in calcium-free medium does not, as shown in Fig.4:15B which is typical of 4 such experiments.

The stimulatory effect of IBMX is rapidly blocked by the adenyl cyclase inhibitor MDL 12,330A when the two compounds are applied simultaneously. Figure 4:16 is an actual record of one of the three experiments of this type. This is a further indication that part of the effect of IBMX is due to stopping the degradation of cyclic AMP which is normally kept at a constant level by being continuously synthesised and broken down within the cell.

Figure 4:14

Effect of calcium-free solution on the secretory response to IBMX. The periods during which calcium (5mM) and IBMX (100uM) were present in the bathing medium are indicated by horizontal bars labelled Ca and IBMX respectively.

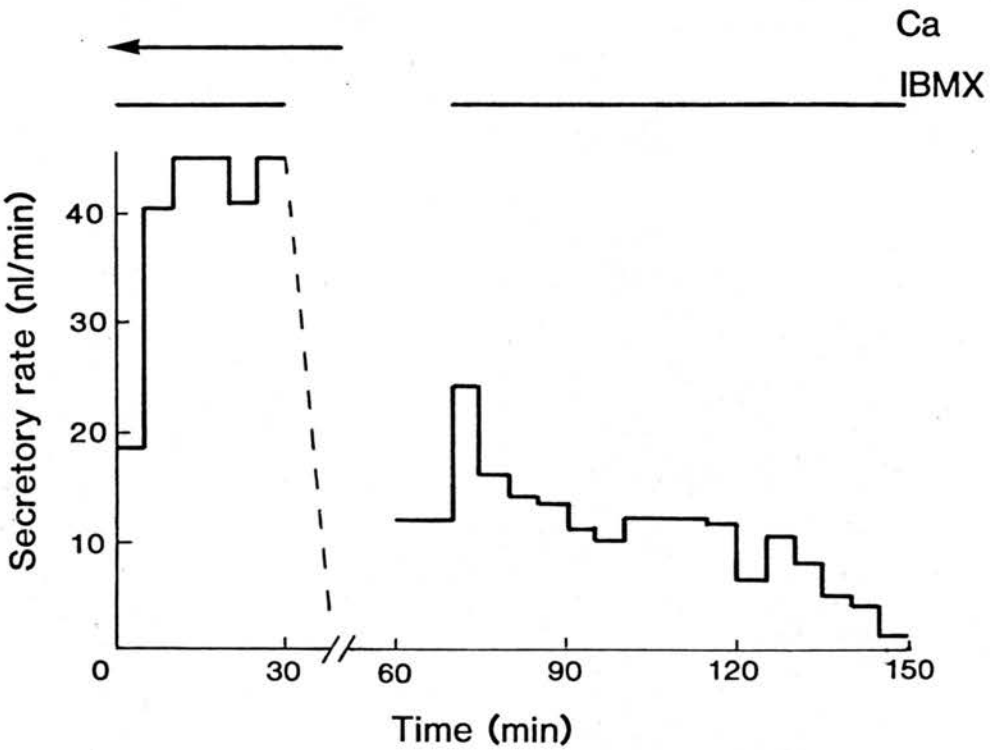

Ca
IBMX
Secretory rate (nl/min)
40
30
20
10
0
30
60
90
120
150
Time (min)

Figure 4:15

a: Effect of dopamine on a gland previously exposed to IBMX in calcium-free solution. In b the treatments were reversed. The periods during which dopamine (1uM) and IBMX (100uM) were present in the bathing medium are indicated by horizontal bars labelled DA and IBMX respectively.

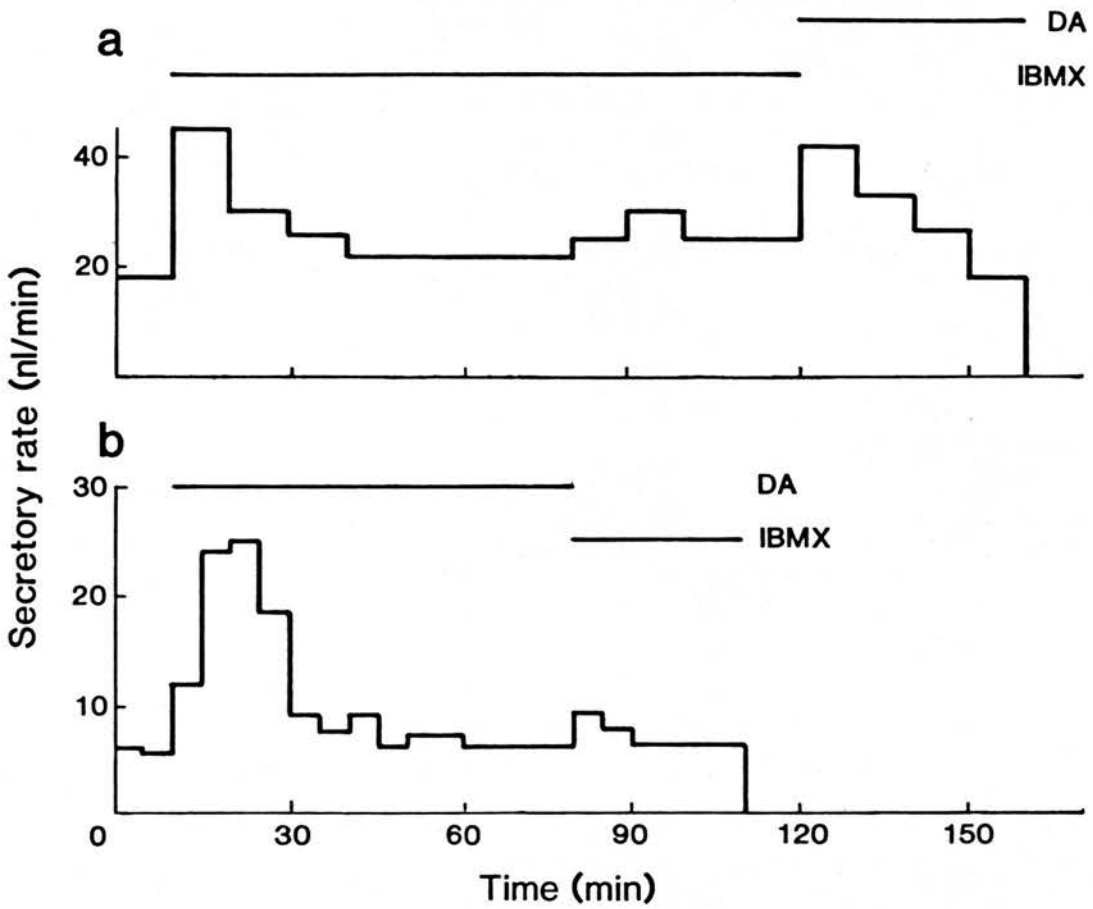
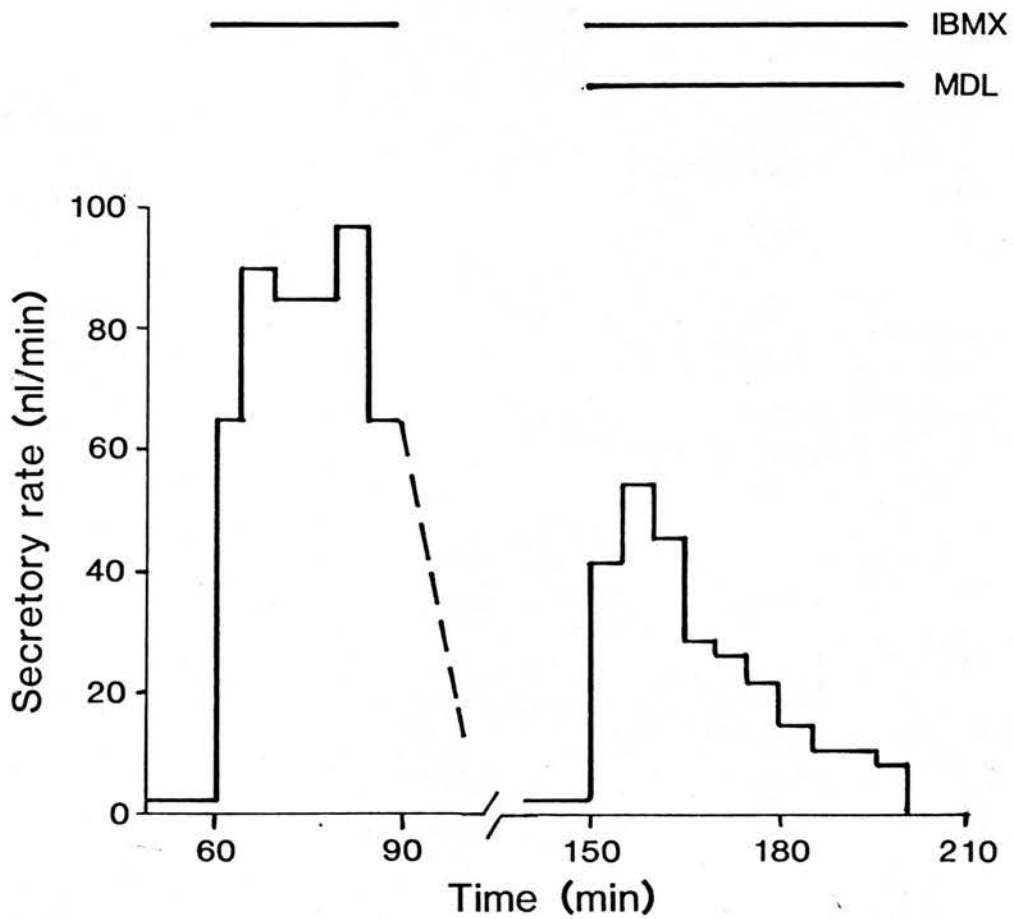

a
DA
IBMX
40
20
b
30
DA
IBMX
20
10
0
30
60
90
120
150
Secretory rate (nl/min)
Time (min)

Figure 4:16

Effect of MDL 12,330A on the secretory response to IBMX. The periods during which IBMX (100uM) and MDL 12,330A (100uM) were present in the bathing medium are indicated by horizontal bars labelled IBMX and MDL respectively.


IBMX
MDL
100
80
60
40
20
0
Secretory rate (nl/min)
60
90
150
180
210
Time (min)

# DISCUSSION

Exogenously applied cyclic AMP is capable of stimulating maximal rates of fluid secretion from the cockroach salivary gland at doses of 0.1-0.5mM, and thereby of mimicing the action of the neurotransmitter, dopamine. It is also known to mimic the actions of many other neurotransmitters and hormones; for example, ACTH (Haynes et.al., 1960), vasopressin (Orloff & Handler, 1962; Grantham & Burg, 1966) and melanocyte stimulating hormone (Bitensky & Burstein, 1965). Most relevant to the present work is the effect of cyclic AMP on the blowfly salivary gland (Berridge, 1975). In that tissue the response to cyclic AMP can equal the maximal response to 5-HT, which is believed to be the neurohormone, although the dose of cyclic AMP required for this is 10mM, much greater than in the cockroach. This discrepancy may be due to permeability differences.

Cyclic AMP is a large, negatively charged, phosphorylated molecule. This may lead to the plasma membrane of some types of cells having only low permeability to it, which may account for the relatively high concentrations required to stimulate many cell types. In some systems derivatives of cyclic AMP may be better able to penetrate the cell membrane, and thus more effective than the parent compound. For example; the dibutyryl derivative is more effective as a stimulant of secretion in rat parotid slices (Bdolah & Schramm, 1965), and in lipolysis in rat adipose tissue (Butcher et.al., 1965) while $N^6$-monobutyryl cyclic AMP is more effective as a stimulant of amylase release from the rat parotid (Babad, et.al., 1967).

As illustrated in Figure 4:2, the steep part of the dose-response curve for cyclic AMP lies between 0.02 and 0.2mM -that is, it is spread over a 10-fold concentration range. This also applies to the blowfly salivary gland, where although the concentrations required are 50 times larger the effective range is from 1-10mM. In the presence of theophylline, the dose response curve for the blowfly gland is shifted to the left, whereas theophylline does not produce such a potentiation in the cockroach gland.

In broken cell preparations the optimal doses of cyclic AMP for its effects on enzymes are mostly around 1uM. In mouse liver extracts the optimal dose for phosphorylation of glycogen synthetase is 1-3uM (De Wulf & Hers, 1961; Walkenbach, Hazen & Larner, 1978); and in skeletal muscle the optimal dose for the phosphorylation of casein is 0.5-1uM (Walsh, Perkins & Krebs, 1968). The high concentrations of extracellular cyclic AMP required to evoke secretion from cockroach and especially blowfly salivary glands may reflect the low permeability of cell membranes to cyclic AMP.

The stimulatory effect of bath applications of cyclic AMP on fluid secretion from the cockroach salivary gland was somewhat surprising in the light of previous obervations that concentrations of up to 1mM cyclic AMP did not evoke secretory or electrical responses from the tissue (C.R. House, M.R. Mitchell, R.K. Smith, unpublished). However, the free acid form of cyclic AMP had been used in these earlier experiments, and despite a number of tests, secretory responses have only been evoked by the sodium salt of cyclic AMP. Interestingly, recent

electrophysiological experiments (C.R. House & B.L. Ginsborg, unpublished) have failed to find any electrical response to doses of the sodium salt which evoke large secretory responses.

Why the gland should respond differently to the two forms of cyclic AMP remains something of a mystery. The preparation is fairly sensitive to changes in the pH of the bathing solution, but at the concentrations used neither form of cyclic AMP had any measureable effect on the pH of the solution. It is possible that a contaminant present in only one form of cyclic AMP could be responsible for the difference in potency. Tests with vanadium, a potential contaminant believed to be present in only the sodium salt of cyclic AMP, showed that it is unlikely to be responsible as its effect on fluid secretion is, if anything, slightly inhibitory.

The fact that phentolamine reversibly blocks the secretory response of the gland to exogenous cyclic AMP may at first suggest that cyclic AMP acts via membrane receptor activation. However Aulich et. al. (1967) found that in rat adipose tissue, phentolamine and other alpha-adrenergic blockers interfere with the lipolytic responses to lipolytic agents including cyclic AMP, possibly at a stage after the accumulation of cyclic AMP in the cells. Phentolamine may therefore have actions unrelated to blockade of neurotransmitter receptors.

In the pancreatic beta cell insulin release is controlled by alpha- and beta-adrenoceptors which mediate divergent effects of adrenaline on the synthesis of cyclic AMP, alpha receptors decreasing and beta receptors increasing it (Porte, 1967; Malaisse, Malaisse-Lagae, Wright et.al., 1967; Gagliardino, Hernandez & Rodriguez, 1968), with the alpha effects usually

predominant. Turtle, Littleton and Kipnis (1967) found that injection of theophylline alone caused release of insulin, injection of theophylline during infusion of adrenaline still caused an eight-fold increase in plasma insulin; when the alpha-adrenoceptors were blocked and the experiment repeated a 30-40-fold increase was observed, but when the experiment was repeated under conditions of beta blockade, the theophylline injection had no effect.

Another possible explanation for the ability of phentolamine to block the secretory response to exogenous cyclic AMP is to suppose that, rather than penetrating the cell and elevating intracellular cyclic AMP concentration directly, exogenously applied cyclic AMP may stimulate membrane sited adenyl cyclases. If the receptors for the neurotransmitter are closely associated with the adenyl cyclase systems, occupation of the receptor by an antagonist could interfere with the activation of the adenyl cyclase by exogenous cyclic AMP. However, against this idea is the finding in rat cerebral cortex slices that the elevation of cyclic AMP induced by the adenyl cyclase activator forskolin is not inhibited by neurotransmitter antagonists including 10uM phentolamine (Seamon, et. al., 1981), although the way in which forskolin activates the enzyme may be quite different from the way in which cyclic AMP does so.

Responses to cyclic AMP in calcium-free solution

As with responses to dopamine (see Chapter 2), the increase in secretory rate in response to exogenous cyclic AMP in calcium free solution is transient. This indicates that extracellular

calcium is required for responses to cyclic AMP to be maintained, but suggests that the cell has some available calcium reserves to support secretion until these reserves are depleted.

Following "exhaustion" of the response to cyclic AMP in calcium-free solution a small transient increase in secretory rate can be elicited by dopamine stimulation; but when "exhaustion" of the response to dopamine stimulation in calcium-free solution is followed by application of cyclic AMP no increase is seen. This suggests that the calcium store available to dopamine is larger than that available to cyclic AMP.

Since artificially increasing the intracellular cyclic AMP level is not sufficient to bypass the calcium-requiring step or steps, these results indicate that calcium must be required at a stage of stimulus-secretion coupling subsequent to cyclic AMP synthesis. However, if one chooses to interpret the stimulation of the gland by exogenous cyclic AMP in terms of stimulation of adenylate cyclase the results discussed above indicate a calcium requirement for cyclic AMP synthesis. Of course these two possible roles for calcium are not mutually exclusive.

The experiments with forskolin, MDL 12,330A and IBMX were carried out in order to discover whether it was possible to place the actions of calcium or cyclic AMP in any sequence in stimulus secretion coupling.

The adenylate cyclase inhibitor MDL 12,330A at 0.1mM effectively inhibits secretory responses to all stimulants tested in the cockroach salivary gland. Guellaen, Mahu, Mavier, Hanoune & Berthelot (1978) have demonstrated non-specific inhibition of a number of membrane-bound enzyme systems in rat liver by this compound. This is cause for concern about how the inhibitor is

acting to prevent fluid secretion. The main precaution taken against non-specific effects in this tissue was to use the minimum effective dose, at which level the inhibition was reversible. The observation that at a low concentration (0.05mM) of MDL a submaximal response to dopamine is still possible, and such a response can be increased by increasing dopamine concentration. This might indicate that the main metabolic processes are not seriously affected by the inhibitor, but further control experiments are needed.

If one can assume that , at the concentration used, MDL is specifically acting on adenylate cyclase the following conclusions may be drawn on the basis of the experiments using this compound. The first is that synthesis of cyclic AMP is an absolute requirement for fluid secretion. Increasing intracellular calcium concentration by means of either the ionophore A23187 (Reed & Lardy, 1972; Mitchell & Martin, 1980) or readmission of calcium following incubation in calcium-free solution (Ginsborg et. al., 1980b;Gray & House, 1982; Chapter 2) did not stimulate fluid secretion in the presence of MDL 12,330A, indicating that the cyclic AMP-requiring step in stimulus-secretion coupling cannot be bypassed in this way. Finally, inhibiting the breakdown of cyclic AMP by exposure to IBMX is not sufficient to induce fluid secretion when adenylate cyclase is inhibited.

Exposure of glands to the phosphodiesterase inhibitor IBMX in control solution is sufficient to cause an increase in secretory rate without further stimulation. The most attractive explanation for this is that, as the level of cyclic AMP in the cell is maintained by a balance of synthesis and degradation of the

nucleotide, inhibiting its degradation permits the concentration of cyclic AMP to rise to a level where fluid secretion occurs. The speed of the response supports this explanation -see Fig.4:10. The dose-response curve for IBMX is very similar to that for cyclic AMP.

The observation that the receptor blocker phentolamine reduces the response to IBMX may suggest some involvement of receptors in the response. However it is possible in this instance, as suggested in the case of exogenous cyclic AMP, that phentolamine may be acting non-specifically, possibly at a stage subsequent to cyclic AMP synthesis. It is also possible that phentolamine interferes with the synthesis of cyclic AMP because the adenylate cyclase may be closely associated with the receptor at which the phentolamine is acting.

If cyclic AMP is indeed a second messenger in this system, and dopamine stimulated fluid secretion is associated with an increase in intracellular cyclic AMP, one would expect that a phosphodiesterase inhibitor would increase the effect of submaximal dopamine stimulation. In fact this is not what is observed -a low concentration of IBMX actually slightly depresses submaximal responses of the gland to dopamine stimulation. One possible explanation for this is that IBMX could be acting as a partial agonist which is less effective at stimulating secretion than dopamine but which displaces dopamine from a proportion of the receptors when the two are present together.

Another possible explanation for the stimulatory effect of IBMX is that IBMX might be liberating intracellularly bound calcium, or promoting an influx of calcium into the cell. This could mimic the action of dopamine -see Chapter 2. However such a

translocation of calcium cannot be the sole cause of IBMX-stimulated secretion because the adenylate cyclase inhibitor blocks the response to IBMX.

The response to IBMX shows the same calcium dependence as the responses to dopamine and to cyclic AMP. As with cyclic AMP stimulation, stimulation with IBMX seems to leave some of the calcium-dependent store available for subsequent response to stimulation by dopamine to be possible, whereas after dopamine stimulation IBMX cannot stimulate a further increase in secretory rate in calcium-free solution. This speaks against the effects of IBMX being due to liberation of calcium, although it does not rule out such a mechanism.

It was hoped to compare the effects of an adenyl cyclase activator in the presence and absence of calcium. However the response of cockroach salivary glands to forskolin was not consistent in control solution. In rat cerebral cortex forskolin causes a rapid and reversible activation of adenylate cyclase and accumulation of cyclic AMP Indeed adenylate cyclases in all rat tissues tested were found to be significantly activated by forskolin (Seamon et. al. 1981). They also found that forskolin activation of membrane adenylate cyclase was not dependent on calcium -all their experiments were carried out in the presence of EGTA. However, calcium is required for activation of adenylate cyclase by hormones and neurotransmitters (Dartt, Torp-Pedersen & Thorn, 1981), and this may reflect the regulatory role of calcium-calmodulin interactions in the activation of brain adenylate cyclase (Cheung, Bradham, Lynch, Lin & Tallant, 1975). Thus two things seem to indicate that the way in which forskolin activates

adenylate cyclase may be different to the way in which the enzyme is activated by a hormone or neurotransmitter; firstly, the difference in calcium requirement mentioned above, and secondly the fact that forskolin stimulation of adenylate cyclase is not inhibited by neurotransmitter antagonists (Seamon et.al., 1981).

As described in Chapter 2, a persistent change takes place in glands stimulated in conditions preventing fluid secretion, which is expressed on return of the missing ions as an increase in secretory rate over and above the normal readmission response. This also occurs when glands are exposed to cyclic AMP in conditions preventing fluid secretion (i.e. in chloride-free solution). The precise nature of the change is not known in either case, although in both cases it has been shown to depend on the presence of extracellular calcium ions during the stimulation period. It seems likely that the change is an accumulation in the cells of a second messenger, possibly either calcium or cyclic AMP.

The fact that the increase in secretory rate is dependent on calcium even when the gland is exposed to cyclic AMP in chloride-free solution indicates that calcium is still necessary, either at a stage subsequent to the accumulation of cyclic AMP, or that calcium is necessary for the synthesis of cyclic AMP by cyclic AMP activated adenylate cyclase; or that the change is due to an accumulation of calcium itself, or some other calcium-dependent second messenger. Whereas the evidence implicating calcium as a second messenger in cockroach glands is indirect, it has been demonstrated that dopamine stimulation increases the cyclic AMP content of the acinar cells in these glands.

# APPENDIX

## Radio immunoassay of cyclic AMP in cockroach salivary gland acinar cells

Some preliminary experiments were carried out in order to measure intracellular cyclic AMP by means of radio immunoassay (RIA). The basic principle of radio immunoassay is the competition between radioactive and non-radioactive antigen for a fixed number of binding sites. If increasing (known) amounts of non-radioactive antigen (the standards) and a fixed amount of radioactive antigen are allowed to react with a constant amount of antibody a decreasing amount of the radioactive antigen is bound to the antibody. This relationship can be expressed as a standard curve. If the reaction is repeated with unknown amounts of non-radioactive antigen (the samples) the concentration of unlabelled antigen can be deduced from the standard curve.

Pre-assay treatments were devised to relate to some of the experimental treatments used in the secretory experiments on the role of cyclic AMP in fluid secretion and stimulus-secretion coupling. It was hoped that it might be possible to correlate increases in cyclic AMP content with conditions stimulating fluid secretion.

### Methods

Tissue Preparation A suspension of acinar cells was prepared

following the method of Grewe and Kebabian (1982). Ten pairs of glands were dissected out, and the acini were carefully teased off the ducts and suspended in 2ml control solution. 60ul of the cell suspension was used in each assay tube.

Pre-assay Treatments The pre-assay treatments were as follows:

| Solution | Drug(s) | Time |
|---|---|---|
| 1 Control | none | |
| 2 Control | dopamine (1uM) | 15 min |
| 3 Control | IBMX (100uM) | 20 min |
| 4 Control | forskolin (100uM) | 20 min |
| 5 Control | dopamine (1uM) + MDL (100uM) | 15 min |
| 6 Chloride-free | dopamine (1uM) | 60 min |

At the end of the incubation period the tubes were boiled for two minutes and then centrifuged.

Assay The assays were carried out using a cyclic AMP radio immunoassay kit
supplied by New England Nuclear, which is adapted from the procedures of Steiner, Parker and Kipnis (1972), with the addition of acetylation of the samples (Harper & Brooker,1975) which increases the sensitivity of the assay, so that it is capable of measuring between 0.0025 and 25.0 picomoles of cyclic AMP per assay tube (manufacturers` specification).

Counting Assay tubes were counted for gamma radiation in an ICN Tracerlab gamma/guard 150 counter for 1 minute per tube.

Tritiated samples (for recovery determination) were counted in an ICN Tracerlab coru/matic 200 counter for 10 minutes per tube.

## Results

The results of the RIA experiments are shown in table 4A:1

Although the number of samples is small, and in some cases there is a considerable variation around the mean, these results suggest that it might be worthwile to continue this method of investigation in order to gain useful information on what happens to the intracellular concentration of cyclic AMP during stimulus-secretion coupling.

Table 4A:1 Results of cyclic AMP determinations. Each value is the mean of 8 determinations, except where indicated otherwise. The standard deviation about the mean is also given.

| | Treatment | Cyclic AMP content (picomoles/gland) |
|---|---|---|
| 1 | Control | 0.08 ± 0.02 |
| 2 | Dopamine | 0.29 ± 0.11 |
| 3 | IBMX | 0.14 ± 0.04 |
| 4 | Forskolin | 0.52 ± 0.1 (n=7) |
| 5 | Dopamine + MDL | 0.1 ± 0.02 |
| 6 | Chloride-free + dopamine | 0.09 ± 0.03 (n=6) |

# CHAPTER 5

# DISCUSSION

The results presented in the preceding chapters are taken as evidence that both calcium and cyclic AMP act as second messengers in stimulus-secretion coupling in the cockroach salivary gland. Not long after proposing the second messenger hypothesis, Sutherland and co-workers set out criteria by which the hypothesis could be verified in any particular case (Sutherland, Robison & Butcher, 1968). Although at the time at which these criteria were formulated cyclic AMP had only been considered as a second messenger in hormone action, they can reasonably be applied to systems where cyclic AMP might mediate the actions of neurohormones or neurotransmitters, for example in the salivary glands of the blowfly and cockroach.

The four original criteria were:

(1) The hormone should be capable of increasing the intracellular level of cyclic AMP in the target cells, while inactive hormones should not. It should be demonstrated that the effect on the level of cyclic AMP occurs at a concentration of the hormone which is at least as small as the minimum capable of producing a physiological response. The increase in cyclic AMP concentration or content should precede, or at least not follow, the physiological response.

(2) It should be possible to potentiate the hormone (i.e. increase the magnitude of the physiological response) by administering the hormone together with methylxanthines or other phosphodiesterase inhibitors.

(3) It should be possible to mimic the physiological effect of the hormone by administering exogenous cyclic AMP.

(4) it should be possible to demonstrate the presence of a hormone-sensitive adenylate cyclase in the plasma membrane fraction of the cell.

A fifth criterion, that a cyclic AMP-dependent protein kinase should be activated, was added later, following the development of the extended second messenger model by Greengard and Kuo (1969) which proposed that all the diverse effects of intracellular cyclic AMP were mediated by a common mechanism, namely the activation of a formerly inactive protein kinase (Greengard, 1978).

Further criteria could also be added, for example it should be possible to mimic the action of the hormone or neurotransmitter by using a specific adenylate cyclase activator, or block the action with an adenylate cyclase inhibitor. However, problems of lack of specificity of available agents, and the possibility that cells may contain a number of adenylate cyclases each specific to a certain stimulus and giving rise to different responses, mean at the present these two criteria cannot be applied.

Not all of the criteria outlined above have been applied to the secretory response of the cockroach salivary gland to dopamine. The radio-immunoassay experiments described in the appendix to Chapter 4 were an attempt to apply the first criterion. The results are regarded as preliminary and they suggest there is an increase in cyclic AMP in the cells which have been treated with substances which stimulate fluid secretion and none in those conditions which inhibit fluid secretion.

Grewe and Kebabian (1982) have measured cyclic AMP concentration in cockroach salivary acinar cells, by the assay

method of Brown, Albano, Ekins, Sgherzi and Tampion (1971). They were not able to show a convincing increase in cyclic AMP in cells exposed to dopamine (0.1mM) unless a phosphodiesterase inhibitor was present. The accumulation of cyclic AMP in acinar cells exposed to dopamine and IBMX increased with increasing dopamine concentration. At 1uM dopamine, a concentration which usually gives rise to a maximal secretory response, the cyclic AMP concentration measured (approx. 25 picomoles/mg protein) was approximately double that in unstimulated cells. IBMX, at a concentration of 1mM, caused a slight increase in the cyclic AMP content of the cells, but when added with 0.1mM dopamine the level of cyclic AMP increased 5-fold. This shows a synergistic effect of the agonist and the phosphodiesterase inhibitor on cyclic AMP level, although the same could not be shown for the physiological response.

A discrepancy between the levels of agonist required to produce a physiological response, and the dose required to produce an increase in cyclic AMP content was observed with regard to ACTH stimulated cyclic AMP production in adrenal cortex cells. The hormone stimulates steroidogenesis at lower concentrations than it stimulates cyclic AMP production (Beall & Sayers, 1972; Honn & Chavin, 1977). To account for these discrepancies four possibilities can be considered.

Firstly, it is possible that the amount of cyclic AMP needed to cause a shift in cyclic AMP-dependent protein kinases from inactive to active forms is very small, requiring more sensitive assay methods than are currently avaiable to detect such small changes. Secondly, increases in cyclic AMP concentration are

likely to be localised within the cell, with little or no change in most parts of the cell.

A third possibility is the "spare receptor" hypothesis. It is believed that the total number of receptors on the cell surface is considerably greater than the number which need to be occupied to elicit a maximum response from the cell. Consequently the remainder of the receptors exist as a "spare" pool, as do the adenylate cyclase molecules. This confers on the cell the ability to accumulate levels of second messengers far in excess of those required for a maximal physiological response (Levitzki, 1976; 1979).

Studies with intact cells are prone to complications arising from the presence of more than one cell type, whose responses, in terms of changes in cyclic AMP content, may differ. For example, adipose tissue contains cells which respond to prostaglandin $E_1$ with an increase in their cyclic AMP content, as well as true adipocytes which respond with a fall in cyclic AMP level (Butcher & Baird, 1968; 1969). Cockroach salivary gland acini do contain more than one cell type (Kessel & Beams, 1963; Bland & House, 1971) but at present there is no evidence about whether the effects of dopamine on cyclic AMP levels are different in the different cell types.

Grewe and Kebabian (1982) also demonstrated that the fourth criterion applies to the response of the cockroach salivary gland to dopamine, by demonstrating the presence of a dopamine-sensitive adenylate cyclase in a cell-free homogenate of the acinar cells. Half-maximal activation (assessed by the amount of cyclic AMP synthesised per minute) occurred with 1uM dopamine.

The experiments described in the early part of Chapter 4 show

that exogenously applied cyclic AMP does mimic the action of dopamine on this preparation, which satisfies the third criterion.

Although not all of the original criteria proposed by Sutherland et.al.(1968) have been met and no attempt has been made to apply the fifth, the latter three have been at least partly satisfied with regard to the secretory response of the cockroach salivary gland to dopamine. This seems to be reasonable evidence that cyclic AMP could be a second messenger in this system.

The criteria set out by Sutherland et.al.(1968) for establishing cyclic AMP as a second messenger are not entirely appropriate for establishing a second messenger role for calcium. Rasmussen (1981) has proposed an alternative set of criteria for calcium. Firstly, it should be possible to demonstrate an increase in cytosolic calcium concentration when the cells are exposed to the agonist. Measurement of intracellular calcium levels has not been attempted in this work, although the chief methods currently available have been outlined in Chapter 3. The quin 2 method developed by Tsien and co-workers (Tsien, 1980; 1981; Tsien, Pozzan & Rink, 1982) seems particularly promising and if applied to the cockroach salivary gland could yield interesting results.

It should also be possible to mimic the action of the agonist by other, less specific means of raising intracellular calcium concentration. In the cockroach salivary gland application of the calcium ionophore A23187 (Reed & Lardy, 1972) mimics both the electrophysiological (Mitchell & Martin, 1980) and secretory effects of dopamine. Moreover, returning calcium to glands conditioned in calcium-free solution evokes electrical and secretory responses (Ginsborg et.al., 1980b; Gray & House, 1982).

The responses in this situation could be due to an influx of calcium through the cell membrane.

Rasmussen`s third criterion is that agents known to inhibit the uptake of calcium by intracellular organelles should enhance the effecct of the agonist, applied at submaximal concentration. This is analogous to the use of phosphodiesterase inhibitors in examining the role of cyclic AMP. No specific agents have yet been found that will inhibit the influx or efflux pathways of calcium in either microsomes or mitochondria *in situ*. Calcium homeostasis is of such fundamental importance to cells that the mechanisms for its maintainance form an intrinsic part of cell function, so that to interfere with them would probably be fatal to the cells.

In those tissues where extracellular calcium is the source of the calcium message, it should be possible to show a direct effect of the agonist on calcium gating in the plasma membrane of the cells. The results presented in Chapter 2 show that there is a requirement for extracellular calcium for maintained fluid secretion from the cockroach salivary gland. This does not fulfil Rasmussen`s fourth criterion but adds weight to the evidence from responses to A23187 and calcium readmission.

The manner in which maintained electrophysiological and secretory responses are dependent on extracellular calcium (Ginsborg et.al., 1980*a*; Gray & House, 1982) suggest that responses to the agonist depend on an influx of calcium to the cytosol from a store which can be replenished only from the outside. This is believed to occur as a result of the agonist binding to receptor sites on the cell membrane.

The fifth criterion proposed by Rasmussen is that it should be possible to demonstrate the presence of calmodulin and/or

calcium-calmodulin mediated effects on isolated membranes or enzymes from tissues where calcium is a second messenger. The present work has been more concerned with the physiological effects of calcium and the absence of calcium than with identifying calmodulin or any other calcium-binding protein within the acinar cells.

The possible mechanisms by which agonist-receptor interaction may bring about an increase in the concentration of either second messenger deserve some consideration here. Taking calcium first, there are several transduction mechanisms by which information carried by the first messenger, the agonist, could be transferred to the second messenger system. As the cytosolic calcium concentration (in physiologically relevant conditions) is always considerably lower than the extracellular concentration, a simplistic explanation is that agonist-receptor interaction renders the cell more permeable to calcium ions. There is evidence for calcium uptake following stimulation from a variety of tissues, for example squid axon (Baker, Meves & Ridgway, 1973), muscle (Caswell & Warren, 1972), frog neuromuscular junction (Miledi, Parker & Schalow, 1980), parotid gland (Putney, 1977), exocrine pancreas (Petersen & Iwatsuki, 1978) and blowfly salivary gland (Berridge, 1980b).

At the molecular level there are two hypotheses about transduction in the calcium system which involve membrane phospholipids. One is that changes in phosphatidylinositol (PI) turnover are the basis of calcium gating. This hypothesis was developed by Michell (1975), and has been supported by many more recent observations , for example those of Fain and Berridge

(1979a;b; Berridge & Fain (1979). PI is present on both plasma and subcellular membrane. Two different functions have been attributed to the PI metabolic pathway. The first involves the interconversion of diphosphoinositide and triphosphoinositide to PI as a means of altering divalent cation binding to the inner surface of the plasma membrane in cells of the nervous system (Hawthorne & White, 1975). The second mechanism involves alteration of calcium in the plasma membrane by altering the turnover of PI, which is caused by agonist-receptor interaction. The site of action of the agonist-receptor complex is considered to be a stimulation of the hydrolysis of PI to diacylglycerol, 1-phosphoinositol and 1,2-cyclic phosphoinositol.

Stimulation of the blowfly salivary gland has been shown to result in the breakdown of pre-labelled PI which correlates in time with an increase in calcium flux across the cells. This breakdown does not depend on extracellular calcium, and is not stimulated by either cyclic AMP or A23187, both of which are capable of stimulating fluid secretion in this tisue (Berridge & Fain, 1979; Fain & Berridge, 1979a;b).

The importance of the PI system has been underlined by two recent developments. Rittenhouse-Simmons, Russell & Deykin (1977) and others have shown that the diacylglycerol derived from PI breakdown is rich in arachidonic acid. Arachidonite is the precursor of prostaglandins and hydroxy fatty acids, each of which may influence cell function, so it is possible that the release of arachidonic acid from diacylglycerol derived from PI may play some type of messenger function in the cells. Also, Takai, Kishimoto, Kikkawa, Mori & Nishizuka, (1979) have shown that calcium-dependent protein kinase requires 1,2-diacylglycerol for its

activation, suggesting that it too may have some messenger function. The other recent development relates to the messenger functions of di- and triphosphoinositide. The release of these substances from the membrane may play a role analogous or complementary to those played by calcium or cyclic AMP; so they may be the final messengers controlling the key intramitrochondrial reactions in steroid biogenesis, for example (Farese Sabir & Vandor, 1979; 1980).

The other possible transduction mechanism involves the conversion of another membrane phospholipid, phosphatidylethanolamine (PE), and S-adenosyl-L-methionine (SAM) to phosphatidyl-N-monomethylethanolamine (PME), followed by the methylation of PME to phosphatidylcholine (PC). The shift from PE to PC invoves the translocation of the phospholipid head group within the two leaflets of the plasma membrane, and leads to an increase in membrane fluidity. This was discovered by Hirata & Axelrod (1980). Increase in membrane fluidity may alter the availability of some receptors to their agonists -for example it may increase the number of available receptors in red cells (Strittmatter, Hirata, Axelrod, Mallorga, Tallman & Henneberry, 1979). Down regulation of receptors may be another feature of this system (Mallorga, Tallman, Henneberry, Hirata, Strittmatter & Axelrod, 1980). In mast cells it has been shown (Ishizaka, Hirata, Ishizaka & Axelrod, 1980) that phospholipid methylation precedes calcium uptake and histamine release.

In some tissues calcium may enter as a result of agonist-receptor interaction via so-called receptor operated channels, which do not cause depolarisation of the membrane, while in others

receptor mediated changes in membrane potential may result in calcium entry through voltage-dependent channels. Both types of channel may exist in the same tissue; for example , a calcium-dependent increase in cyclic GMP content can be caused by the interaction of carbachol with muscarinic receptors, and also by depolarisation by high external potassium concentration (Study, Breakfield, Bartafai & Greengard, 1978). As the effects of both were blocked by the calcium channel blocker D600 it remains possible that the same channel may be opened by two means.

There may be a fundamental difference between the calcium channels found in tissues such as parotid, liver and salivary gland on one hand and those in nerve endings and heart and chromaffin cells on the other, as the latter type are blocked more or less specifically by verapimil and its derivatives, whereas they do not have the same effect on calcium entry in the former tissues (Rasmussen, 1981). A review of the current literature on calcium channels has been published by Hagiwara & Byerly (1981).

The evidence from the cockroach salivary gland, presented in Chapter 2 indicates that the primary source of calcium for stimulus-secretion coupling may be a cellular calcium store. In this respect transduction in the calcium system in this tissue may resemble that in skeletal muscle, where a message at the cell surface is transduced into a second message generated at a different membrane, the sarcoplasmic reticulum. In this case there may be some type of electrical coupling between the two membranes. In the heart there is considerable evidence that release of calcium from SR may be triggered by calcium entering across the cell membrane (Fabiato & Fabiato, 1979). In some smooth muscle cells calcium may be mobilised from an internal pool by low

concentrations of agonists, while higher concentrations alter the entry of calcium into the cells (Bolton, 1979).

In other cell types endoplasmic reticulum may be a primary source of calcium for stimulus-response coupling -studies in a number of cell types, for example nerve terminals (Henkart & Nelson, 1979); cultured L cells (Reaven & Axline, 1973) and fibroblasts (Moore & Pastan, 1977) suggest that surface membrane to endoplasmic reticulum coupling may be a widespread cell property, coupling plasmamembrane excitation to the generation of a calcium second messenger signal. The experiments with the fluorescent probe chlorotetracycline described in Chapter 3 are an initial step towards identifying the location of the calcium store in cockroach salivary gland cells.

Having considered possible transduction mechanisms by which the calcium second messenger signal may be generated it is now time to consider the possible function of increased cytosolic calcium. The mechanism of action of calcium was first studied in muscle contraction. A group of tropomyosin-associated proteins were discovered in the mid 1960s, one of which binds calcium with high affinity (Ebashi, Ebashi & Kodama, 1967; Ebashi & Endo, 1968; Ebashi, Endo & Otsuki, 1969). This calcium receptor protein binds calcium and interacts with the tropomyosin complex. It does not appear to have enzyme properties, but it is presently believed that interaction with the protein leads to a conformational change in the tropomyosin complex (Gusev & Freidrich, 1980) so that it no longer blocks binding sites on the actin molecule, thus allowing actin and myosin to interact (Weber & Murray, 1973;

Potter, Nagy, Collins, Siedel, Leavis, Lehrer & Gergely, 1976). This mechanism also operates in cardiac muscle (Chapman, 1979), however in other smooth muscle types the mechanism of calcium action in excitation-contraction coupling is different -the calcium receptor protein is calmodulin (Dabrowska & Hartshorne, 1978; Barron, Barany & Barany, 1979). The binding of calcium was found to lead to phosphorylation of a light chain of myosin -that is, there was a calcium dependent protein kinase as a component of the myosin complex. This was an important discovery as it showed that calcium could act as an activator of protein kinases in the same way as cyclic AMP.

There are now known to be many instances where the action of calcium is mediated through a homologous class of binding proteins which serve as receptors for cacium ions (Kretsinger, 1976; Watterson, Harrelson, Keller, Sharief & Vanaman, 1976). The increase in calcium concentration resulting from stimulation causes the binding protein to form an active complex with calcium ions. This complex may then combine with an apoenzyme or effector protein to trigger a biochemical reaction which culminates in a physiological response. The mechanism of action of calmodulin was first established in the phosphodiesterase system (Lin, Liu & Cheung, 1974; Teo & Wang, 1973; Kakiuchi, Yamazaki, Teshima & Uenishi, 1973), which underlines the close links between calcium and cyclic AMP as second messengers. Calmodulin is now known to regulate many other cell functions -see Cheung (1980) for a review. Although calmodulins isolated from different species may not be absolutely identical they do have similar amino acid sequences, and share several basic characteristics.

The precise role played by cytosolic calcium in fluid

secretion is as yet unclear. In the cockroach salivary gland fluid secretion is believed to be driven by sodium transport, sodium being actively extruded into the acinar lumen by Na-K pump activity (Smith & House, 1979). If the rate at which the pump operates is somehow related to intracellular sodium concentration, intracellular calcium could promote secretion by increasing the rate at which sodium enters the cell across the basolateral membrane. This could be achieved either by a mechanism exchanging intracellular calcium ions for extracellular sodium ions, or by calcium influencing the membrane permeability to sodium.

The observation that increasing intracellular concentration may lead to increased sodium influx or transport has been made in a number of tissues. It has been interpreted by some workers as evidence of sodium-calcium exchange (e.g. Lee, Uhm & Dresdner, 1980; Ozaki & Urakawa, 1979); and by others as evidence of calcium influencing membrane permeability (e.g. Parod & Putney, 1980; Brismar, 1980). Studies with the fluorescent calcium-sensitive dye quin 2 in rabbit alveolar macrophages have indicated that intracellular calcium concentration influences membrane permeability to calcium (Kesteven, 1982). When the cells are loaded with calcium chelator there is a rapid influx of calcium to maintain a normal intracellular free calcium concentration.

As already indicated, the calcium-calmodulin system may be one method of linking receptor activation to the generation of a cyclic AMP signal. Alternatively, the receptor may form a subunit of the adenylate cyclase system in the membrane, so that when the agonist binds to the receptor the adenylate cyclase system is stimulated. A detailed model of the steps involved from agonist-

receptor interaction to cyclic AMP synthesis has been proposed by Ross and Gilman (1980); this is a modified version of the models discussed by Cassel and Selinger (1977) and Levinson and Blume (1977). The agonist-sensitive cyclase system consists of at least three separate proteins, a receptor subunit, a catalytic subunit and a regulatory subunit. The catalytic subunit is relatively inactive in the free state, but is activated when agonist-receptor interaction causes an exchange of guanine nucleotides at a nucleotide binding site on the regulatory subunit. The active moiety which converts ATP to cyclic AMP is the complex formed between the catalytic subunit and the regulatory protein with its substituted guanine nucleotide. The vast literature on transduction in the cyclic AMP system has been reviewed by Rasmussen (1981).

The precise role played by cyclic AMP in fluid secretion has not yet been defined. In many systems, including hepatic glycogenolysis where cyclic AMP was discovered, its effect is on a class of protein kinases, influencing the enzyme systems of the cell. In the kidney argenine vasopressin (AVP) acts via cyclic AMP to influence water flow in different parts of the nephron. It has been proposed (see Rasmussen, 1981) that AVP acts on an adenyl cyclase in the basolateral membrane, leading to a rise in intracellular cyclic AMP concentration which activates a protein kinase in the luminal membrane. Activation of the protein kinase leads to the phosphorylation of one or more proteins which are responsible for the change on the permeability properties of the membrane. As is the case with all polar cells, it is not clear how the cyclic AMP signal generated at the basolateral membrane is transmitted to the luminal membrane, in light of the levels of

phosphodiesterase activity in the cells.

The evidence presented in the preceding chapters indicates that the effects of calcium and cyclic AMP on stimulus-secretion coupling in the cockroach salivary gland are intertwined, as is the case in many other systems. Examples can be found in muscle contraction, glycogen metabolism and stimulus-division coupling as well as in other secretory systems. Each messenger influences the activity of the other in a complex relationship which controls the response of the cell to the stimulus. The precise mechanisms involved in the cockroach salivary gland have yet to be uncovered, but the available evidence from this and other systems suggests that stimulus-secretion coupling in the cockroach salivary gland may resemble the model illustrated in figure 5:1. In this model the receptor at the cell surface is part of the adenyl cyclase system, and is also functionally linked to the phospholipids in the membrane which are responsible for calcium gating. These in turn are linked to an intracellular store of calcium. There may also be a direct link between receptor and calcium store. When the agonist, dopamine, interacts with the receptor this causes an increase in cytosolic calcium, possibly by altering phospholipid turnover in the membrane. The adenyl cyclase system is also activated, causing an increase in cytosolic cyclic AMP. The two messengers complex with their receptor proteins, which are probably calmodulin and a protein kinase respectively, to influence membrane permeability and sodium and water transport, possibly by the phosphorylation of certain membrane-associated proteins.

The calcium-receptor protein complex may modify adenylate

Figure5:1

Diagrammatic representation of the roles of calcium and cyclic AMP in stimulus-secretion coupling in the cockroach salivary gland acinar cell.

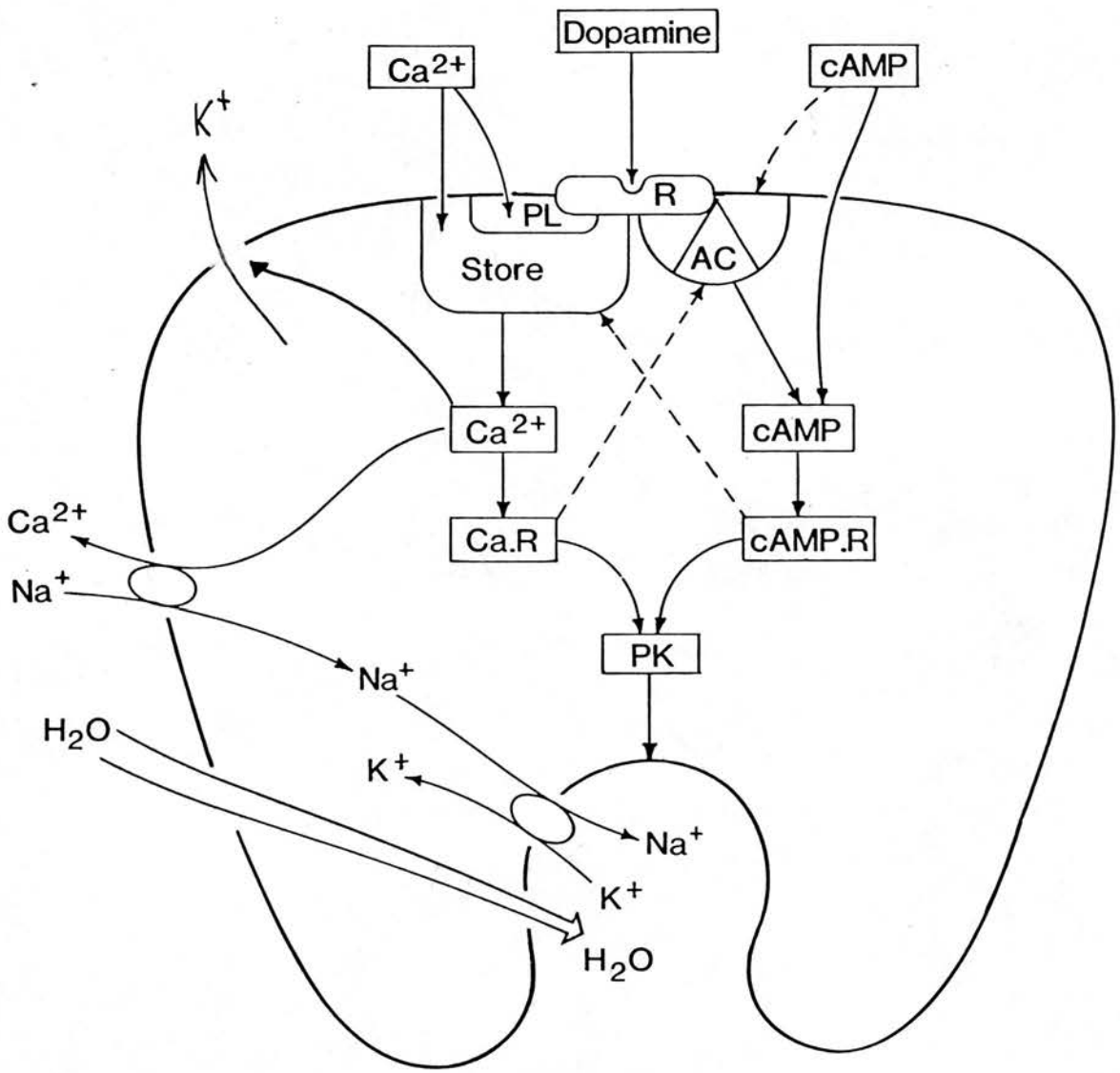
R = receptor PL = membrane phospholipids (PI or PE)

Store = calcium store AC = adenylate cyclase

Ca.R = calcium-receptor protein complex

cAMP.R = cAMP-receptor protein complex

PK = protein kinase


Dopamine
Ca2+
cAMP
K+
R
PL
AC
Store
Ca2+
cAMP
Ca2+
Ca.R
cAMP.R
Na+
PK
Na+
H2O
K+
Na+
K+
H2O

cyclase activity, and the formation of cyclic AMP from ATP (Carnes et.al., 1980) or the cyclic AMP-receptor protein complex may influence release of intracellularly bound calcium and calcium gating via membrane phospholipids. These feedback mechanisms are indicated in the model, as is the possible role of calcium in Ca-Na exchange at the basolateral membrane, which may stimulate sodium, and therefore water, transport. *

Evidence is accumulating that in some tissues where calcium and cyclic AMP act synarchically as second messengers there may in fact be two pharmacologically distinguishable classes of receptor for the agonist. One class, when activated brings about the generation of a calcium signal, the other is linked to the adenyl cyclase system. This has been shown in tissues as diverse as the striatum of the brain (Kebabian & Calne, 1979) and the blowfly salivary gland (Berridge & Heslop, 1981). In the present study no attempt has been made to characterise the receptors of the salivary acinus in these terms, although it would be of interest to do so. Whether dopamine is found to act on one or two classes of receptors need not necessarily alter most of the model presented in figure 5:1.

As more and more research is carried out into the mechanism of stimulus-response coupling in different tissues the models proposed are becoming more similar, and it seems likely that eventually a mechanism will be worked out which is common to a wide variety of systems.

* While it is almost certain that Na/K pump activity occurs in the cockroach salivary gland cells there is as yet no available evidence as to its localisation. For the purpose of the model it is convenient to place it at the luminal membrane, although this is contrary to evidence from mammalian epithelia which indicates that the Na/K pump is located in the basolateral membranes of these tissues.

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PUBLICATIONS

Some of the work presented in this thesis has already been published:

Gray, D.C. & House, C.R. (1982) The influence of calcium on the control of fluid secretion in the cockroach salivary gland. Quarterly Journal of Experimental Physiology 67: 639-654

Gray, D.C. & House, C.R. (1983) Chlorotetracycline fluorescence associated with plasma membranes of cockroach salivary gland cells. Quarterly Journal of Experimental Physiology 68: 105-121

Reprints of these papers are bound in at the back of this volume. A third paper on the role of cyclic AMP in stimulus-secretion coupling in the cockroach salivary gland has been submitted for publication.

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# THE INFLUENCE OF CALCIUM ON THE CONTROL OF FLUID SECRETION IN THE COCKROACH SALIVARY GLAND

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## SUMMARY

When cockroach salivary glands are bathed in calcium-free medium the basal rate of fluid secretion increases from about 1 nl/min to about 10 nl/min; maintained dopamine stimulation elicits a further rise in secretory rate which gradually declines. Evidence is presented which indicates that magnesium is unable to substitute for calcium in this system. When calcium is returned to the bathing solution after a period of calcium deprivation there is a transient increase in secretory rate. Stimulation of the glands in certain conditions which inhibit the secretory response leads to some kind of calcium-dependent active state in the secretory cells which can outlast the interaction of the agonist with its receptors. It is concluded that stimulus–secretion coupling in this gland involves a calcium–dependent second messenger system.

## INTRODUCTION

Both calcium ions and cyclic AMP have been proposed as 'second messengers' in secretory tissues as diverse as the blowfly salivary gland (Berridge, 1979) and mammalian parotid gland (Leslie, Putney & Sherman, 1976). How these second messengers control the rate of secretion of enzymes, ions and water is not thoroughly understood. However, there is evidence from pancreatic acinar cells (Petersen & Iwatsuki, 1978) and nerve cells (Meech, 1972) that calcium mediates changes in the permeability of cell membranes to ions. In the blowfly salivary gland calcium probably controls the chloride permeability of the secretory cells and the rate of active potassium transport into the saliva (Prince, Berridge & Rasmussen, 1972). A calcium-dependent rise in the potassium permeability of cockroach salivary gland acinar cells has also been reported (Ginsborg, House & Mitchell, 1980*a*, *b*).

Observations of declining responses to stimulation in calcium-free media have been made in a number of tissues, e.g. cockroach salivary gland (Ginsborg *et al.* 1980*a*), blowfly salivary gland (Prince & Berridge, 1973), rat parotid gland (Petersen & Pedersen, 1974; Rudich & Butcher, 1976; Petersen, Ueda, Hall & Gray, 1977) and exocrine pancreas (Agent, Case & Scratcherd, 1973; Petersen & Ueda, 1976; Kanno & Yamamoto, 1977; Iwatsuki & Petersen, 1977*a* and Laugier & Petersen, 1980). These have led to the proposal of intracellular calcium-dependent stores of some substance (possibly calcium itself) essential for the response to stimulation.

Certain characteristics of this calcium-dependent store in the cells of the cockroach salivary gland have been examined by recording the secretory response of the isolated perfused preparation developed by Smith & House (1977, 1979), and the results are presented in this paper.

The present experiments also investigated whether dopamine stimulation under conditions

preventing fluid secretion can produce some kind of active state in the secretory cells which outlives the interaction of the agonist with its receptors. The calcium-dependence of this state was also investigated. It is concluded that stimulus–secretion coupling in this gland involves a second messenger system which is calcium-dependent.

## METHODS

### *Preparation*

The paired salivary apparatus was dissected from adult cockroaches and mounted in a channel in a Perspex slab, through which varying solutions were perfused (Smith & House, 1977). One of the salivary ducts was ligated near its end, passed through a small hole in the side of the channel into a pool of liquid paraffin, and anchored by the ligature. A puncture was made in the duct posterior to the ligature to allow the secreted fluid to escape.

### *Volume of secretion*

The volume of secretion was measured by taking up the droplet of saliva from the duct with a micropipette and transferring it to another pool of liquid paraffin, where it assumed a spherical shape. The diameter could then be measured and the volume calculated.

### *Composition of bathing fluids*

The control solution contained (mM): NaCl, 160; KCl, 10; HCl, 4; $CaCl_2$, 5; Tris(hydroxymethyl)aminomethane, 5 and glucose 20. For the calcium-free EGTA solution, $CaCl_2$ was replaced by 1 mM-EGTA. The free calcium concentration of such a solution has been estimated to be less than $10^{-9}$ M (Hubbard, Jones & Landau, 1968). For brevity this will be referred to as 'calcium-free solution' throughout. For the calcium-free magnesium solution, $CaCl_2$ was replaced by 5 mM-MgCl. For the sodium-free solution, NaCl was replaced by 160 mM-choline chloride (repurified before use). The composition of the chloride-free solution was (mM): sodium methylsulphate, 160; $K_2SO_4$, 5; $CaSO_4$, 1; Tris, 5; $H_2SO_4$, 2; glucose, 20, with 1 mM-EGTA replacing the $CaSO_4$ in the case of Cl-, Ca-free solution. The pH of all solutions used was in the range 7·3–7·7.

### *Protocol*

At both the beginning and end of each experiment the gland was exposed to a test dose of 1 $\mu$M-dopamine in control solution. If the final response was significantly smaller than the first, it was assumed that the preparation had in some way been damaged during the experiment and the results were discarded. Variations in the protocol according to the aims of each experiment are detailed in the Results.

## RESULTS

### *Calcium store*

The effects of calcium-free medium on the secretory response of the isolated cockroach salivary gland are shown in Fig. 1. The important feature of the tissue's behaviour during the withdrawal of extracellular calcium is that the secretory response to maintained dopamine stimulation gradually declines (see section headed '*Decline*'). It was also noted that the basal or unstimulated secretory rate increases slightly (see '*Basal rate*') in the absence of extracellular calcium ions. When calcium is returned to the bathing fluid there is a transient secretory response in the absence of dopamine stimulation (see '*Readmission response*').

After a short recovery period in control solution the gland is capable of giving a response as large as that obtained in control solution prior to calcium-free treatment; therefore the effect of calcium-deficient medium cannot be attributed to permanent damage to the tissue (Fig. 1).

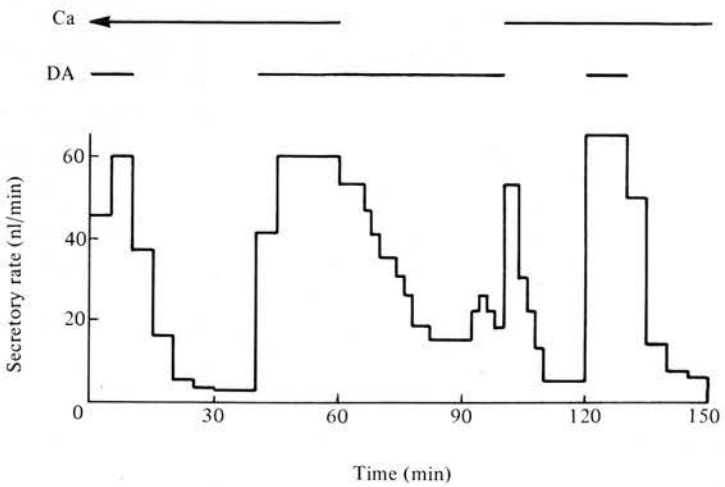


Fig. 1. Effect of calcium-free medium on the secretory response to dopamine. The periods during which calcium (5 mM) and dopamine (1 $\mu$M) were present in the bathing medium are indicated by horizontal bars labelled Ca and DA respectively.

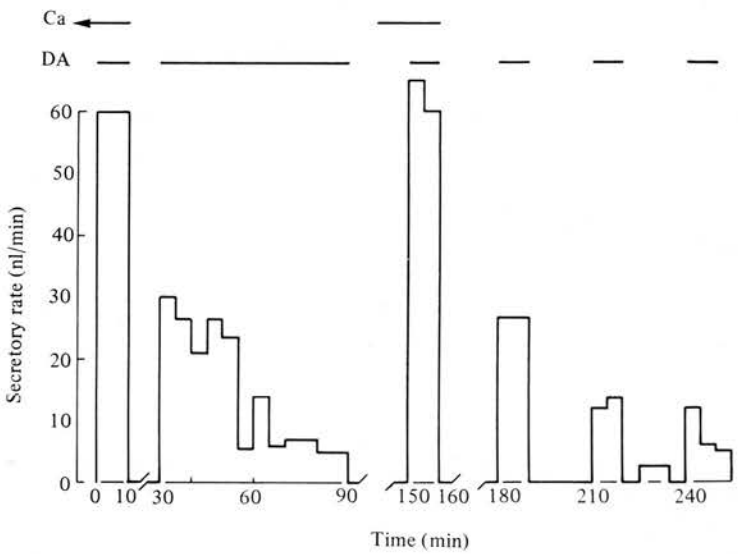


Fig. 2. Effect of calcium free medium on secretory responses to dopamine. The periods during which calcium (5 mM) and dopamine (1 $\mu$M) were present in the bathing medium are indicated by horizontal bars labelled Ca and DA respectively.

*Decline.* Fig. 2 shows that a declining response occurs when the preparation is stimulated after exposure to calcium-free solution has started, and that the decline occurs whether the stimulation is prolonged or given in 10 min pulses. Other experiments of this type have been done and have shown that the order of presentation of pulsed and prolonged stimulation is not important. The declining response to dopamine is consistent with the idea that

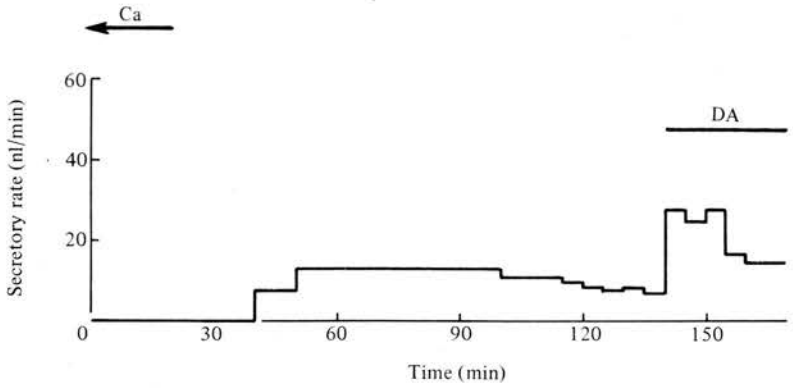


Fig. 3. Effect of calcium-free medium on the basal secretory rate. The periods during which calcium (5 mM) and dopamine (1 $\mu$M) were present in the bathing medium are indicated by horizontal bars labelled Ca and DA respectively.

stimulation in calcium-free conditions causes depletion of an intracellular store of some substance necessary for secretion. The decreasing responses to repeated pulses of stimulation in calcium-free conditions indicate that no significant replenishment of the store occurs between periods of stimulation.

*Basal rate.* It was observed that, after exposing the glands to calcium-free medium, the basal rate of secretion began to rise without any stimulation. Further experiments were carried out to study this effect more closely and to see whether this increased rate of secretion would have any effect on the subsequent response to stimulation. Fig. 3 shows the gradual increase in basal rate which begins after a short incubation in calcium-free medium (typically 20 min) and eventually reaches a plateau. Fig. 3 also shows that it is still possible to obtain an increase in secretory rate in response to stimulation after 120 min exposure to calcium-free medium but that the rate is significantly reduced. This preparation gave a satisfactory response after a recovery period in control solution (see Methods: '*Protocol*') indicating that no permanent damage had occurred.

A range of pre-stimulation times was tried on twenty-five glands and there appeared to be a trend towards a reduction in response with longer pre-stimulation periods, indicating that the increased basal rate secretion possibly may be connected with the reduction in the response to dopamine. Six attempts were made to extend the range of pre-stimulation periods to include 180 min, and in none of these experiments was any secretion collected in response to dopamine. In all cases the basal secretory rate dropped to zero during the pre-stimulation period, and recovery responses after the return of calcium were very poor.

*Magnesium.* Magnesium is believed to displace calcium from membranes (e.g. Petersen & Ueda, 1976) or to compete with calcium for membrane binding sites (e.g. Smith & House, 1979). Experiments were carried out to determine whether 5 mM magnesium could (i) substitute for 5 mM calcium, or (ii) displace calcium from an intracellular store. As shown in Fig. 4 the response to stimulation in calcium-free magnesium medium is small, indeed smaller than might be expected in calcium-free solution without magnesium. This indicates that not only is magnesium unable to substitute for calcium but that there may be a slight inhibitory effect (Smith & House, 1979), since the secretory rate rises slightly, even before

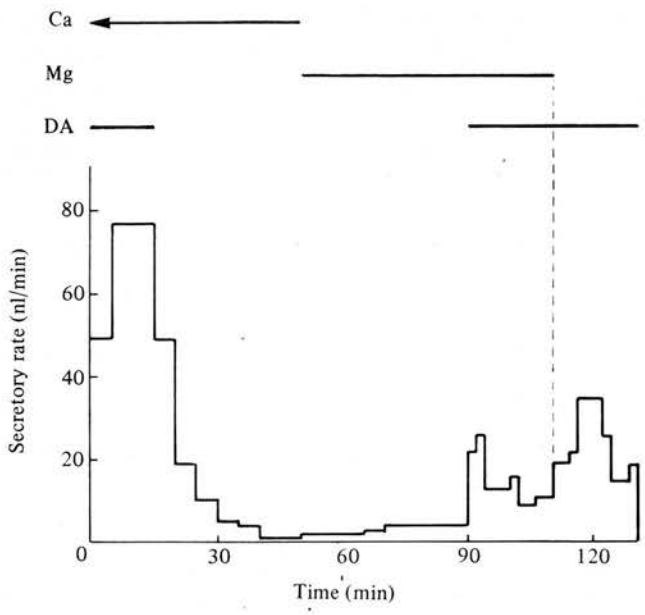


Fig. 4. Effect of calcium-free medium containing 5 mM-magnesium on the secretory response to dopamine. The periods during which calcium (5 mM), magnesium (5 mM) and dopamine (1 $\mu$M) were present in the bathing medium are indicated by horizontal bars labelled Ca, Mg and DA respectively.

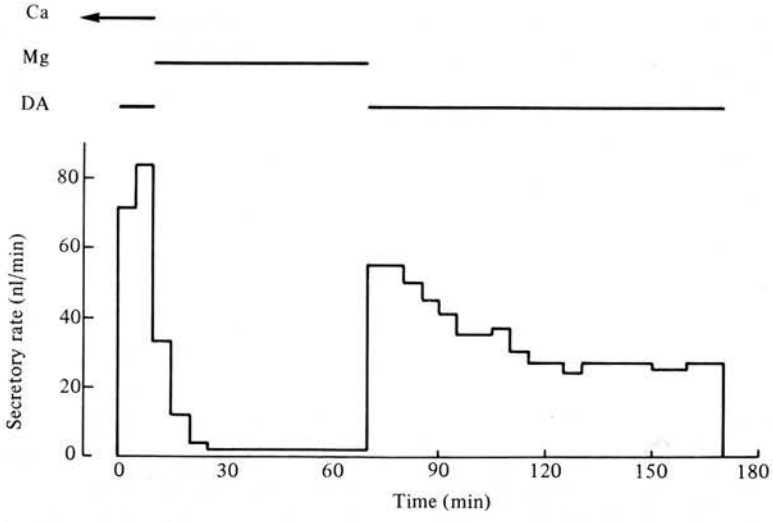


Fig. 5. Effect of pre-incubation in calcium-free medium containing 5 mM magnesium on the subsequent secretory response to dopamine in calcium-free medium. The periods during which calcium (5 mM), magnesium (5 mM) and dopamine (1 $\mu$M) were present in the bathing medium are indicated by horizontal bars labelled Ca, Mg and DA respectively.

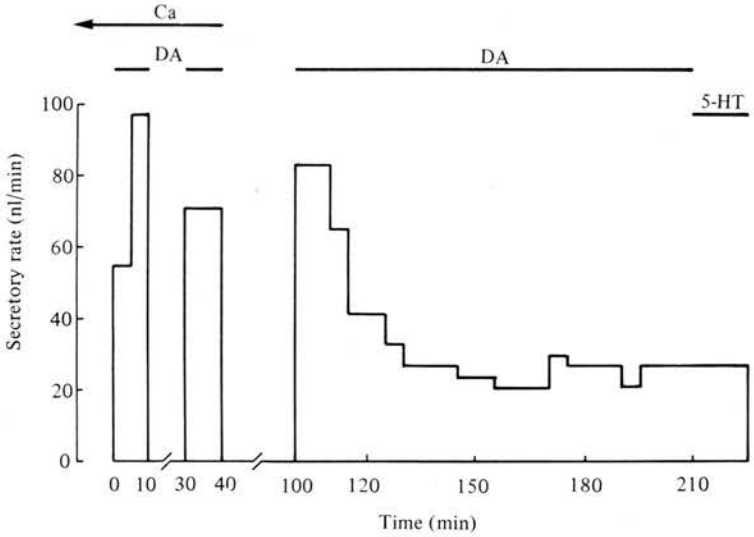


Fig. 6. Effect of 5-hydroxytryptamine stimulation on fluid secretion from a gland previously stimulated by dopamine in calcium-free medium. The periods during which calcium (5 mM), dopamine (1 $\mu$M) and 5-HT (1 $\mu$M) were present in bathing medium are indicated by horizontal bars labelled Ca, DA and 5-HT respectively.

replacement of calcium, when magnesium is withdrawn. When the gland is stimulated in calcium-free medium after incubation in calcium-free magnesium medium (Fig. 5), the response obtained is within the range expected for that pre-stimulation exposure time in calcium-free solution without magnesium; thus magnesium has not had a lasting effect on the calcium-dependent store. An increase in basal rate during exposure to calcium-free magnesium solution was observed less frequently and was smaller than the rise during incubation in calcium-free solution.

*5-Hydroxytryptamine.* 5-Hydroxytryptamine (5-HT), an agonist evidently stimulating the gland via receptors other than those activated by dopamine (House, Ginsborg & Silinsky, 1973; Bowser-Riley, House & Smith, 1978), was used to test whether more secretion could be elicited from a preparation 'exhausted' by dopamine stimulation in calcium-free conditions. As Fig. 6 shows, no increase in secretion was caused by the application of 1 $\mu$M-5-HT to an 'exhausted' preparation.

*Second messenger*

The second part of the work reported here was an investigation of the possible calcium-dependence of a second messenger in stimulus–secretion coupling in the cockroach salivary gland. The object of the experiments was to look for the effect of a build-up of second messenger in the cells when they were stimulated in the absence of an essential ion for salivary secretion. It was anticipated that such a build-up might be expressed, when the missing ion was returned, as a transient secretory reponse larger than the normal readmission response to that ion.

The experiments described below were prompted by our finding that the replacement of sodium or chloride ions to the bathing solution after a period of dopamine stimulation

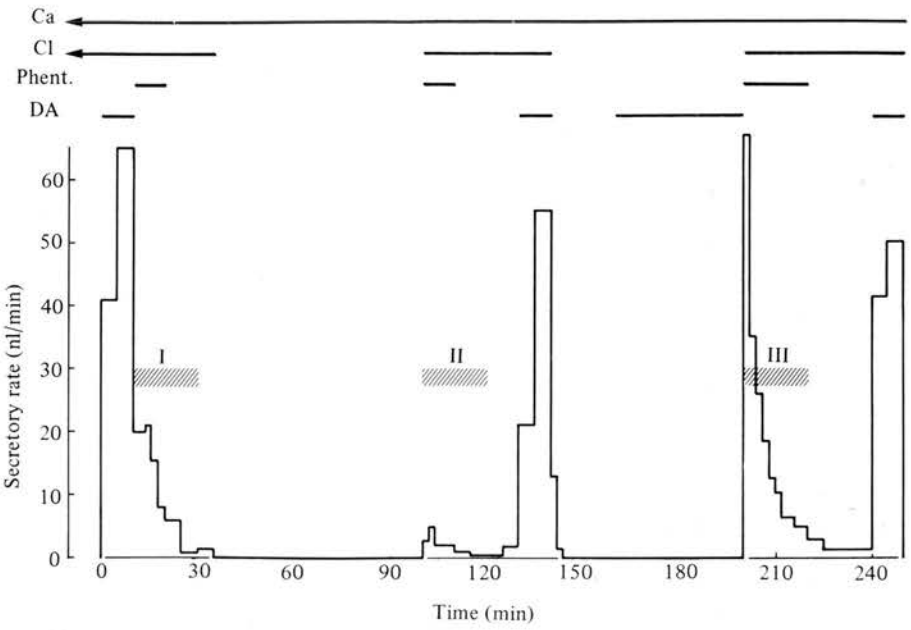


Fig 7. Effect of chloride free solution containing calcium on the secretory response to dopamine. The periods during which calcium (5 mM), chloride (184 mM), phentolamine (100 $\mu$M) and dopamine (1 $\mu$M) were present in the bathing medium are indicated by horizontal bars labelled Ca, Cl, Phent. and DA respectively. See text for further details.

caused a transient secretion of fluid lasting up to 20 min. It is unlikely that residual dopamine in the medium produced this response because the time to change solutions within the bath is less than 40 s, moreover the response occurred even when the missing ion was replaced up to 10 min after the end of dopamine stimulation.

The protocol of these experiments was designed with a control part built into each experiment to enable direct comparison of experimental and control results to be made for each gland. Following the initial test stimulation, the 'switching-off' phase was measured (i.e. the secretion during the 20 min immediately following withdrawal of dopamine; phase I in Figs. 7, 9 and 10). The control solution was then changed for one of the test solutions (chloride-free; chloride- and calcium-free; sodium-free; and sodium- and calcium-free) and the preparation incubated in this solution for 60 min. The control solution was then returned and the readmission response measured, again for 20 min (phase II in the Figures). That completed the control part. While the preparation was perfused with control solution another test response was obtained to check that the condition of the preparation was similar to that at the start of the experiment. Then the ion-deficient test solution was returned and after 20 min the preparation was stimulated in test solution for 40–60 min. A prolonged period of dopamine stimulation was chosen so that the cytosolic concentration of the suspected second messenger might reach an optimum (cf. Heslop & Berridge, 1980); in separate experiments it was shown that the 'switching off' phases after brief and prolonged stimulation periods were similar. At the end of this stimulation period the missing ion was returned and the response over the next 20 min was recorded as the experimental response (phase III in the Figures). Lastly a test response to 1 $\mu$M-dopamine was obtained after a recovery period in control solution, as a final check on the condition of the

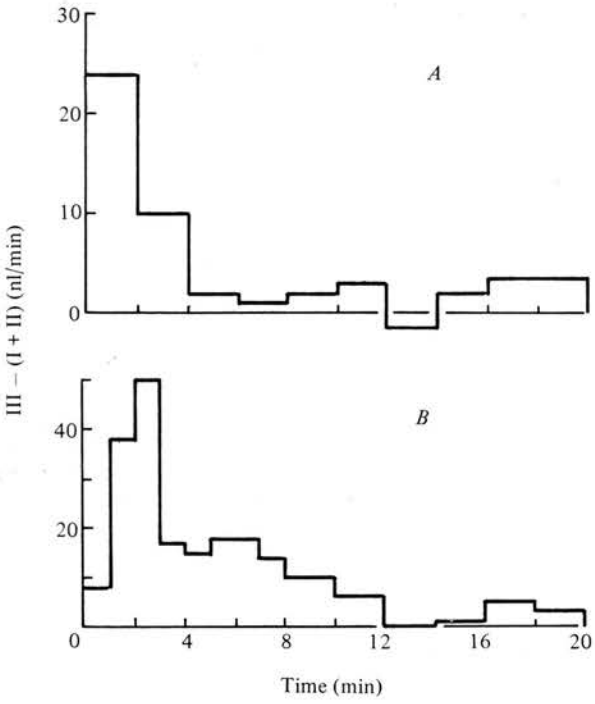


Fig. 8. Analysis of the results in Fig. 7. The difference between phase III and the sum of phases I and II has been plotted as a function of time in *A*. The results of a similar experiment are plotted in *B*.

preparation. This protocol was repeated on three preparations for each of the test solutions specified above. When calcium was withdrawn as well as either sodium or chloride, calcium was returned some time after the other ion, to separate the responses. In order to ensure that no contribution to the crucial part of the results could be made either by dopamine remaining in the bath, or by release of transmitter from the nerve endings and gland cells, 100 $\mu$M-phentolamine, an effective antagonist of dopamine's action (House & Smith, 1978) was included in the medium during phases I, II and III.

Fig. 7 shows one of the experiments with chloride as the missing ion and with calcium present throughout. There is a small readmission response (II) in the control part, and a much larger response to the return of chloride following stimulation in the experimental part (III).

Since the fluid secretion during both the 'switching-off' (phase I) after stimulation, and the readmission (phase II) of the missing ion might contribute to the experimental response (III) the experiments were analysed by subtracting the sum of phases I and II from phase III. Fig. 8 shows the results of this analysis plotted for two of the chloride-free experiments. Fig. 8*A* is the result of the analysis for the experiment shown in Fig. 7. The positive difference between III and the sum of I and II indicates that a part of the experimental response must be due to the preceding period of stimulation.

When the experiment was repeated with calcium withheld as well as chloride there was no large response on the return of chloride following stimulation in the test solution (Fig. 9) and phase III was not greater than the sum of phases I and II.

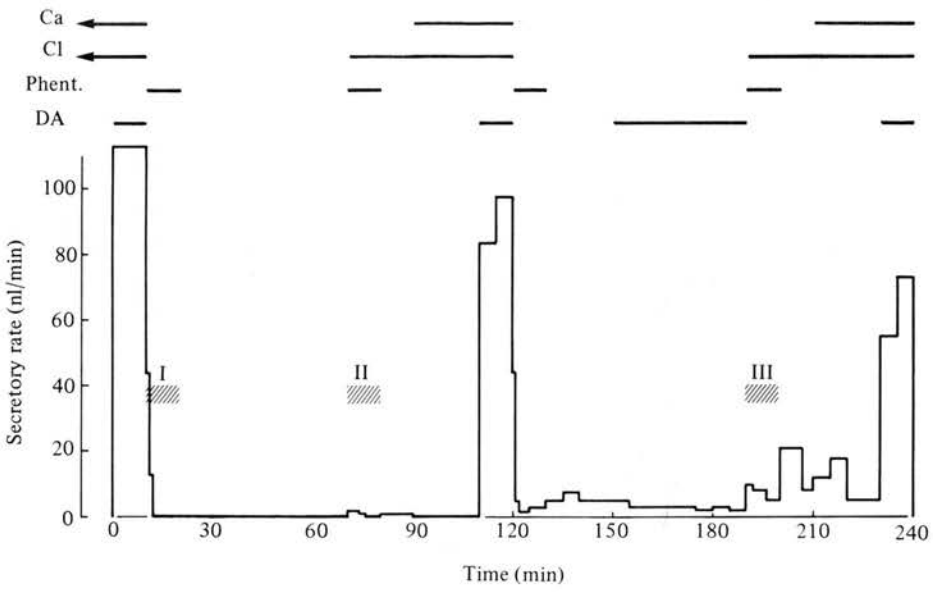


Fig. 9. Effect of chloride-free, calcium-free solution on the secretory response to dopamine. The periods during which calcium (5 mM), chloride (184 mM), phentolamine (100 $\mu$M) and dopamine (1 $\mu$M) were present in the bathing medium are indicated by horizontal bars labelled Ca, Cl, Phent. and DA respectively. See text for further details.

These experiments were also repeated using sodium as the missing ion, with or without calcium (Fig. 10*A*, *B*). However, in this case phase III was approximately equal to the sum of phases I and II (in the presence of calcium) because in all of the experiments the sodium readmission response (phase II) was so large that it defeated the purpose of the comparison.

*Readmission responses.* As can be seen in Fig. 11*A*, when calcium is returned to the medium after a period of calcium deprivation, there is a transient secretory response. This has also been observed when other ions, for example chloride or sodium, have been withheld and then replaced. Fig. 11 also shows examples of readmission responses to chloride and sodium ions.

## DISCUSSION

### *Calcium store*

The decline of the response to maintained stimulation in calcium-free solution can be interpreted as further evidence of the role played by calcium in stimulus–secretion coupling in the cockroach salivary gland, and for a calcium-dependent store of a necessary substance in the gland cells. It is possible that the necessary substance is calcium itself and that the calcium store is associated with the plasma membrane of the acinar cells. Such a store could give up calcium ions to the cytosol during stimulation, and be replenished from the exterior (Ginsborg *et al.* 1980*a*). Recent experiments (D. C. Gray & C. R. House, unpublished) with chlorotetracycline, a fluorescent calcium probe (Caswell, 1979), have demonstrated the

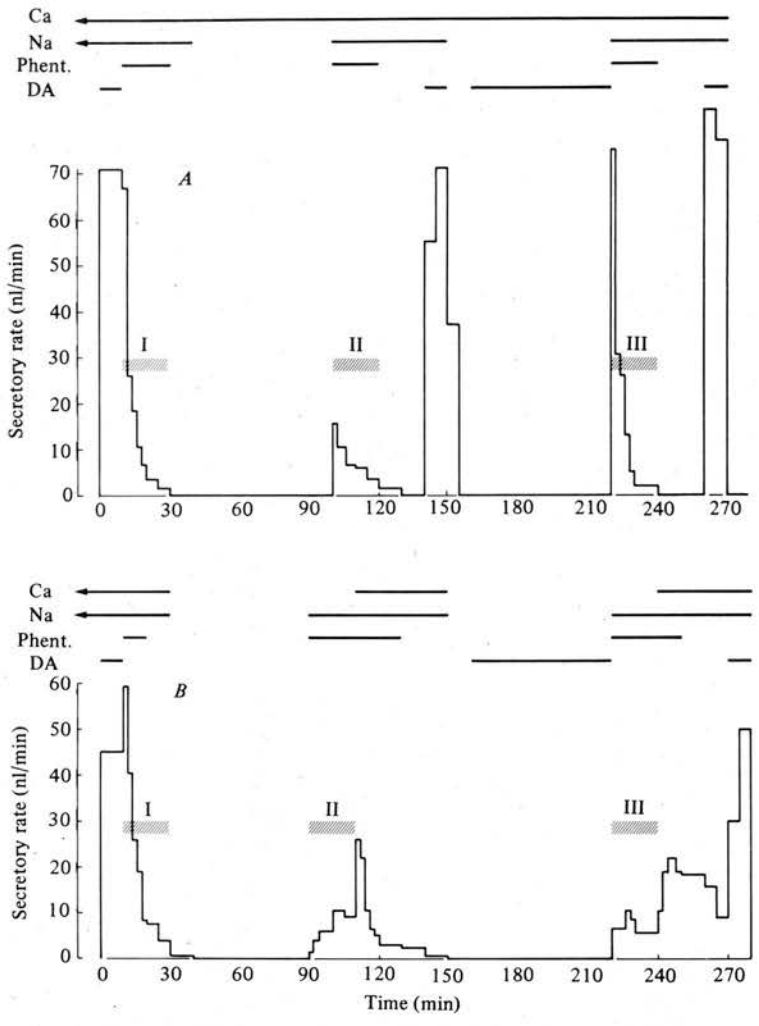


Fig. 10. Effect of sodium-free solution in the presence (*A*) or absence (*B*) of calcium, on the secretory response to dopamine. The periods during which calcium (5 mM), sodium (160 mM), phentolamine (100 $\mu$M) and dopamine (1 $\mu$M) were present in the bathing medium are indicated by horizontal bars labelled Ca, Na, Phent. and DA respectively. See text for further details.

presence of calcium associated with the plasma membranes of the peripheral cells (Bland & House, 1971) which probably are solely responsible for ion and water secretion into the acinar lumen (Maxwell, 1981).

Cellular calcium stores have previously been proposed on the basis of several different types of evidence (see Table 1). Many of the observations of declining responses to stimulation in calcium-free media have been interpreted as the result of stimulation causing release of a limited amount of calcium from a store in the cell, which brings about a transient increase in cytosolic free calcium and secretory rate, both declining when the calcium from the store is used up.

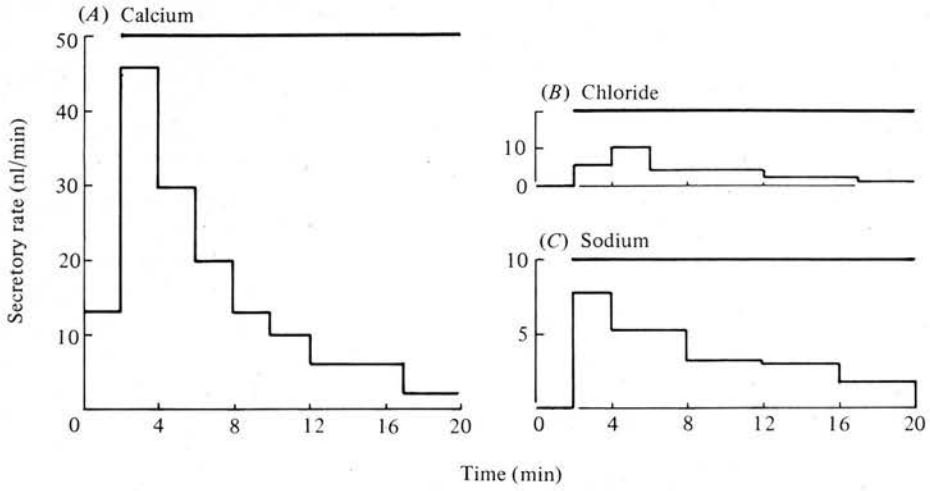


Fig. 11. Examples of secretory responses to the replacement of calcium (*A*), chloride (*B*) or sodium ions (*C*) in the bathing medium. The periods during which calcium (5 mM), chloride (184 mM) and sodium (160 mM) were present in the bathing media are indicated by horizontal bars.

Table 1. *Observations of declining responses to stimulation in calcium-free conditions*

| Tissue | Response | Stimulus | Reference |
|---|---|---|---|
| Cockroach salivary gland | Hyperpolarization of acinar cells | Ionophoretic dopamine | Ginsborg *et al.* 1980*a* |
| | Hyperpolarization of acinar cells | Ionophore A23187 | Mitchell & Martin, 1980 |
| Blowfly salivary gland | Fluid secretion | 5-HT | Prince & Berridge, 1973<br>Prince *et al.* 1972 |
| | Transpithelial potential | 5-HT | Prince & Berridge, 1973<br>Berridge & Prince, 1975 |
| | Calcium efflux | 5-HT | Prince *et al.* 1972 |
| Rat parotid gland | Amylase release | ACh & α-adrenergic | Petersen *et al.* 1977 |
| | $K^+$ efflux | Substance P | Rudich & Butcher, 1976 |
| | Amylase release | Eledoisin<br>Cyclic nucleotides | |
| Mouse parotid gland | Hyperpolarization of acinar cells | Epinephrine | Petersen & Pedersen, 1974 |
| Mouse and rat pancreas | Amylase release | ACh | Petersen & Ueda, 1976 |
| | Depolarization of acinar cells | ACh | Petersen & Ueda, 1976<br>Laugier & Petersen, 1980<br>Iwatsuki & Petersen, 1977*b* |
| Rat pancreas | Amylase release | CCK-PZ* | Kanno & Yamamoto, 1977 |
| | Fluid secretion | CCK-PZ | Kanno & Yamamoto, 1977 |
| Cat pancreas | Amylase release | ACh | Argent *et al.* 1973 |

*CCK-PZ: cholecystokinin-pancreozymin.

*Basal rate in calcium-free medium.* Prince & Berridge (1973) reported a significant rise in secretion from blowfly salivary glands exposed to a calcium-free solution containing 5 mM-EGTA. This increase in the 'unstimulated' secretory rate, although of greater amplitude, follows the same time course as that reported here for cockroach glands bathed in calcium-free solution containing 1 mM-EGTA. Also, as is the case with the cockroach gland, the response of the blowfly gland to the agonist was reduced after this period of enhanced secretion.

Treatments which increase the cytosolic free calcium concentration without activating receptors on the cell membrane are capable of mimicking the stimulation of secretion. In the cockroach salivary gland the ionophore A23187, which is known to transport calcium ions (Reed & Lardy, 1972) produces a hyperpolarization due to increase in membrane potassium permeability (Mitchell & Martin, 1980) similar to that produced by stimulation of the gland by dopamine (House, 1973; Ginsborg, House & Silinsky, 1974). Rose & Loewenstein (1975) found an increase in cytosolic calcium concentration in *Chironomus* salivary gland cells exposed to calcium-free medium. This increase in cytosolic free calcium is presumably supplied by intracellular calcium stores – for example the mitochondria may release calcium (Carafoli, Tiozzo, Lugli, Crovetti & Kratzing, 1974).

It is also possible that EGTA itself may have a stimulatory effect by causing some redistribution of intracellular calcium. Huddart and Saad have found that the rate of efflux of radioactive calcium from the cells of rat ileum placed in calcium-free medium is significantly increased when EGTA is added to the medium (H. Huddart, personal communication). This rise in efflux has a time course similar to that of the increase in basal secretory rate observed in cockroach salivary glands exposed to calcium-free EGTA solution, reaching a peak about 20 min after addition of EGTA. The fact the EGTA induced an increase in calcium efflux from rat ileum might indicate that EGTA is bringing about the release of bound calcium. If a similar phenomenon occurs in the cockroach gland it could produce a rise in basal secretion if calcium efflux is linked to sodium influx.

*Magnesium.* The observation reported here, that the response to stimulation in calcium-free magnesium solution is less than that observed in control medium, indicates that magnesium cannot simply replace calcium in this secretory system. The same conclusion was reached for fluid secretion from the blowfly salivary gland (Prince & Berridge, 1973).

Magnesium is believed to have the ability to displace calcium from membranes (Petersen & Ueda, 1976). Although there is some evidence that a small hyperpolarization of cockroach salivary gland acinar cells occurs by displacement of calcium by magnesium (Mitchell & Martin, 1980), it seems unlikely that such a process contributes greatly to fluid secretion, since the basal rate is very low in calcium-free magnesium solution; moreover magnesium apparently is unable to displace calcium from the cellular store which can be exhausted by dopamine stimulation (Fig. 5).

*5-Hydroxytryptamine.* The inability of 5-HT to elicit an increase in secretory rate from a gland previously 'exhausted' by dopamine stimulation in calcium-free solution indicates that, although the two agonists evidently stimulate the gland through different receptors (Bowser-Riley *et al.* 1978) they operate a common post-receptor mechanism, and there is no store of calcium accessible to 5-HT which is not accessible to dopamine. Parod & Putney (1978) also suggested a common calcium-requiring post-receptor mechanism operated by both $\alpha$-adrenergic and muscarinic receptors mediating the control of potassium permeability in the rat lacrimal gland.

*Second messenger*

The results presented in the second part of this paper are evidence that when cockroach salivary glands are stimulated in conditions which inhibit fluid secretion, a change takes place within the glands which persists, and which can be expressed as an increase in secretory rate after the removal of both agonist and inhibition. This change is probably the build-up of a second messenger within the cell. The observation that, in the absence of calcium in the bathing fluid, this change does not occur indicates that calcium is necessary for the build-up of second messenger or may itself be the second messenger. The amount of calcium that can be released from the cellular stores by stimulation in calcium-free solution is apparently not sufficient to permit the concentration of second messenger in the cells to increase sufficiently to cause secretion when the inhibition is removed.

Berridge (1979) has proposed a scheme for excitation–secretion coupling for the blowfly salivary gland which involves both calcium and cyclic AMP as second messenger with rather complex feed-back control. The normal biphasic cyclic AMP response to 5-HT stimulation is curtailed in the absence of calcium, and the conclusions drawn from this are that the initial increase in cyclic AMP is due to mobilization of internally stored calcium, and that calcium may play an important part in the differential activation of adenylate cyclase and phosphodiesterase (Heslop & Berridge, 1980). They have also presented evidence for two separate 5-HT receptors, one acting through calcium and one through cyclic AMP as second messengers (Berridge & Heslop, 1981).

Kebabian & Calne (1979) have discussed pharmacological evidence for two types of dopamine receptor, one of which is linked to adenylate cyclase and the synthesis of cyclic AMP. Apparently no evidence has yet been found that cyclic AMP is able to stimulate fluid secretion in the cockroach salivary gland. Nevertheless, in the presence of a phosphodiesterase inhibitor, stimulation with dopamine does increase the concentration of cyclic AMP in the gland cells (J. W. Kebabian, personal communication), so it is possible that cyclic AMP may be involved, along with calcium, as a second messenger in the cockroach salivary gland.

*Readmission responses.* Transient electrical and secretory responses to the return of calcium after a period of calcium deprivation have been reported in a number of preparations, namely the adrenal chromaffin cell (Douglas & Rubin, 1961), exocrine pancreas (Petersen & Ueda, 1976; Kanno & Yamamoto, 1977) and salivary gland (Mitchell, Ginsborg & House, 1980; Ginsborg *et al.* 1980*a*, *b*). These may be further evidence for the role of calcium as, or in the regulation of, a second messenger. In the cockroach salivary gland there is always a marked increase in secretion in response to the return of calcium after a period of calcium deprivation (Fig. 11). The electrical response to calcium readmission is a large transient hyperpolarization (Ginsborg *et al.* 1980*a*, *b*) which reflects an increase in potassium permeability (Ginsborg *et al.* 1980*b*).

The electrical response to dopamine stimulation is also a hyperpolarization due to increased potassium permeability (Ginsborg *et al.* 1974) and it seems likely, therefore, that receptor activation causes an increase in the concentration of cytosolic calcium, which opens potassium channels in the plasma membrane of acinar cells as found in other cells (Meech & Strumwasser, 1970).

The precise role played by cytosolic calcium in fluid secretion is as yet unclear. Fluid secretion in the cockroach salivary gland is believed to be driven by sodium transport, sodium being actively extruded into the acinar lumen by Na–K pump activity (Smith & House, 1979). If the rate at which the Na–K pump operates is somehow related to

intracellular sodium concentration, intracellular calcium could promote secretion by increasing the rate at which sodium enters the cell across the basolateral membrane. This could be achieved either by a mechanism exchanging intracellular calcium ions for extracellular sodium ions, or by calcium influencing the membrane permeability to sodium.

The observation that increasing intracellular calcium concentration may lead to increased sodium influx or transport has been made on a number of tissues, and interpreted by some workers as evidence of sodium–calcium exchange (e.g. Lee, Uhm & Dresdner, 1980; Ozaki & Urakawa, 1979), and by others as evidence of calcium influencing membrane permeability (e.g. Parod & Putney, 1980; Brismar, 1980).

A third possible mechanism by which intracellular calcium might play a regulatory role in fluid secretion is by involvement with the regulation of cyclic AMP levels in the acinar cell. Further evidence about the role, if any, of cyclic AMP in the secretory response of the cockroach salivary gland is needed before any conclusion about the role of calcium in relation to such a system can be drawn.

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# CHLOROTETRACYCLINE FLUORESCENCE ASSOCIATED WITH PLASMA MEMBRANES OF COCKROACH SALIVARY GLAND CELLS

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## SUMMARY

The fluorescent compound chlorotetracycline (CTC) enters the cells of the cockroach salivary gland. The acinar peripheral cells and the non-secretory duct cells become preferentially labelled by CTC. Microscopic examination of the intracellular distribution of CTC indicates that this compound labels the highly folded apical plasma membranes of the peripheral cells and the deep infolds of the basal plasma membranes of the non-secretory duct cells. Lanthanum blocks the entry of CTC into all of the gland cells and in this condition the CTC labels the basal surfaces of the acini and ducts. The results of this investigation support the idea that CTC labels calcium ions in the vicinity of plasma membranes.

## INTRODUCTION

Many exocrine glands require calcium for maintained secretion of enzymes and fluid in response to agonists (Case, 1978). For example, in the absence of extracellular calcium ions the rates of fluid secretion by isolated blowfly and cockroach salivary glands stimulated respectively by 5-hydroxytryptamine and dopamine gradually decline to basal values (Prince & Berridge, 1973; Gray & House, 1982). Possibly this exhaustion reflects the depletion of a calcium-dependent store of second messenger necessary for stimulus–secretion coupling. In blowfly glands both cyclic AMP and calcium ions have been proposed as second messengers (Berridge, 1979) whereas in cockroach glands the identity of second messengers is uncertain. Not only secretory responses to agonists decline in the absence of calcium ions. The electrical responses of acinar cells in cockroach salivary glands to a sequence of ionophoretic pulses of dopamine also decline progressively (Ginsborg, House & Mitchell, 1980). This finding suggests the presence of an intracellular calcium store possibly located in the plasma membranes of the acinar cells. A similar conclusion has been drawn for other exocrine glands on the basis of different evidence (Petersen, 1980).

As a first step to establishing whether there is a plasma membrane store of calcium, we have investigated the distribution of the fluorescent antibiotic chlorotetracycline within cockroach salivary gland cells, since this compound may be a useful indicator of membrane calcium ions (Caswell, 1979).

## METHODS

The paired salivary glands and reservoirs were dissected from adult cockroaches, *Nauphoeta cinerea*, and bathed at room temperature in a solution containing (mM): NaCl, 160; KCl, 10; $CaCl_2$, 1; glucose, 20; Tris, 5; HCl, 4. This medium is referred to as normal solution and in one series of experiments $MgCl_2$ was added to it to give a final magnesium concentration of 5 mM.

A calcium-free solution was made by replacing $CaCl_2$ in normal solution by 1 mM-EGTA. The free calcium concentration of such a solution has been estimated to be less than $10^{-9}$ M (Hubbard, Jones & Landau, 1968).

A sodium-free solution was made by replacing NaCl in normal solution by 160 mM choline chloride (re-purified before use).

The composition of a chloride-free solution was (mM); sodium methylsulphate, 160; $K_2SO_4$, 5; $CaSO_4$, 1; glucose, 20; Tris, 5; $H_2SO_4$, 2.

The pH of all solutions lay in the range 7·3–7·7. Chlorotetracycline (Sigma Chemical Co.) and lanthanum nitrate (Taab Laboratories, Reading) were added to solutions to give the final concentrations cited in the text. In some experiments where measurements of salivary secretion were made the glands were stimulated by adding dopamine (Sigma) to the bathing solutions.

*Secretory measurements*

Isolated glands were mounted in a channel in a Perspex block through which various solutions were perfused (House & Smith, 1978). One of the salivary ducts was ligated near its end, passed through a small hole in the side of the channel into a pool of liquid paraffin and anchored by the ligature. A puncture was made in the duct posterior to the ligature to allow secreted fluid to escape. The volume of secretion was measured by removing the droplet of saliva from the duct with a micropipette and transferring it to another pool of liquid paraffin where it assumed a spherical shape. The diameter of the sphere was measured and the volume calculated.

*Microscopy*

Glands were examined by transmitted visible or ultra-violet light on a Carl Zeiss Universal microscope fitted with fluorescence illuminator and filters.

Pilot experiments showed that solutions containing 100 $\mu$M chlorotetracycline (CTC) gave optimal staining within a convenient experimental period. The results of each treatment described in Results were obtained on at least six glands. Representative light and fluorescence pictures of CTC-stained glands and tissue sections (see *Histology*) are shown because the results obtained under the different conditions were consistent and no other reliable way of giving the results is available. All fluorescence pictures, except those in Fig. 5, were obtained under the same photographic conditions and have been reproduced by an identical procedure for the sake of comparison. Fluorescence pictures of control glands and tissue sections incubated in normal solution are shown in Fig. 1; these pictures were obtained under the conditions of photography and printing which were used for the experimental groups in our study. The corresponding control pictures obtained for other experimental solutions containing CTC have not been reproduced because as shown in Fig. 1 these glands also had insufficient autofluorescence to make them visible under these conditions.

*Histology*

Glands bathed in various solutions containing 100 $\mu$M-CTC were fixed in 10% formalin. After wax imbedding, 15 $\mu$m sections were prepared for examination by fluorescence microscopy. In most experiments photographs were made of wax sections because the process of de-waxing reduced the fluorescent staining of the sections. In some experiments where high power light and fluorescence pictures were desired, glands were exposed to 500 $\mu$M-CTC, fixed and the sections were de-waxed and mounted in Gurr's U.V.-inert mountant. After fluorescence pictures of these sections were obtained the cover slips were floated off the slides by prolonged immersion in xylene and the tissue sections stained with Ehrlich's Haemotoxylin and Eosin and finally mounted in DPX (Raymond A. Lamb) for examination by light microscopy.

*Electron microscopy*

Glands from control and experimental groups were fixed at 4 °C in 4% glutaraldehyde in 0·05 M sodium cacodylate buffer with added sucrose (28 g/l). The glands were then processed conventionally for electron microscopy and stained with uranyl acetate and lead citrate. Ultra-thin sections (gold) were cut from araldite blocks and examined through a Philips 400 electron microscope.

## RESULTS

The uptake of CTC into cockroach salivary gland cells is relatively slow as observed in other cells (Chandler & Williams, 1978*b*; Naccache, Showell, Becker & Sha'afi, 1979; House & Bland, 1983). Fig. 2 shows light and fluorescence pictures of an acinus and duct bathed in

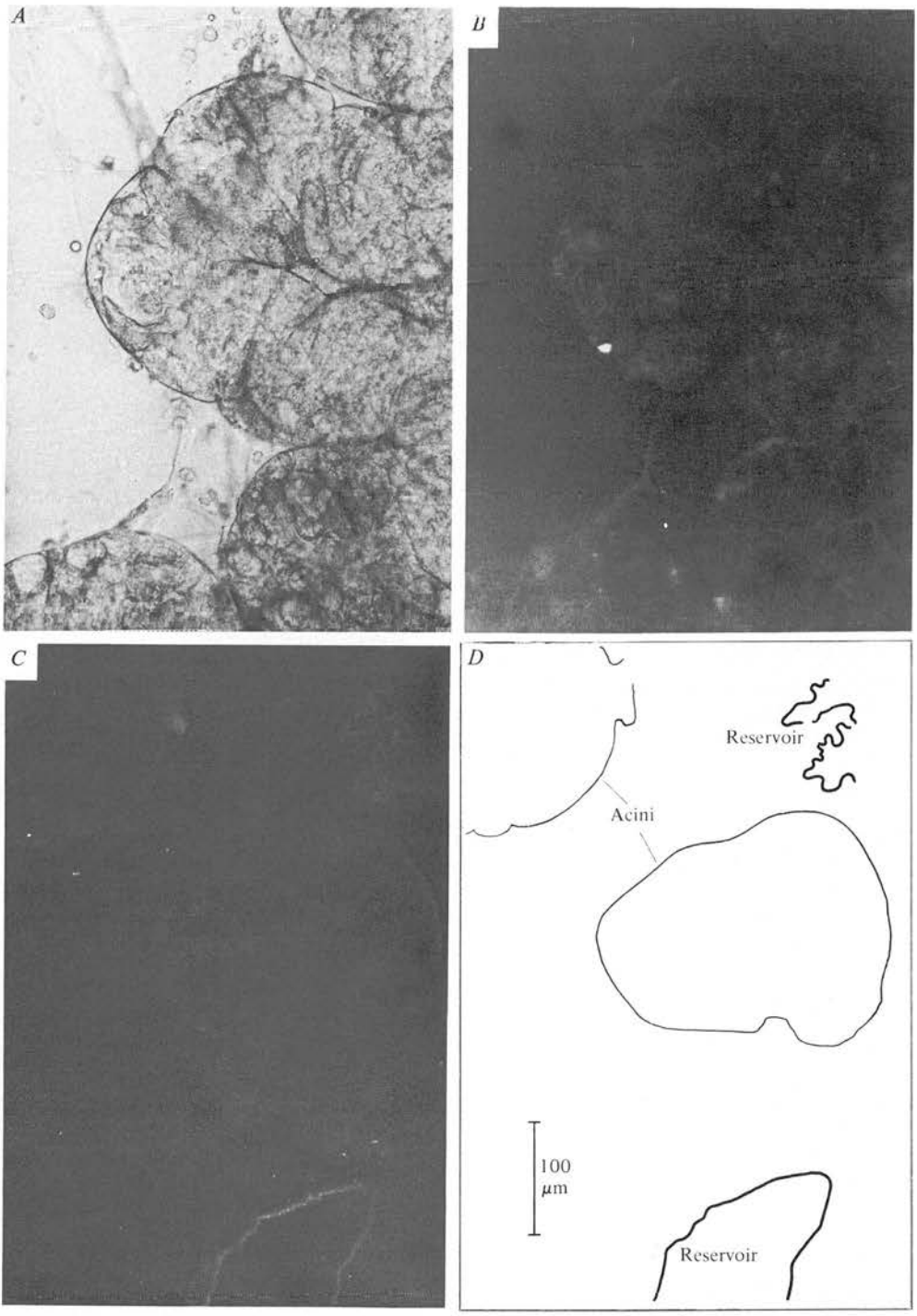


Fig. 1. Light and fluorescence pictures of salivary gland acini incubated in normal solution. *A* and *B* are respectively light and fluorescence pictures of living acini. *C* is a fluorescence picture of a wax section of acini from another control gland; *D* is a line drawing showing the boundaries of the acini and reservoirs in *C*.

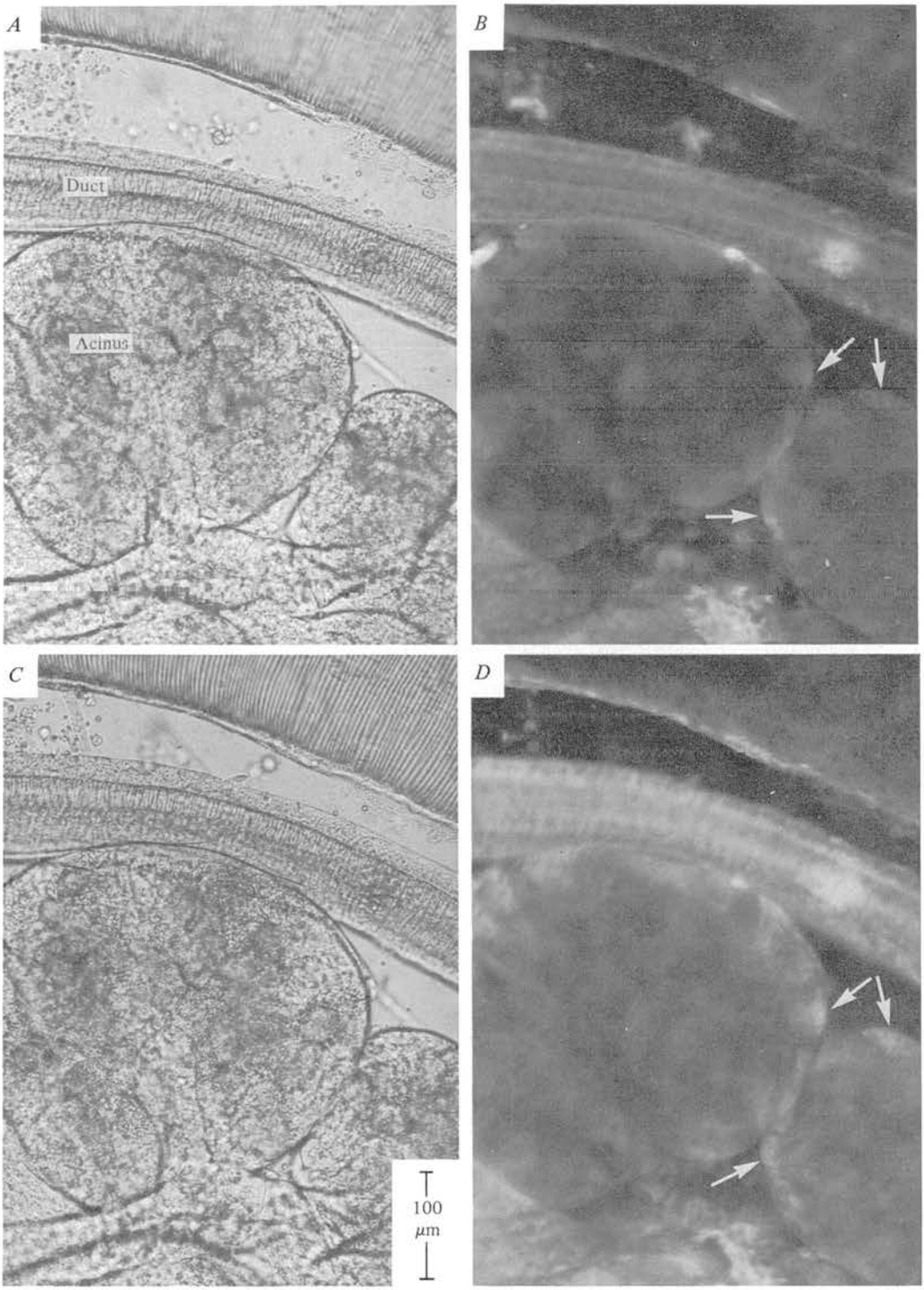


Fig. 2. Light and fluorescence pictures of living gland cells during uptake of CTC. *A* and *B* show corresponding light and fluorescence pictures of an acinus and a duct about 5 min after immersion in normal solution containing 100 μM-CTC. *C* and *D* show the same acinus and duct about 10 min later. The fluorescent CTC is taken up into duct cells and preferentially into acinar cells with a pyramidal shape (arrows).

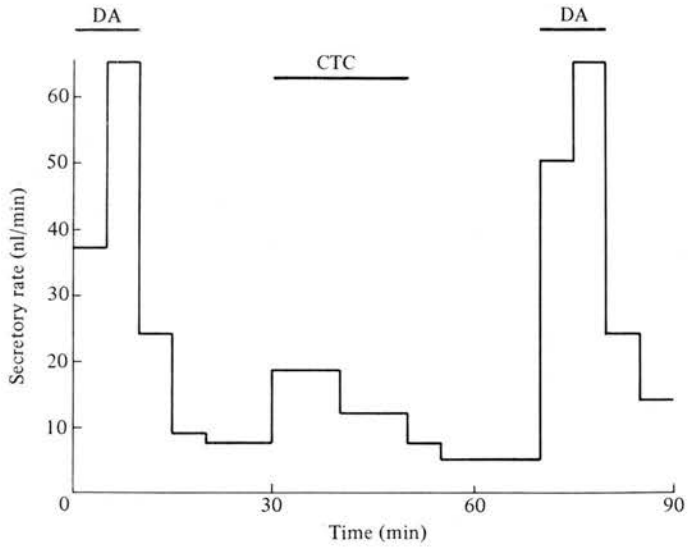


Fig. 3. Effect of CTC on the rate of fluid secretion by an isolated salivary gland. Dopamine (DA) and CTC were present in the bathing solution at concentrations of 1 $\mu$M and 100 $\mu$M respectively for the periods indicated by the horizontal bars.

normal solution containing 100 $\mu$M-CTC. Within about 5 min of exposing the gland to CTC the acinus and duct were weakly fluorescent. In the acinus the fluorescent label occurred at particular places (arrows, Fig. 2 *B*). When the same field was examined 10 min later the intensity of fluorescence in the acinus and duct had increased. Whereas the labelling of the duct did not vary along its length the labelling of the acinus was clearly concentrated in triangular shaped regions (Fig. 2 *D*). The area of these fluorescent regions (arrows) suggested that CTC preferentially had labelled the acinar peripheral cells (Bland & House, 1971) which have a characteristic pyramidal shape. The intensity of fluorescence continued to increase during further incubation in CTC solution, the gland cells being optimally labelled after 60–90 min. Examples of maximal labelling are shown below.

It was important to establish whether CTC interfered with the anatomy or physiology of gland cells. An electron microscopical study was made and the results are discussed below (see *Ultrastructure of CTC-stained cells*).

A check on the physiological function of the CTC stained gland was made as follows. The isolated gland secretes fluid in response to nerve stimulation or the presence of dopamine in the bathing fluid (House & Smith, 1978), and the effect of CTC on the power of the gland to secrete fluid was investigated. The results of a representative experiment are shown in Fig. 3. The gland was stimulated by passing a solution containing 1 $\mu$M dopamine through the chamber holding the gland. The rate of fluid secretion rose from a basal rate of about 1 nl/min (not shown) to about 60 nl/min during dopamine application. After recovery from dopamine stimulation a solution containing 100 $\mu$M-CTC was passed through the chamber. At this concentration CTC itself evoked secretion and did not depress the ability of the gland to secrete fluid in response to subsequent dopamine stimulation. In other experiments we found that dopamine could produce an increase in secretion in the presence of CTC.

*Sites of CTC staining*

The fluorescent staining in glands incubated in normal solution containing 100 $\mu$M-CTC for periods of 60–90 min was confined to certain acinar cells and duct cells. Fig. 4 shows a representative pattern of acinar labelling by CTC where the fluorescence originates from pyramidal-shaped cells (arrows, Fig. 4*B*). To establish the site of CTC deposition with greater precision we fixed CTC-labelled glands in 10% formalin and then processed the tissue for the preparation of wax sections. Evidently these procedures do not remove a substantial fraction of CTC from the cells because the tissue sections remained fluorescent. Fig. 4*C* shows a section from a gland incubated for 30 min in 100 $\mu$M-CTC; fluorescent regions can be seen in acinar cells (small arrows) and duct cells (large arrows). Fig. 4*D* shows a section from a gland incubated for 60 min in normal solution containing 100 $\mu$M-CTC; the fluorescence of acinar cells is more intense than in Fig. 4*C* but the subcellular distribution is similar. In an effort to determine the site of CTC staining within acinar cells we examined sections of stained cells at higher magnification after incubation in normal solution containing 500 $\mu$M-CTC for 2 h. Fig. 5 shows examples of acinar cells where the CTC staining occurred in a ring-like structure within the cells. These cells were confirmed to be peripheral cells by subsequently staining the section with haemotoxylin and eosin. Thus the ring structure, which is an origin of intense CTC staining, is the microvillar border formed by the prominent folding of the apical plasma membrane (Fig. 5*C* and *D*). The other type of acinar cell is the central cell with numerous granules and endoplasmic reticulum (Bland & House, 1971); evidently CTC does not stain granules in this kind of cell but the adjacent regions containing endoplasmic reticulum are fluorescent.

The idea that the acinar sites of CTC staining are associated with regions of prominent foldings of plasma membranes is compatible with our observation that certain duct cells are also intensely stained since these have prominent basal membrane infolds (Bland & House, 1971).

*Magnesium solution*

The enhanced fluorescence of CTC depends on the presence of divalent cations. Our normal solution contained calcium but not magnesium. Was the pattern of CTC labelling modified by the addition of magnesium to the normal solution? Fig. 6*A* and *B* show respectively the CTC staining in glands incubated in normal solution and normal solution containing 5 mM-$MgCl_2$. It is clear that magnesium ions partially reduce the CTC staining of gland cells and it seems unlikely that trace amounts of magnesium are the source of the relatively intense staining of glands bathed in normal solution. Nevertheless, the CTC staining present in glands incubated in solution containing magnesium is still associated with the same structures in cells (small arrows, Fig. 6*C*) as those seen in glands in normal solution (Fig. 4*D*).

*Calcium-deficient solution*

In glands incubated in calcium-deficient solution for several hours CTC labelling persists (Fig. 7) although it is less intense than in glands bathed in normal solution. The staining

Fig. 4. Light and fluorescence pictures of glands labelled by CTC. *A* and *B* show pictures of living acini incubated for about 40 min in normal solution containing 100 $\mu$M-CTC. *C* is a fluorescence picture of a section of acini and ducts (large arrows) incubated for 30 min in normal solution containing 100 $\mu$M-CTC; the CTC is non-uniformly distributed within some acinar cells (small arrows). *D* is a fluorescence picture of a section of acini and reservoir (*R*) incubated for 60 min in normal solution containing 100 $\mu$M-CTC; as in *C* the CTC is non-uniformly distributed (small arrows).

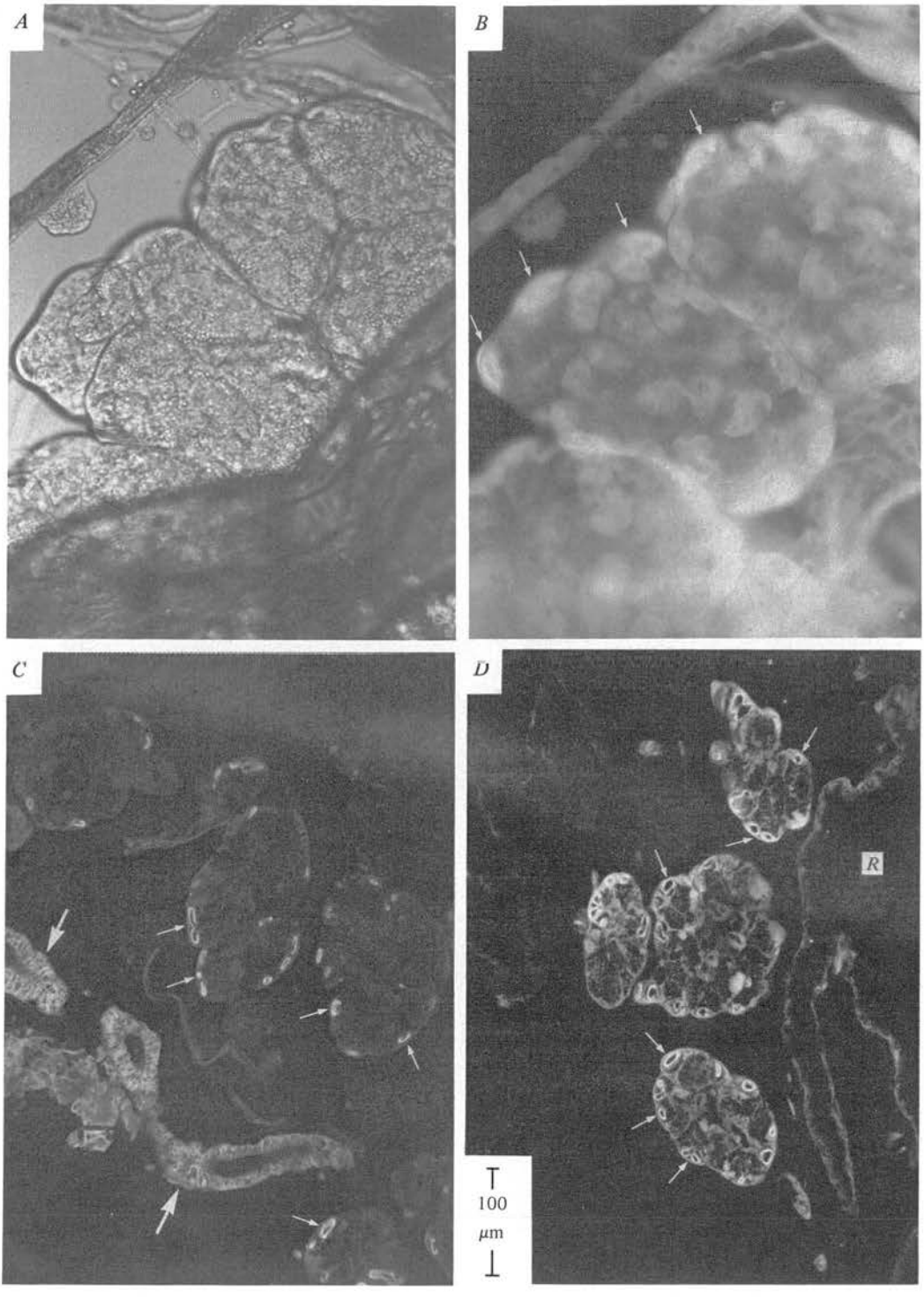


Fig. 4.

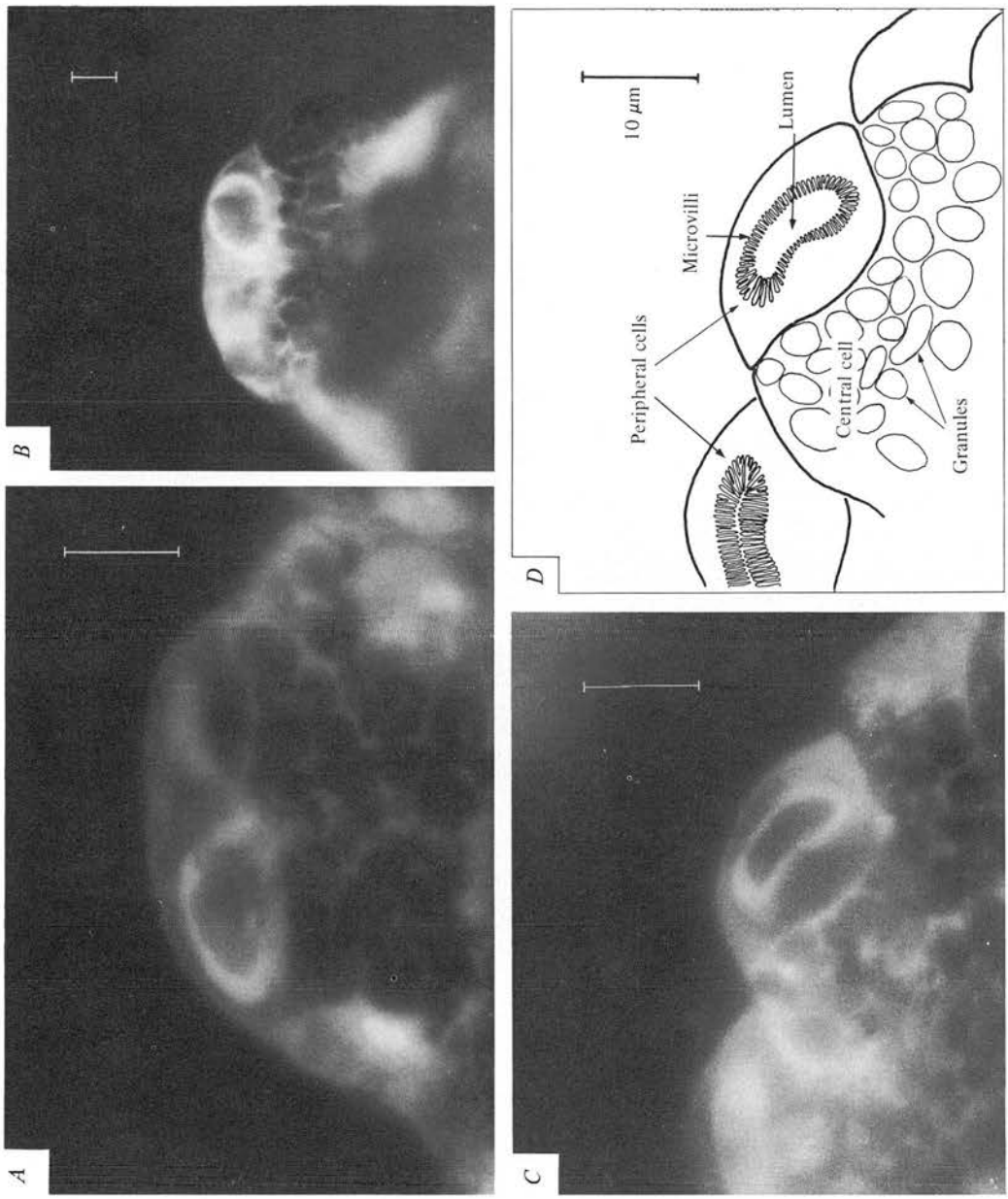


Fig. 5. Fluorescence pictures of sections of gland cells incubated in normal solution containing 500 μM-CTC. *A*, *B* and *C* show examples of acinar cells each containing intense CTC labelling of a ring-like structure. Subsequent histochemical examination revealed that these labelled cells were peripheral cells. *D* is a line drawing of the acinar cells in *C*. The scale bar in each part of the Figure indicates 10 μm.

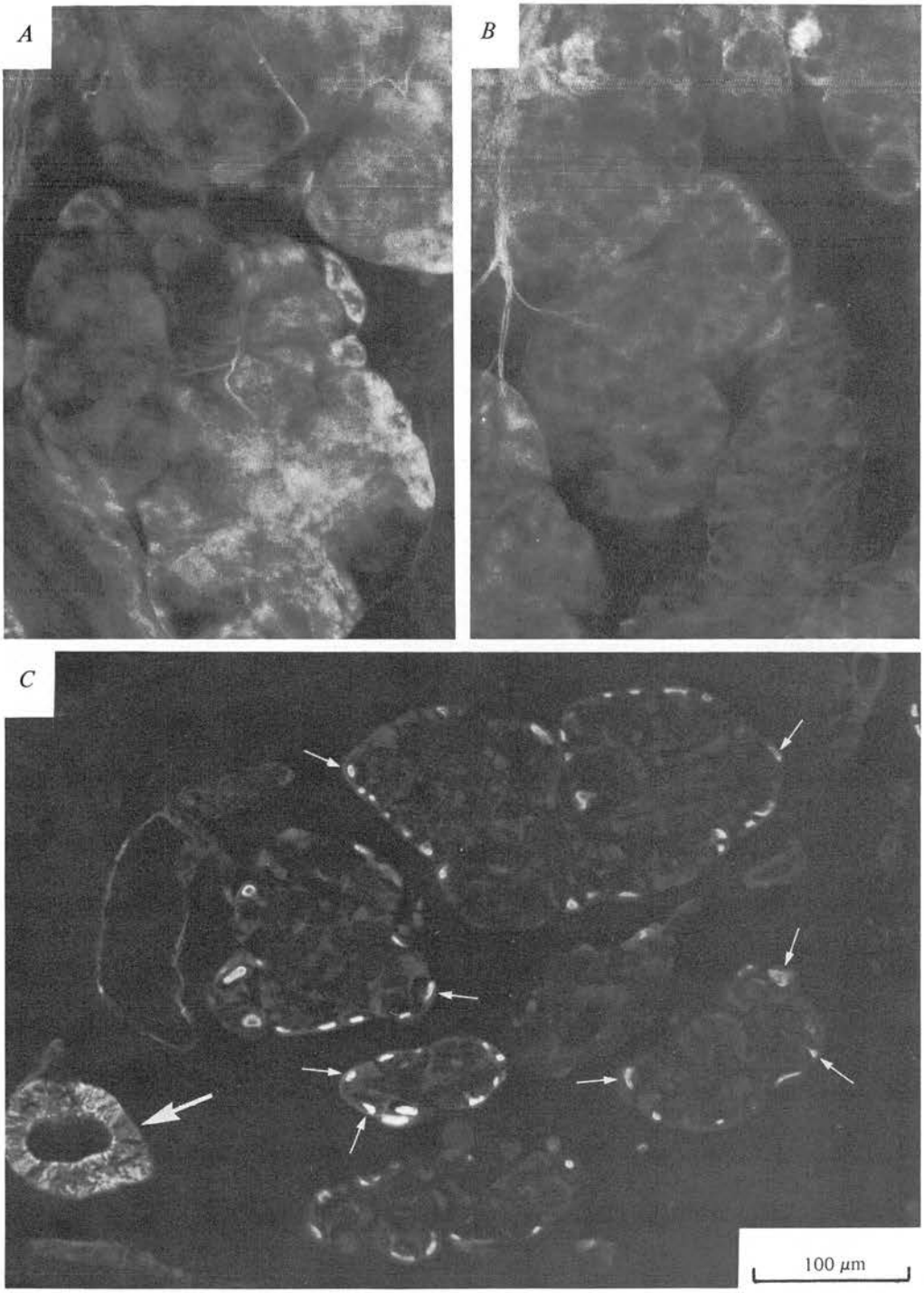


Fig. 6. Fluorescence pictures of gland labelled by CTC. *A* and *B* show pictures of living acini in glands incubated for 60 min respectively in normal solution containing 100 mM-CTC and in normal solution containing 5 μM-Mg and 100 μM-CTC. *C* shows a picture of a section of acini and duct (large arrow) in a gland incubated for 60 min in normal solution containing 5 mM-Mg and 100 μM-CTC.

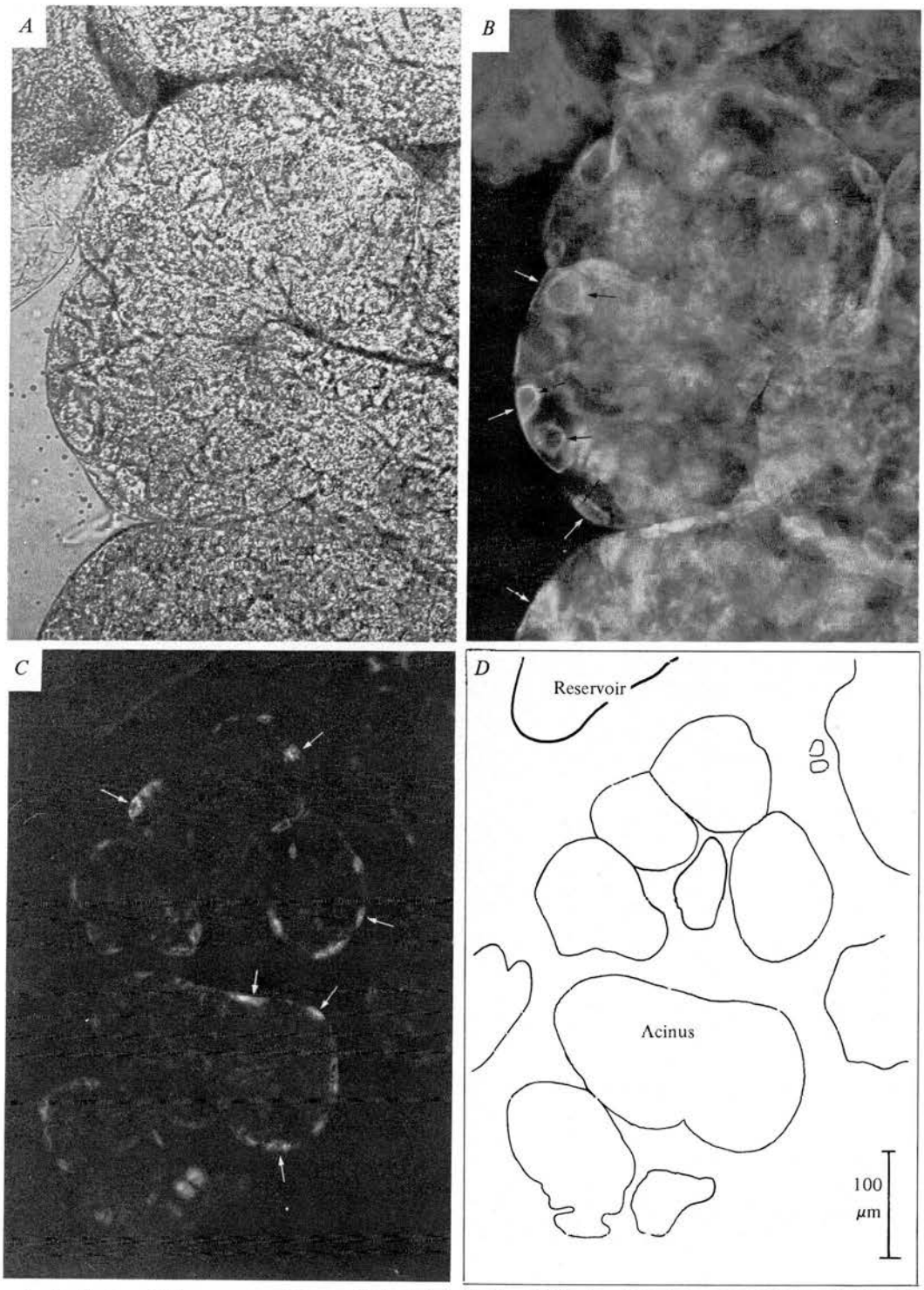


Fig. 7. Light and fluorescence pictures of glands labelled by CTC. *A* and *B* show corresponding light and fluorescence pictures of an acinus in a gland incubated for 60 min in calcium-free solution containing 100 μM-CTC. The fluorescence is located at the surface (white arrows) and intracellular structures (black arrows) of some acinar cells in *B*. *C* is a fluorescence picture of a section of acini from a gland incubated for 60 min in calcium-free solution containing 100 μM-CTC. *D* is a line drawing showing the boundaries of the acini and reservoir in *C*.

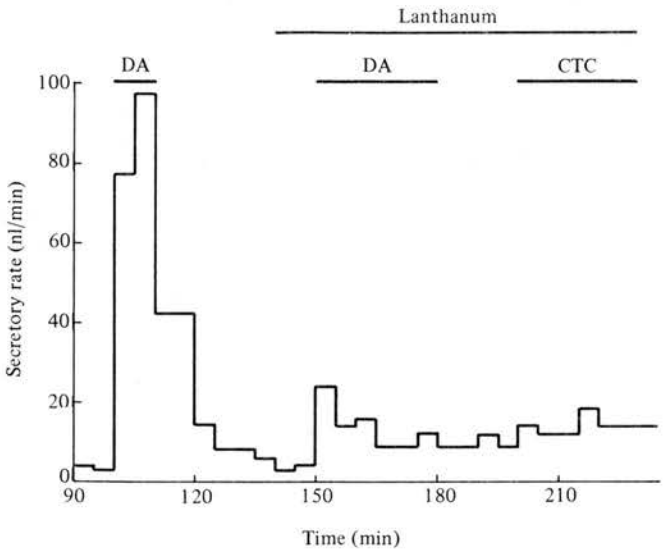


Fig. 8. Effect of lanthanum ions on the rate of fluid secretion by an isolated salivary gland. Dopamine (DA), CTC and lanthanum nitrate were present in the bathing solution at concentrations of 1 $\mu$M, 100 $\mu$M and 2 mM respectively for the periods indicated by the horizontal bars.

pattern in both whole glands and tissue sections strongly suggests that CTC labels the peripheral cells in the acini (white arrows, Fig. 7*B* and *C*). The labelling is relatively intense in the foldings of the apical membranes of these cells (black arrows, Fig. 7*B*).

*Solutions preventing fluid secretion*

Previous work (Smith & House, 1979) has shown that cockroach salivary glands bathed in sodium free or chloride free solutions do not secrete fluid in response to dopamine. Another extracellular ion which is necessary for maintained, but not transient, fluid secretion is calcium (Gray & House, 1982). One way of reducing calcium movement across the plasma membrane is to add lanthanum ions to the bathing solution (e.g. Herchuelz & Malaisse, 1978). In the blowfly salivary gland, for example, lanthanum ions appears to block both the influx of calcium ions and the fluid secretion normally produced by 5-HT stimulation (Prince & Berridge, 1973; Hansen Bay, 1978). Lanthanum ions also block dopamine-stimulated (or CTC-stimulated) fluid secretion by isolated cockroach glands (Fig. 8).

Glands bathed in sodium-free, chloride-free or normal solution containing lanthanum were examined. Each solution contained 100 $\mu$M-CTC and all glands were exposed to CTC for 60 min. Fig. 9 shows corresponding whole glands and tissue sections for chloride-free (*A* and *B*), sodium-free (*C* and *D*) and lanthanum solutions (*E* and *F*). The CTC labelling of glands incubated in chloride-free solution is similar to that observed in calcium deficient solution. In sodium-free solution CTC labelling is relatively weak; however, there appears to be labelling of peripheral cells (white arrows, Fig. 9*C*). In glands bathed in normal solution containing 2 mM lanthanum nitrate the pattern of CTC labelling was substantially different from that found in all other solutions used in this study. Fig. 9*F* shows that CTC was confined to the surface and extracellular space in the acini (small arrows) and ducts (large arrows). This surface labelling was non uniform and examination of whole acini (Fig. 9*E*) revealed patches of fluorescence which occurred in pairs.

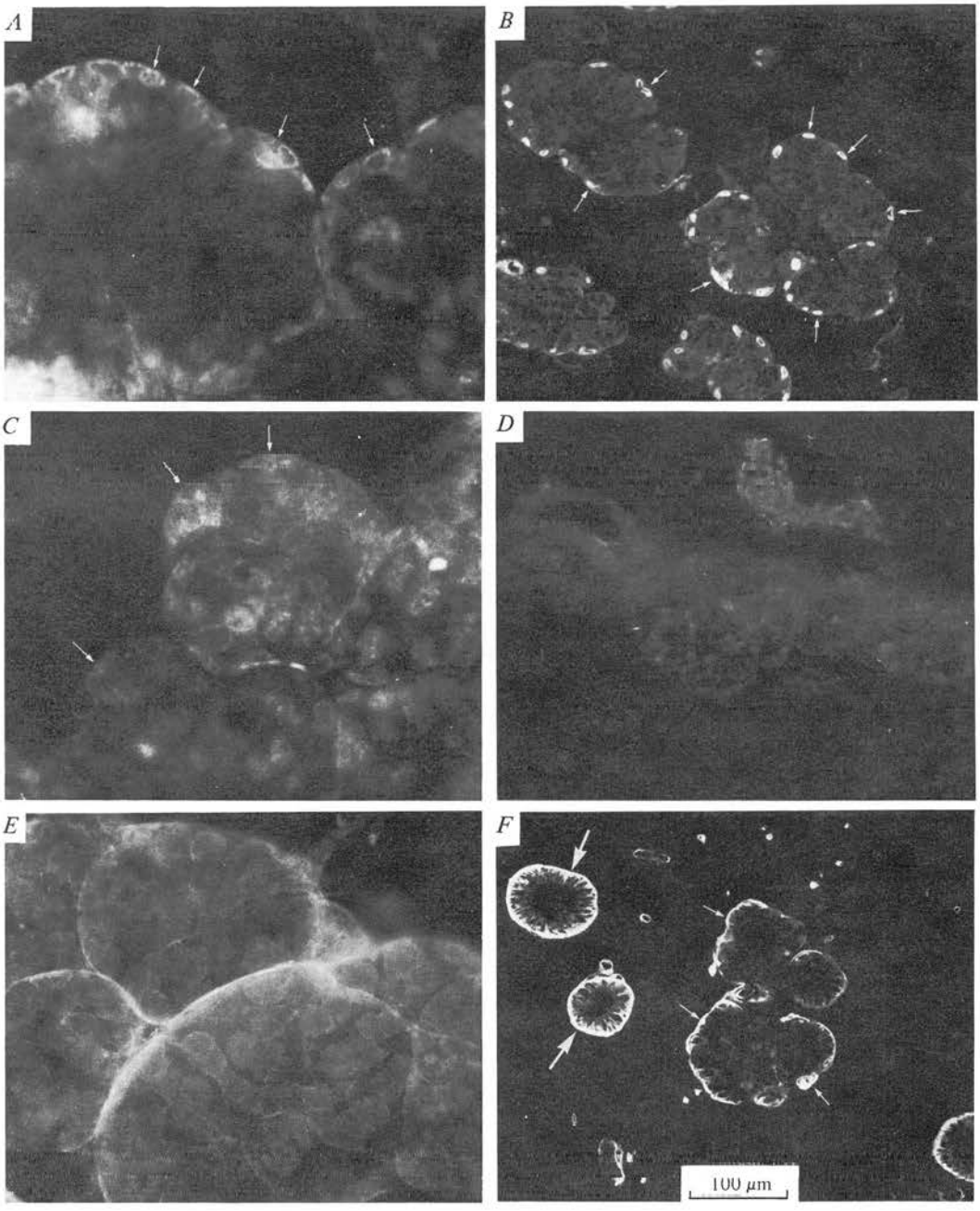


Fig. 9. Fluorescence pictures of glands labelled by CTC in solutions preventing fluid secretion. *A*, *C* and *E* show pictures of living acini and *B*, *D* and *F* show pictures of sections from glands incubated under the same conditions. *A* and *B* show CTC labelled acini incubated for 60 min in chloride-free solution containing 100 μM-CTC; selective uptake of CTC occurred (arrows). *C* and *D* show CTC-labelled acini incubated for 60 min in sodium-free solution containing 100 μM-CTC; selective uptake of CTC occurred (arrows). *E* and *F* show labelled acini incubated for 60 min in normal solution containing 2 mM lanthanum nitrate and 100 μM-CTC. In *F* the CTC labelling is located evidently at the surfaces of ducts (large arrows) and acini (small arrows).

*Effects of dopamine stimulation on CTC staining*

An important aim of the present study was to establish whether the plasma membranes of cockroach salivary gland cells had an associated store of calcium ions. Another aim of great interest was to discover whether such a store might be depleted by dopamine stimulation. Isolated glands were bathed in calcium-free solution containing 1 mM dopamine for three hours to deplete the calcium stores (see Gray & House, 1982). Another group of glands were bathed in calcium-free solution for the same period. Stimulated and unstimulated glands were bathed in calcium-free solution containing 100 $\mu$M-CTC for the last hour of the incubation and subsequently examined by fluorescence microscopy. No significant reduction in the CTC staining of stimulated glands compared to unstimulated glands was noted.

*Ultrastructure of CTC-stained cells*

Light and fluorescence pictures of CTC-stained glands shown above (Figs. 2, 4–7, 9) have no signs of gross cellular damage. Moreover, there is no evidence that CTC causes ultrastructural damage in mammalian pancreatic acinar cells (Chandler & Williams, 1978*b*) and eggs (House & Bland, 1983). Nevertheless, it seemed prudent to compare the ultrastructure of CTC-stained and normal cells of cockroach glands. No significant differences in the ultrastructure of normal and stained cells were noted. Fig. 10 shows representative areas from electron microscopical sections taken from acini bathed for 60 min in normal solution containing: 50 $\mu$M (*A*) or 100 $\mu$M-CTC (*B*). Both electron micrographs show regions of peripheral (*p*) and central cells (*c*) which are indistinguishable from pictures of normal *p* and *c* cells already published (Bland & House, 1971).

## DISCUSSION

*Distribution of CTC within cells*

The pattern of CTC labelling of the acinar and duct cells of the cockroach salivary gland strongly resembles the distribution of folded plasma membranes of the gland cells (Kessel & Beams, 1963; Bland & House, 1971; Whitehead, 1971).

In acinar peripheral cells the basal plasma membrane has shallow infolds of about 1 $\mu$m whereas the apical plasma membrane forms an elaborate surface of microvilli extending about 5 $\mu$m into the lumen (Fig. 10). The peripheral cells contain numerous mitochondria. Our finding that CTC staining of peripheral cells is particularly intense, especially in the region of the microvillar surface (Fig. 5), indicates that CTC labels plasma membranes and probably also mitochondrial membranes. Evidence for plasma membrane labelling by CTC has also been obtained in mammalian erythrocytes (Chandler & Williams, 1978*b*), pancreatic islet cells (Täljedal, 1978) and eggs (House & Bland, 1983). Mitochondrial labelling by CTC is also probably the origin of the fluorescent spots in isolated liver cells (DuBuy & Showacre, 1961; Caswell, 1972; Chandler & Williams, 1978*b*).

In contrast to peripheral cells the central cells do not have prominently folded plasma membranes and most of their volume is occupied by granules destined for secretion. The central cells are not well stained by CTC except in sparse cytoplasmic regions where there is endoplasmic reticulum between granules (Fig. 5). Indirectly the poor staining of the central cell supports the idea that CTC is associated with membranes. In these cells the site of staining is probably the endoplasmic reticulum in accord with the finding that in

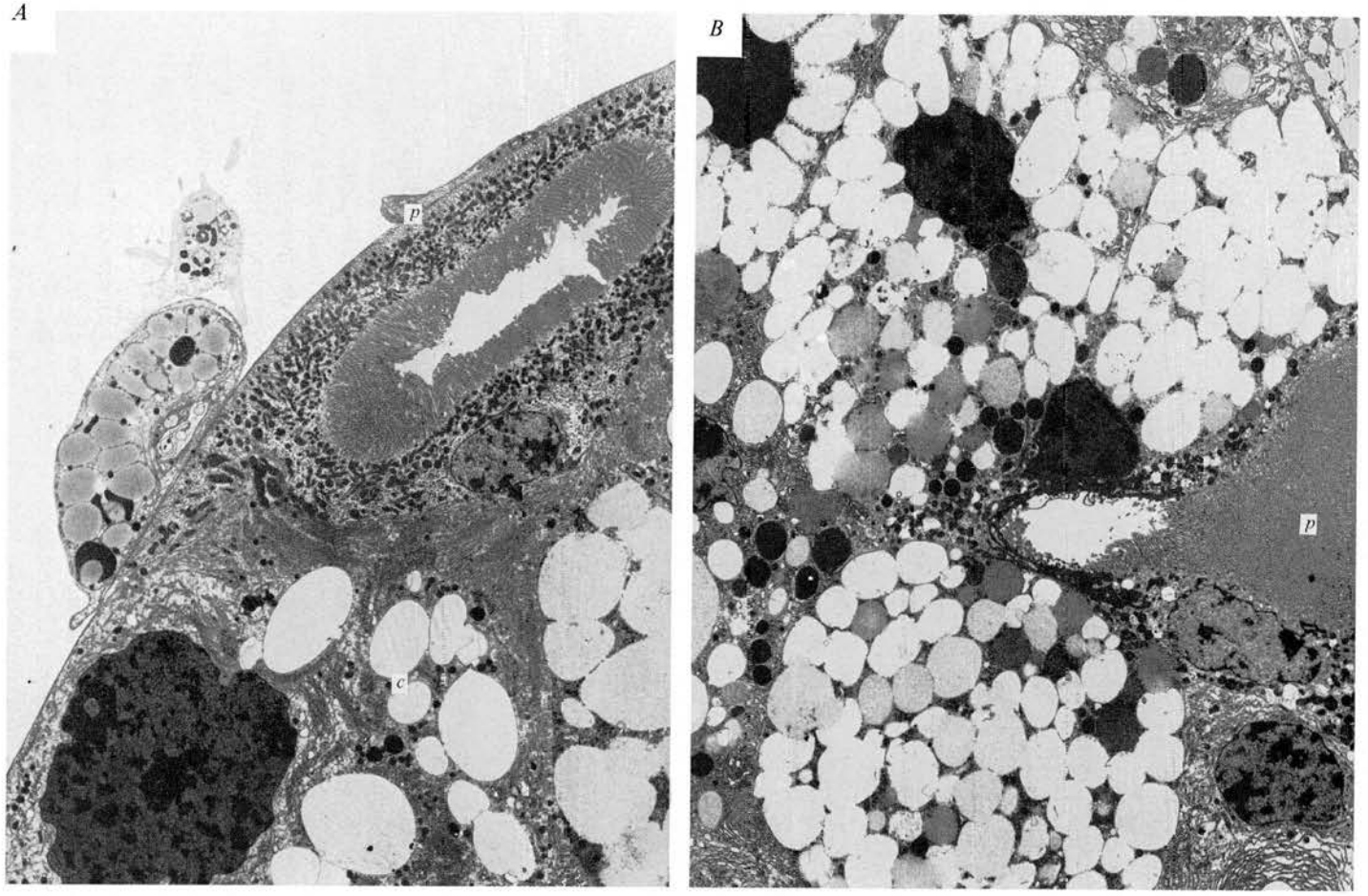


Fig. 10. Electron micrographs of acinar cells in glands incubated for 60 min in normal solution containing CTC prior to fixation. *A* shows peripheral (*p*) and central (*c*) cells in a gland exposed to 50 μM-CTC. *B* shows similar acinar cells in a gland exposed to 100 μM-CTC.

pancreatic acinar cells the regions containing rough endoplasmic reticulum (or Golgi vesicles) are labelled by CTC (Chandler & Williams, 1978*b*).

The secretory ducts adjacent to acini are comprised of cells which apparently make, store and secrete a mucoid substance (Bland & House, 1971). The ultrastructural features of these secretory cells are similar to those of the acinar central cells and make them unlikely candidates for intense labelling of plasma membranes.

Another type of duct cell is found at some distance from the acini in this gland. It contains numerous mitochondria interleaved between long infolds of the basal plasma membrane. The apical plasma membrane of this cell has short infolds. Apparently the intense but non-uniform labelling by CTC of this type of duct cell is consistent with a pattern of plasma membrane labelling expected from our observations on the acinar peripheral cells.

Thus it appears that the pronounced foldings of the plasma membranes of particular cells in the cockroach salivary gland are the origin of strong CTC fluorescence. Compartments in rod photoreceptors containing folded disc membranes are equally intense sources of CTC fluorescence (Chandler & Williams, 1978*b*). Plasma membrane in an unfolded state is not such an effective source of fluorescence although in the early stages of the labelling of mammalian pancreatic islet cells (Täljedal, 1978) and eggs (House & Bland, 1983) discrete patches of fluorescence have been observed at the surface of these cells. That this surface labelling originates in the plasma membrane has not been established beyond doubt. However, when both cell types are incubated for about 60 min in solutions containing CTC and lanthanum the surface labelling becomes relatively intense. It seems that lanthanum ions interfere with the entry of CTC into these cells and thereby enhance the relative intensity of labelling at the cell surface as has been found in the present study (Fig. 9*E* and *F*).

*Influence of calcium on CTC staining*

When CTC binds to calcium or other divalent cations in aqueous solution the complex formed has an enhanced fluorescence. The enhancement is increased further if binding occurs in a less polar medium such as membrane lipid. Thus CTC may act as an indicator of divalent cations stored in the vicinity of cell membranes.

Our results suggest that calcium rather than magnesium ions increase the fluorescence of CTC associated with the plasma membranes of cockroach salivary gland cells. The intensity of labelling is reduced in glands incubated in calcium-free solution or normal solutions containing magnesium. Thus magnesium cannot substitute for calcium at the sites where CTC fluorescence originates in the cells. Nor can magnesium substitute for calcium as an agent influencing the control of fluid secretion by this gland (Gray & House, 1982).

Possibly CTC is a calcium ionophore as suggested by Täljedal (1978) for pancreatic islet cells. It produces an apparent increase in insulin release from pancreatic islet cells (Täljedal, 1978) and a rise in the calcium influx into these cells (Sehlin & Täljedal, 1979). A similar phenomenon might also occur in cockroach salivary glands since CTC evokes fluid secretion (Fig. 3). Lanthanum ions block fluid secretion evoked by CTC or dopamine (Fig. 8) and also reduce the entry of CTC into gland cells (Fig. 9*E* and *F*). Poor cytoplasmic labelling by CTC in the presence of lanthanum has been observed also in mammalian pancreatic islet cells (Täljedal, 1978) and eggs (House & Bland, 1983). Perhaps lanthanum blocks a co-entry process mediating the influx of calcium and CTC. In this connexion it is interesting to note that the acinar surface labelling by CTC in normal solution containing lanthanum seems to occur in patches similar in size and distribution to the basal surfaces of peripheral cells.

Our results suggest that the plasma membranes of cockroach gland cells have a store of calcium ions. Is this calcium stores affected by dopamine stimulation? Our experiments on this point were inconclusive and perhaps a quantitative method of monitoring the CTC fluorescence from areas of plasma membrane would yield definitive results. Fluorometric measurements of CTC fluorescence from whole isolated cells, including pancreatic acinar cells (Chandler & Williams, 1978*a*), islet cells (Täljedal, 1978) and neutrophils (Naccache *et al.* 1979) indicate that stimulation by various agonists causes calcium release from intracellular stores. The subcellular location of these stores, however, remains to be identified.

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