ROLE OF CALCIUM, CATECHOLAMINES AND CYCLIC AMP IN CONTROLLING SALIVARY FLUID SECRETION IN THE LONE STAR TICK

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Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
May, 1978

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ROLE OF CALCIUM, CATECHOLAMINES AND CYCLIC
AMP IN CONTROLLING SALIVARY FLUID
SECRETION IN THE LONE STAR TICK

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PREFACE

Salivary glands of female ixodid ticks perform several very crucial functions that facilitate successful host-parasite interaction. One of the most critical and interesting physiological processes of this tissue involves regulation of ion and water balance while embibing a substantial bloodmeal. In this regard, considerable research effort has focused on salivary gland and nervous system morphological characteristics and control of salivation itself with respect to the primary gland stimulus; however, very little attention has been given to detailed study of how the primary nerve signal is translated into cells responsible for fluid secretion.

The objective of the present research is to determine if calcium plays a role as an intracellular regulator or cofactor in controlling salivation in rapidly engorging female ixodid ticks and what interrelationship(s) might exist with respect to the primary stimulus, cyclic AMP and calcium. If temporal changes in cytosolic calcium concentrations are necessary for altered cell activity, then information about the source of this cation (intracellular, extracellular or both) and when "flux" occur would provide some valuable insights into the mechanisms involved in fluid secretion. Ultimate elucidation of the exact roles of the primary stimulus, calcium and cyclic nucleotides in controlling ion and water movement is desired but beyond the scope of this study.

The author would like to express his gratitude to Dr. Calvin G. Beames, Jr., Professor and Acting Chairman, Department of Physiological Sciences for his valuable advice and encouragement during this study and I would like to thank Dr. Lester L. Rolf, Jr., Assistant Professor, Department of Physiological Sciences, for his assistance and helpful comments during my program. Appreciation is also extended to Dr. Jerry Young, Professor, Department of Entomology, for his guidance on many statistical aspects of this study. I greatly appreciate the assistance of Dr. Jakie A. Hair, Professor, Department of Entomology, in providing ticks and allowing unhindered access to his pH meter. All these individuals served on the author's graduate committee.

Thanks go to Kathy Dart, Kila Roggow, Carlene King, Robert Morris, Pam Needham and the people at the Medical Entomology Lab for their valuable technical assistance.

I would like to offer a very special "thank you" to Dr. Pete Teel, John Riner and Wendell Johnson for their continued interest, frequent encouragement and stimulating bull sessions. And thanks go to Harold McMullen for his persistence in helping me to develop patience and for his continued friendship.

The author expresses his sincerest gratitude to Dr. John R. Sauer, Professor, Department of Entomology, who while serving as Graduate Committee Chairman, provided unfailing encouragement, patience and direction.

Finally to my parents, Bob and Dollie, and my sisters, Pam and Nancy, I extend my deepest appreciation for their love and understanding throughout my persuit of a career in science.

This research was supported in part by NSF Grant Nos. PCM 74-24140 AO_2 and PCM 74-24140 AO_1 from the National Science Foundation and by the Oklahoma Agricultural Experiment Station.

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CHAPTER I

INTRODUCTION

Bloodsucking ixodid ticks face a major task in maintaining salt and water balance while embibing large bloodmeals. During the rapid phase of engorgement (last 12-24 hours of feeding) the lone star tick, *Amblyomma americanum* (L.) more than doubles (350 to 800 mg) in weight while excreting approximately one-third of the ingested blood (in the form of excess ions and water) through the salivary glands back to the host (Sauer and Hair, 1972). The salivary glands were shown to be the ticks' chief excretory organs by Gregson (1967, 1969), Balashov (1965), Tatchell (1967a) and Kaufman and Phillips (1973a). The watery fluid secreted by the glands contains high concentrations of sodium and chloride (Kaufman and Phillips, 1973c, Hsu and Sauer, 1975) there being some evidence of active transport of one or both ions (Kaufman and Phillips, 1973c; Needham and Sauer, 1975; Kaufman et al. 1976).

Recent findings on control of salivary fluid secretion show that glands are stimulated to salivate when pilocarpine (a cholinomimetic) or catecholamines (adrenaline, noradrenaline and dopamine) are injected into the feeding tick (Kaufman and Phillips, 1973b; Hsu and Sauer, 1975). In addition, *in vitro* salivary gland preparations are sensitive to catecholamines but not pilocarpine. These results suggest cholinergic involvement in glandular control at sites distant from the glands themselves. In support of this, Teel et al. (in preparation), found that

Fenthion®, an organophosphate insecticide, stimulates or inhibits salivation in female lone star ticks to produce what has been termed a "blistered" (abnormally swollen) tick or a dessicated tick, respectfully. The poisoned tick probably responds according to the degree of acetylcholine esterase inhibition that occurs. It is interesting to note that these ticks are very loosely attached, indicating that cement secretion was antagonized by the organophosphate. In support of the above Megaw (1974) suggested that salivation is normally initiated by the firing of cholinergic nerves which in turn synapse with adrenergic secretomotor nerves.

There is now considerable evidence implicating nervous control of salivary fluid secretion rather than hormonal control: (1) No hormonal factor(s) have been found in the hemolymph which stimulate *in vitro* salivation (Kaufman and Phillips, 1973b; Kaufman, 1976). (2) Adrenergic pharmacological agents (catecholamines) induce fluid secretion *in vivo* and *in vitro* (Kaufman and Phillips, 1973b; Kaufman, 1976; Kaufman, 1977; Needham and Sauer, 1975). (3) Nerves can be seen leading from the synganglion and its branches to the salivary glands and their individual alveoli (Obenchain and Oliver, 1976; Megaw, 1977). (4) Noradrenaline and dopamine are found in high concentrations in both the synganglion and salivary glands (Megaw and Robertson, 1974; Binnington and Stone, 1977). (5) Monoamine oxidase activity is present in the synganglion and salivary glands of *Boophilus* (Atkinson et al., 1974). Determining the actual neurotransmitter associated with stimulating salivation and characterization of the receptor site(s) has been the subject of considerable interest and investigation (Kaufman and Phillips, 1973b; Kaufman, 1976, 1977; Sauer, 1977; Sauer et al., in preparation).

Kaufman (1977) determined that tick salivary glands contain one or several receptors differing pharmacologically from mammalian α-adrenergic, β-adrenergic and dopaminergic receptors. Interestingly, dopamine is thought to innervate the insect (cockroach) salivary gland (Robertson, 1975) and could also be the neurotransmitter at the tick neuroglandular junction because of its very low stimulatory threshold concentration (Kaufman, 1976; Sauer et al., in preparation).

Tick salivary gland control is apparently multifaceted and most research to date has focused on whether the primary stimulus is neural or hormonal. Indeed, it is still not known what factor(s) initiate the salivation process and maintainance of ion and water balance during feeding. In Rhodnius prolixus (L.) stretch receptors in the abdominal wall are sensitive to distention. When a bloodmeal is taken these receptors stimulate neurosecretory cells in the fused mesothoracic mass to release diuretic hormone from nearby neurohemal organs (Maddrell, 1966). Diuretic hormone causes the Malphighian tubules to initiate diuresis (Maddrell, 1964).

At this stage I think it is important to emphasize that invertebrate systems at the cell level are often times functionally comparable with vertebrate systems and therefore frequent references will be made to information concerning a vertebrate cell or tissue type without hesitation.

Most investigations have focused on control of the salivary glands, that is, the primary stimulus and resultant increase in fluid secretion. There are probably many events that occur between this initial step and the final result. Considerable information has been gathered on the initial gland stimulus and its pharmacological characteristics; however,

virtually no studies have been conducted on the intracellular events which are responsible for salivation in ticks.

Calcium and cyclic nucleotides (cyclic AMP and cyclic GMP are the main components of an internal messenger system in many cells with the importance of each dependent on the tissue. The primary function of each is to negotiate the cells response to external stimuli. Generally the plasma membrane receives the external signal and conducts the message into the cell by rapidly changing intracellular concentrations of calcium or cyclic nucleotides or both. One feature that Berridge (1975) feels is basic to second messenger homeostasis is the very rapid turnover of the messengers. This turnover enables the signaling system to respond to small changes in the signal input arriving at the cell.

In the following, second messenger homeostasis with emphasis on calcium metabolism within the cell will be discussed. In addition, references will be made to the cyclic nucleotides and the role they may play. Calcium's importance in biological systems has been appreciated since the early experiments of Sidney Ringer (1890). We now know that calcium is essential in cellular adhesion and cell or membrane permeability. Calcium modulates monovalent cation permeability in excitable tissues and neutrotransmitter release, muscle contraction, blood coagulation, energy production as it relates to glycogenolysis, respiration and electron transport in mitochondria and is involved in stimulus division coupling and regulation of the activity of many enzyme systems (Rubin, 1974). Each of these processes or properties involving calcium seem to possess their own characteristics as it relates to controlling movement and concentrations of calcium.

Cell calcium can be characterized as follows (Rassmussen, 1975;

Rasmussen and Goodman, 1977). (1) Its distribution in the cell is not homogeneous, but is compartmentalized into isolated subcellular fractions. (2) Most cellular calcium is tied up as a readily exchangeable non-ionic phosphate salt. (3) The dynamic state of calcium is dependent on many interrelated factors such as electrical potential differences between cell compartments, extracellular pH and phosphate concentration.

Calcium compartmentalization can be divided further into (1) an interstitial fraction; (2) membrane fractions, which include plasma membrane systems and endoplasmic reticulum (microsomal); (3) other cellular organelles, including mitochondria secretory granules and lysosomes (Rubin, 1974). Cell calcium is bound to acidic phospholipids, basic components of membranes (Dawson and Hauser, 1970) or more importantly tied up as a non-ionic phosphate complex in mitochondria which is readily exchangeable with mitochondrial calcium and thus with the calcium ions in the cell's cytosolic and extracellular fluids (Rasmussen, 1975).

Mitochondria actively take up calcium by a stoichiometric coupling of calcium accumulation to electron transport (Chance, 1965; Lehninger, 1970). The avidity of this transport was demonstrated by Patriarca and Carafoli (1968) where the majority of radiocalcium injected in vivo was concentrated by the mitochondrial fraction of tissues they examined. However, the importance of mitochondria in storing calcium varies from tissue to tissue (Carafoli and Lehninger, 1971).

The explanation of cell calcium homeostasis thus far has been very general and extremely simplified but there are many complicating factors

that may alter the effects described above. When one considers the many inorganic and organic species present in the cell and how they interact through various feedback and buffering complexes, it is not surprising that the phrase "cell ionic net" is used to describe this situation (Rasmussen, 1975; Rasmussen and Goodmann, 1977). This means that a change in any ionic or molecular species can potentially lead to a change in the distribution of all or part of those species present.

Processes responsible for removal of calcium from the cell's cytosol are as important as the signals which initiate an increase in cell calcium. Free cytoplasmic calcium is the important fraction and in the resting cell the intracellular concentration is between 10^{-5}M to 10^{-7}M (measured in nerve and muscle cells; Hodgkin and Keynes, 1957, Portzeh et al., 1964; Luxoro and Yanez, 1968), while the extracellular level is approximately 10^{-3}M. The existence of this chemical gradient and an electrical gradient permits calcium entry into the cell. When cells are at rest, however, calcium entry is very slow despite this substantial electrochemical gradient, with the plasma membrane of most cells relatively impermeable to calcium. Altering membrane permeability to calcium seems to be responsible for excitability of many cells and thus controlling cytoplasmic levels of calcium.

Berridge et al., (1975) discusses at least two ways of altering calcium permeability: (1) the stimulant induces a change in membrane permeability with little change or hyperpolarization in the membrane potential (i.e., ACTH in the adrenal cortex; insect salivary gland); (2) the action of the stimulant is coupled to changes in the membrane potential (usually depolarization). High potassium concentrations can be used to activate cells via depolarization and thus allow calcium influx,

Following either an extracellular influx or intracellular release, or both, from calcium reservoirs, the cell may recover by active uptake or extrusion mechanisms (Brinley, 1973). In various excitable cells (nerve or heat muscle) calcium may be extruded from the cell by a forced exchange with sodium (Reuter and Seitz, 1968; Blaustein and Hodgkin, 1969; Baker, 1970). Nonexcitable cells may utilize a Ca-activated ATPase to pump calcium from the cell (Schatzmann, 1975).

The role of calcium as a universal intracellular second messenger has been accepted rather slowly. This is due to several factors. A major hinderance has been that the calcium ion concentration of the cell cytosol cannot be measured easily. Additionally, there have previously been no means of controlling cytosolic levels of calcium, enabling alteration of Ca-dependent intracellular systems.

Recent development of rather specific calcium ionophores A23187 and X-537 (see Reed and Lardy, 1972; Scarpa and Inese, 1972; Babcock et al., 1976; Pfeiffer and Lardy, 1976) and drugs such as verapamil or D-600 which is capable of blocking cell calcium flux (Fleckenstein, 1971; Kohlhardt et al., 1972, Malaisse et al., 1977) or mitochondrial calcium uptake blockage by ruthenium red (Carafoli et al., 1975) has opened a new era in the study of cellular calcium and cell activation. Also, the antibiotic filipin has helped differentiate between the involvement of mitochondria and the plasma membrane as it pertains to cell calcium entry and efflux (Babcock et al., 1976).

Further inforamtion about cell calcium homeostasis has evolved by studying isolated cell organelles such as mitochondria (Chance, 1965; Borle, 1975; Scarpa, 1975) and microsomal fractions (Ebashi and Endo, 1968).

Some of the most comprehensive information gathered thus far has been obtained using radiocalcium-45 and the chemicals and techniques mentioned above (Prince et al., 1973; Thorn, 1974).

There has been considerable confusion however, when one begins to compare tissue concentrations of calcium and this is due partially to differences in methods of measuring its level. Many techniques have been utilized, including conventional spectrophotometry (Grossman and Furchgott, 1964) fluorescence spectrophotometry (Jaanus and Rubin, 1971), atomic absorption spectrometry (Hilmy and Somjen, 1968) and dual-wavelength spectrophotometry with the calcium complexing dye murexide (Onishi and Ebashi, 1963; Chaplin and Grace, 1976). The most interesting and possibly the most important new technique is one employing a photoprotein aequorin, which emits light in the presence of free calcium (Blinks et al., 1976; Rose and Loewenstein, 1976). Another promising method of qualitating and quantitating specimens *in situ* is electron-probe x-ray microanalysis using either "conventional" or scanning electron microscopy (Hall, 1975).

Tissue levels of cyclic AMP (cAMP) are somewhat easier to measure than calcium and this has facilitated a more rapid understanding of its involvement as an intracellular second messenger. The intracellular concentration of cAMP is determined by a dynamic balance between synthesis and degradation. The original hypothesis of Robinson, Butcher and Sutherland (1967) describes a receptor unit translating the primary extracellular signal to the catalytic unit (adenyl cyclase) in the plasma membrane. Adenyl cyclase acts to convert ATP to cAMP. Cyclic AMP then activates a protein kinase which catalyzes the phosphorylation of a

protein to alter some intracellular process. The enzyme phosphodiesterase (PDE) is responsible for degradation of cAMP to 5'-AMP. The important point is the dynamic nature of this cyclic nucleotide and how very minor changes in either the synthetic or degradative processes may alter intracellular concentrations drastically. There undoubtedly are complicated feedback mechanisms that regulate levels of cAMP (and calcium).

PDE is inhibited by methylxanthines which has afforded scientists an effective tool for altering cAMP levels without a primary stimulus. Theophylline and caffeine allow one to separate effects due to the primary stimulus from effects caused by elevated levels of cAMP. However, one must view the results of these experiments with caution because these drugs have been shown to have affects on cells not related to PDE inhibition.

A recent review by Berridge (1975) has brought to light the importance of cyclic nucleotide and calcium interaction with the cell. Because these second messengers exist in the cell together and usually affect the same processes, it is difficult to envision their activities as being independent. On the contrary, intricate feedback mechanisms must be operating within the cell.

Calcium seems to exert its affect on cyclic nucleotides by activating PDE through the phosphodiesterase activator (PDEA) and calcium may also lower the cAMP by inhibiting adenyl cyclase. Alteration of PDE activity by calcium (through PDEA) is most clearly demonstrated in brain (Kakiuchi et al., 1973) and heart muscle (Teo and Wang, 1973). Adenyl cyclase inhibition by calcium is shown where calcium-free solutions potentiate higher levels of cAMP (Nagata and Rasmussen, 1970; Prince, Rasmussen and Berridge, 1972).

Berridge (1975) is convinced that cAMP's ability to modulate calcium homostasis is perhaps the most significant feedback relationship within the cell. He states that "cyclic AMP can act by stimulating either the mechanisms responsible for removing calcium or it can increase the influx of extracellular or the release of intracellular calcium." The sarcoplasmic reticulum in cardiac muscle sequesters calcium via cAMP stimulation (Entman et al., 1969). In smooth muscle and other cells cAMP stimulates a plasma membrane pump which helps remove calcium.

During cell stimulation, cAMP may act to signal calcium entry either from the outside or from intracellular stores. Calcium entry across the cell membrane in nerves and heart is well documented and calcium release from intracellular reservoirs (mitochondria) has been shown in the blowfly salivary gland (Berridge, 1975; Berridge et al., 1975). The activated release of $^{45}Ca^{++}$ during label studies is probably due to efflux of calcium from these reservoirs.

In an attempt to classify cells according to the type of control (Goldberg et al., 1974), the terms mono- and bidirectional were introduced. Initially the theories included reference to the primary stimulant(s) and interaction of cyclic nucleotides (cAMP and cGMP) intracellularly; however, with the increased importance of calcium becoming evident, Berridge (1975) has now incorporated this divalent cation into the description of these two control systems.

Briefly, the monodirectional control system are regulated by a single external stimulant. This stimulatn increases cell activity, and calcium is usually the second messenger; additionally, cAMP may act to enhance the calcium signal. The bidirectional control systems are generally found in cells regulated by two opposing stimulants. One of the

stimulants increases cell activity while the second stimulant opposes or removes the initial stimulant. Berridge (1975) supports the idea that calcium is the second messenger for the first stimulatn while cAMP mediates the action of the second stimulant. The major differences between these two systems is the relationship between cAMP and calcium. cAMP enhances the calcium signal in the monodirectional system, but opposes the calcium signal in the bidirectional system.

With respect to tick salivary glands, most research to date has been concerned with the sensitivity of glands to changes in bathing media factors and pharmacologically active substances in an effort to characterize the primary stimulus and its specific role in fluid secretion. However, little is known about intracellular events controlling salivation, except that high levels of exogenous cAMP stimulate in vitro glands to secrete (Needham and Sauer, 1975; Sauer et al., 1976; Kaufman, 1976). Cyclic AMP stimulates in vitro salivary fluid secretion in the lone star tick if the nucleotide is accompanied by theophylline (Sauer et al., 1976); by cAMP alone if glands are pre-exposed to a catecholamine in a Ringer-solution (Needham and Sauer, 1975) and by cAMP alone if the glands are bathed in modified tissue culture medium 199 (Kaufman, 1976; Sauer et al., in preparation). In addition, cAMP and theophylline stimulate ^{36}Cl uptake in isolated glands (Sauer et al., 1974). Preliminary tests show the presence of phosphodiesterase (PDE) activity in the salivary gland of female ixodid ticks, (McMullen and Sauer, in preparation) and that dopamine causes an increase in gland cAMP (Sauer et al., in preparation).

CHAPTER II

MATERIALS AND METHODS

Experimental Animals

Ticks (*A. americanum*) were reared in the laboratory by methods described by Patrick and Hair (1976). Salivary glands were dissected from female lone star ticks undergoing rapid engorgement and used within one hour after removal of the tick from the sheep. Glands in this physiological condition are thought to be most active with respect to fluid secretion because during this period, the last 12-24 hours spent on the host, females more than double in body weight. Also, glands are more responsive to *in vitro* exposure of catecholamines and cAMP/theophylline in the rapid engorgement phase.

Support Media

Four support media were used to bathe glands during various *in vitro* experiments. All solutions were made up with 10 to 15 megaohm deionized water unless specified otherwise and were oxygenated for 30 min. prior to use with 100% O_2. Also, Penicillin G sodium (Mann Research, New York) (30 mg/l) and streptomycin sulfate (ICN) (100 mg/l) were added to each solution to inhibit possible microbial contamination. Glands were dissected in the same type of solution in which the experi-

ment was performed, except in calcium deletion and alteration studies where calcium containing salines were utilized.

The solution referred to as tick saline with phosphate buffer (TS/PO_4) contained the following reagent grade constituents in mg/l: NaCl, 10,000; $Na_2HPO_4H_2O$, 2,350; $NaH_2PO_4 \cdot 7\ H_2O$, 2,350; $KHCO_3$, 1,920; $CaCl_2$, 390; $MgSO_4$, 340; inositol, 470; D-glucose 5,800 and bovine albumin, 100. The pH was 7.0 $\pm$ 0.2.

In the second medium 10 mM MOPS buffer (morpholinopropane sulfonic acid; Sigma Chem. Co., St. Louis, Mo.) was substituted for the mono- and dibasic sodium phosphate in TS/PO_4. This solution is referred to as tick saline with MOPS or $TS/MOPS_{10}$. While 30 min. oxygenation of this solution caused some $alkalinization_{13}$ (due to the loss of CO_2 from this bicarbonate buffer), the resultant pH of 7.5 $\pm$ 0.2 seemed to have no affect on the secretory ability of glands. This was tested by subjecting glands to alternate exposures of TS/MOPS with dopamine (10^{-5}M) adjusted to pH 7.0 and 7.5. Additionally, Kaufman and Phillips (1973b) found that a pH range of 6.0 to 8.0 is optimum for secretion by isolated glands of <u>Dermacentor</u> <u>andersoni</u>.

The third medium was the same as $TS/MOPS_{10}$ except that 20 mM MOPS was used to prevent extensive alkalinization ($TS/MOPS_{20}$). The pH was adjusted to 7.1 $\pm$ 0.1 using HCl.

The fourth medium was modified tissue culture medium 199 (Morgan et al., 1950) with 10 mM MOPS (TC 199/MOPS). Sodium chloride was added to replace sodium lsot due to the deletion of $NaHCO_3$. The pH was adjusted to 7.0 $\pm$ 0.1 with NaOH or HCl. This medium contained many components including Hank's balanced salt solution (mg/l): NaCl, 8,000;

$Na_2HPO_4 \cdot H_2O$, 48; KCl, 400; KH_2PO_4, 60; $CaCl_2$, 140; $MgSO_4$, 98; D-glucose, 1600 and phenol red, 10.

Pharmacological Agents and Chemicals

Solutions containing pharmacologically active drugs were prepared just prior to use in all experiments.

Drugs or chemical were obtained from the following suppliers: Noradrenaline (NA) ICN; dopamine·HCl(DA); adenosine 3'; 5' cyclic monophosphate (cAMP), theophylline; ethylene glycol bis-(amino-ethyl)tetra-acetic acid (EGTA), from Sigma Chemical Co. Verapamil·HCl (VA) was a gift of Knoll Pharmaceutical Co. (Whippany, New Jersey) and the ionophore A23187 (acid salt) was kindly supplied by Dr. Robert Hamill of the Eli Lilly Co. (Indianapolis, Ind.).

Because verapamil is difficult to get into solution Windholtz, 1976) the dissolving medium must be acidified (pH 4-6) with 1N HCl then warmed. Once verapamil was in a solution, the pH was readjusted upward to 7.0 - 7.1 using 1M NaOH; dopamine was then added with subsequent dilution to the correct volume.

Two 10 mM stock solutions of ionophore A23187 were prepared. A23187 (5.23 mg/ml) was added to a 1 ml volumetric flask and diluted in absolute ethanol. With constant stirring for ~ 2 hours, A23187 was completely dissolved. The other stock solution was prepared using the same amount of A23187 but dimethyl sulfoxide (DMSO) was the diluent. Care was taken not to expose this material to light as the ionophore may be slightly sensitive to light. Quantities of A23187 were added to $TS/MOPS_{20}$ with constant agitation of a Genie® vortex stirrer. Constant mixing was achieved by use of a magnetic flea and stirrer.

Dissection and Preparation of Glands

Salivary glands are removed from feeding females and fluid secretion monitored *in vitro* by methods described by Needham and Sauer (1975). Briefly, the dissected gland is surrounded by a support medium and submerged in liquid paraffin. The main salivary duct is drawn out into the paraffin and secured to the roughened surface of a minutin pin. Five minutes of incubation is followed by three minutes of rinsing and measurement. The secreted droplet is collected, its diameter measured in the liquid paraffin and converted to rate of secretion in nanoliters/minute. Temperatures of solutions were maintained at $24 \pm 2^{\circ}C$.

Calcium Determinations

Gland calcium was determined using standard atomic absorption techniques with a Beckman 440 Spectrophotometer. $LaCl_3$ (0.5% w/v) was present in each unknown sample and standard curve solution to limit interference from extraneous substances (Zettner and Seligson, 1964).

Salivary gland wet weight was determined (after removing excess fluid from the gland) by weighing on a Mettler balance sensitive to 10 μg.

Radiocalcium Efflux

Glands were dissected in modified TC 199/MOPS (Kaufman, 1976), then soaked for one hour at room temperature in the same type solution containing $^{45}CaCl_2$ (New England Nuclear). After cooling ($4^{\circ}C$) and rinsing, the glands were transferred to a $37^{\circ}C$ water bath and allowed to equilibrate for five minutes. Paired glands from the same tick were

then incubated in 200 µl of TC 199/MOPS with or without NA (10^{-5}M) for either 5, 10 or 15 min. Samples (10 µl) were removed from the bathing medium and radioactivity was counted by a Nuclear-Chicago 4342 Gas-Flow Planchet Counter.

Radiocalcium Uptake

Glands were dissected and allowed to equilibrate for at least 15 min. in TS/PO_4, and then blotted on glass slides and weighed. Glands were transferred to TS/PO_4 (99 µl at 37°C) or TS/PO_4 with dopamine (10^{-5} M) and incubated with 1 µl of a solution containing 2 µCi of $^{45}CaCl_2$. After incubation (37°C) in a Precision Shaker water bath, tubes with glands were immediately transferred to an ice bath where cold saline was added to each tube. Glands were rinsed four times with aliquots of cold saline and then weighed. Concentrated HNO_3 (100 µl) was used to digest glands overnight and 0.9 ml of 4 mM $CaCl_2$ solution was added to prevent adherence of ^{45}Ca to the glass container. Samples were counted on a Nuclear-Chicago 4342 Gas Flow Planchet counter. Results are expressed as percent change ^{45}Ca in uptake per mg wet weight by experimental glands as compared to control glands.

CHAPTER III

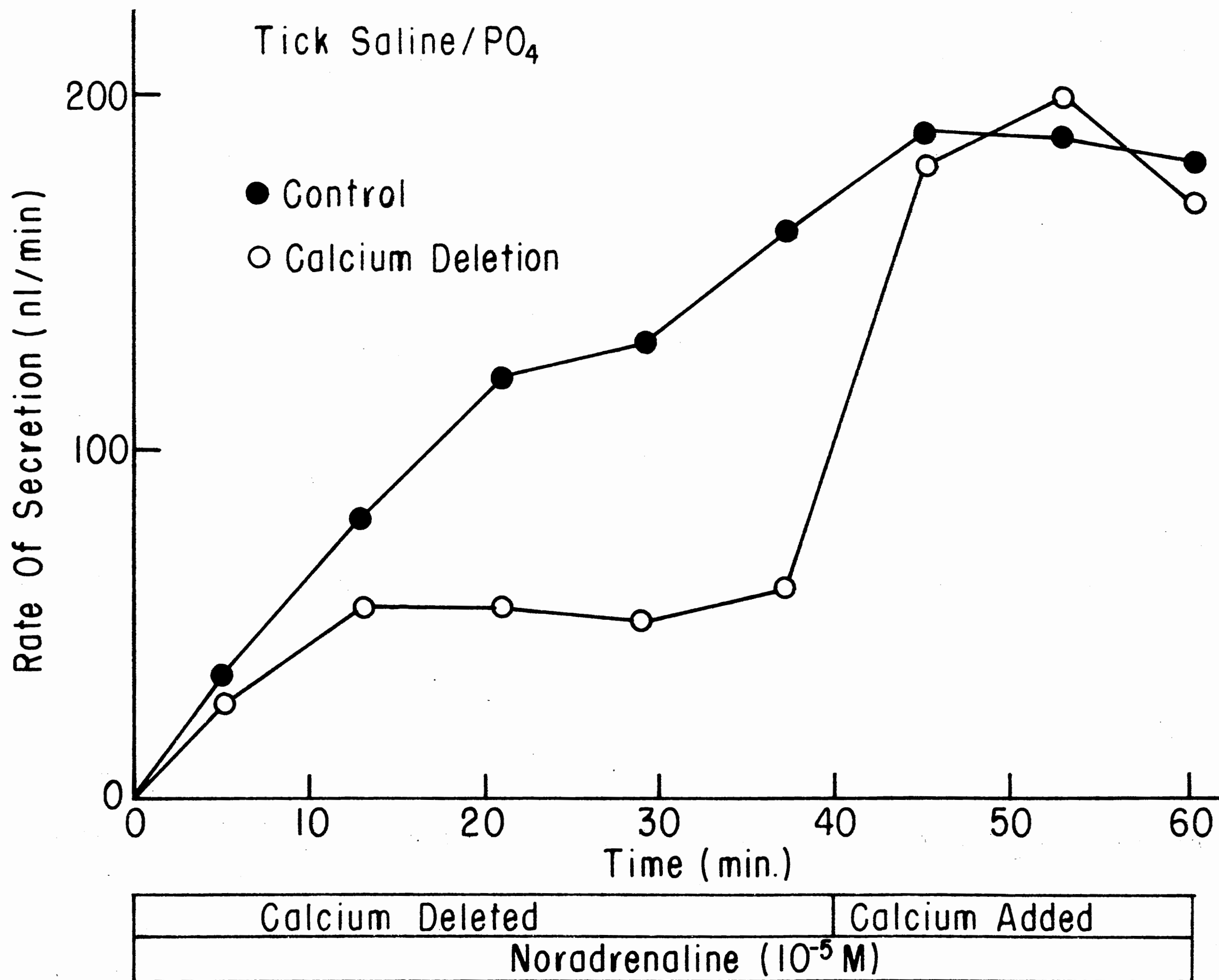
RESULTS

Effects of Calcium Deletion on Fluid Secretion

In an effort to determine whether calcium plays a role in controlling tick salivary fluid secretion, calcium was deleted from the bathing medium (TS/PO_4) to see if its presence (especially an exogenous source) is necessary to support *in vitro* secretion (Figure 1). Preliminary experiments revealed a depression of NA stimulated secretion (as compared to controls), but glands still secreted at measureable rates. Most importantly, addition of 3.5 mM calcium (at 40 min.) stimulated a significant ($0.01 < P < 0.005$) three-fold increase, bringing the rates close to values of control glands.

With this in mind, it seems possible that chelation of calcium by a high concentration of phosphate in TS/PO_4 could be having an important inhibitory effect on the secretory process. Utilizing standard formulas for phosphate precipitation of calcium at pH 7.0 and ignoring the effects of other ionic species (Freiser and Fernando, 1966), it was estimated that TS/PO_4 contained 10^{-7} to 10^{-6}M free calcium. A chelating effect was also reported by Kaufman (1976), who observed that citrate substantially decreased fluid secretion when added to modified TC199. To overcome this, MOPS buffer (10 mM) was substituted for mono- and dibasic sodium phosphate ($TS/MOPS_{10}$); the freezing point depression

Figure 1. Effect of Calcium Deletion and Addition on the Rate of Fluid Secretion in Tick Saline with Phosphate Buffer (TS/PO_4); ● (n=5); o (n=4)

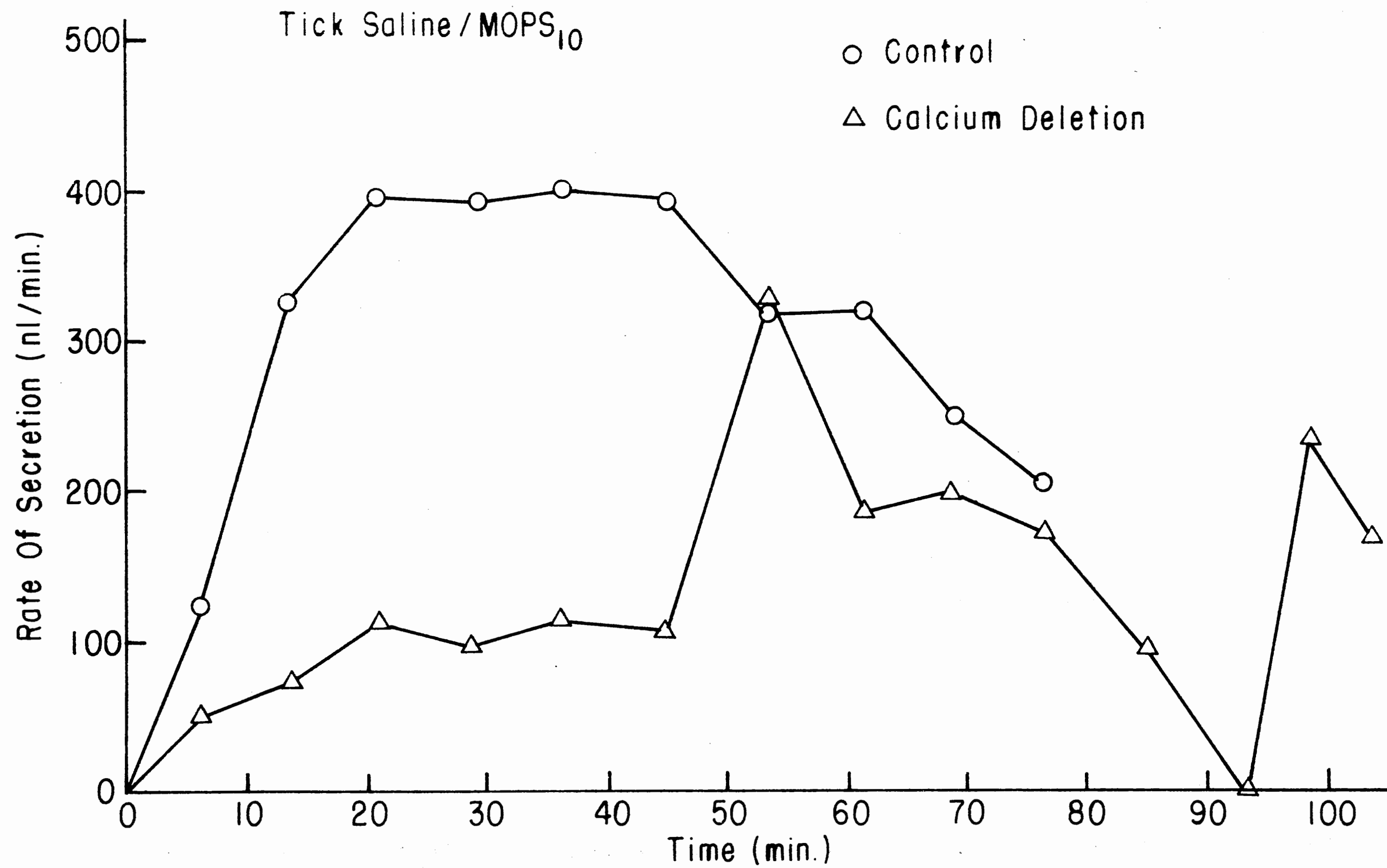

Tick Saline/PO_4
200
100
0
Control
Calcium Deletion
Rate Of Secretion (nl/min)
0
10
20
30
40
50
60
Time (min.)
Calcium Deleted
Calcium Added
Noradrenaline (10^{-5} M)

was not altered significantly. This change dramatically increased the fluid secretory capacity of the glands (Figure 2). In a set of controlled experiments MOPS was added to TS/PO_4 but this addition did not alter the gland's secretory response to DA or NA. As before, removal of calcium from the bathing medium (NA in $TS/MOPS_{10}$) reduced the glands secretory response (Figure 2). Addition of calcium (3.5 mM) again produced an approximate three-fold increase ($P < 0.0005$) in the rate of secretion to values near those observed in control glands (48-53 min. interval).

Because NA and DA are found in the synganglion and salivary glands of an ixodid tick (Megaw and Robertson, 1974) and each stimulates secretion _in vivo_ in the lone star tick (Needham and Sauer, unpublished), _in vitro_ experiments were performed with DA as the stimulating drug using $TS/MOPS_{10}$ as the support medium. With calcium missing from the support medium, DA stimulates an initial rate of secretion close to control rates, but later (after 13 minutes) the rate drops sharply (Figure 3). By contrast, the rate increases gradually when NA is used to stimulate glands (Figure 2). The introduction of calcium (48-53 min. interval) causes a marked increase in the rate of secretion ($P < 0.0005$) to values substantially higher than those observed in control glands Figure 3). Interestingly, most glands secrete only slightly when calcium is returned to the support medium in the absence of DA (88-93 min).

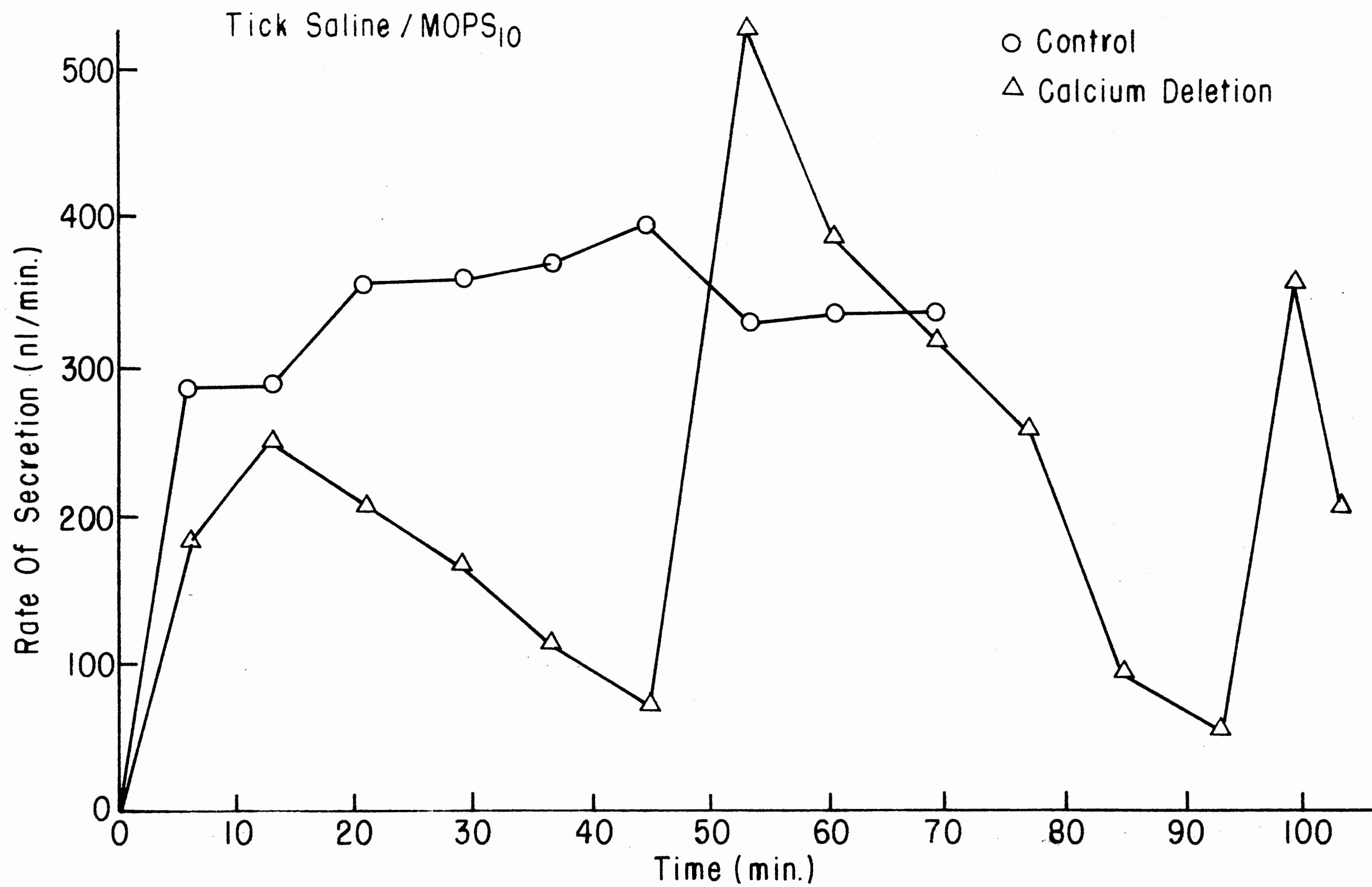
Cyclic AMP/theophylline (10 mM) in $TS/MOPS_{20}$ free of calcium (5 mM EGTA) induces absolutely no salivation, and addition of calcium at 40 min. produces no secretion. Exposure of these glands (n=3) to DA (10^{-5}M) stimulates an average rate of secretion of 37 $\pm$ 16 (S.E.M.)

Figure 2. Effect of Adding Calcium to a Calcium Deleted Support Medium ($TS/MOPS_{10}$) on the Rate of Fluid Secretion by *In Vitro* Salivary Glands Stimulated by Noradrenaline (Δ, n=5). Rates of secretion by control glands (o n=4) where calcium was present in the support medium throughout are included for purposes of comparison. Addition of calcium produced a significant increase in salivation (48-53 min.)



Calcium Deleted	Calcium Added	Cal. Deleted	Calcium Added
Noradrenaline (10^{-5} M)			Noradren

Figure 3. Effect of Adding Calcium to a Calcium Deleted Support Medium ($TS/MOPS_{10}$) on the Rate of Fluid Secretion by *In Vitro* Salivary Glands Stimulated by Dopamine (Δ, n=5). Rates of secretion by control glands (o, n=7) where calcium was present in the support medium throughout are included for purposes of comparison. Addition of calcium produced a significant increase in salivation (48-53 min.)


Tick Saline / MOPS10
○ Control
△ Calcium Deletion
Rate Of Secretion (nl/min.)
500
400
300
200
100
0
0
10
20
30
40
50
60
70
80
90
100
Time (min.)
Calcium Deleted
Calcium Added
Cal. Deleted
Calcium Added
Dopamine (10-5 M)
Dopamine

(72-93 min.) with a single maximum high of 124 nl/min. and average maximum of 70 ± 37 (S.E.M.)

Comparison of Bathing Media

Four support media were compared with DA as the primary stimulating drug (Figure 4). Additional information became available during the course of the study, showing that tick weight is a reliable indicator of maximum fluid secretory ability of glands (Kaufman, 1976; Sauer et al., in preparation). Glands from lone star ticks weighing 300 mg or more are thought to be capable of secreting maximally, while glands from ticks weighing less than this or those obtained from replete ticks secrete at lower rates. Comparisons of the effectiveness of the four media to support secretion are made using glands from engorging ticks weighing near 300 mg (± 25 mg) to near repletion.

The major factor in the ability of each medium to support dopamine stimulated secretion appears to be available calcium. This is indicated by higher rates of secretion exhibited by glands bathed in the three salines buffered with MOPS, allowing for millimolar quantities of unprecipitated calcium rather than micromolar amounts that were present in TS/PO_4 ($P < 0.0005$) (Table I, Figure 4). Other media differences that could also affect gland sensitivity to DA are listed in Table I.

When comparing the ability of cAMP/theophylline to stimulate fluid secretion in the three bathing media TC199/MOPS, TS/PO_4 and $TS/MOPS_{20}$, there is a significant difference between TC199/MOPS and the two tick salivas after 29 min. ($P < 0.0005$) but not TS/PO_4 and $TS/MOPS_{20}$. Prior to this time there is no apparent difference in the secretory rates

TABLE I

DIFFERENCES IN COMPONENTS AND ABILITIES OF FOUR MEDIA TO SUPPORT DOPAMINE (10^{-5}M) STIMULATED SECRETION

Medium	pH	HCO_3^-	$Na^+:K^+$ (mM)	Ca^{++} (mM)	Average Response[5] (nl/min)	Average Maximum Response (nl/min)
TS/PO_4[1]	7.0 ± 0.1	yes	207:19	0.0001 to 0.001	53(36)	110(4)
TC199/MOPS[2]	7.0 ± 0.1	no	137:5	1.3	204(54)	372(6)
TS/$MOPS_{20}$[3]	7.1 ± 0.1	yes	170:19	3.5	267(54)	381(6)
TS/$MOPS_{10}$[4]	7.5 ± 0.2	yes	170:19	3.5	372(54)	487(6)

[1] 3.5 mM Ca^{++} was added, but because of precipitation by phosphate the Ca^{++} concentration was estimated to be 10^{-7} to 10^{-6}M.

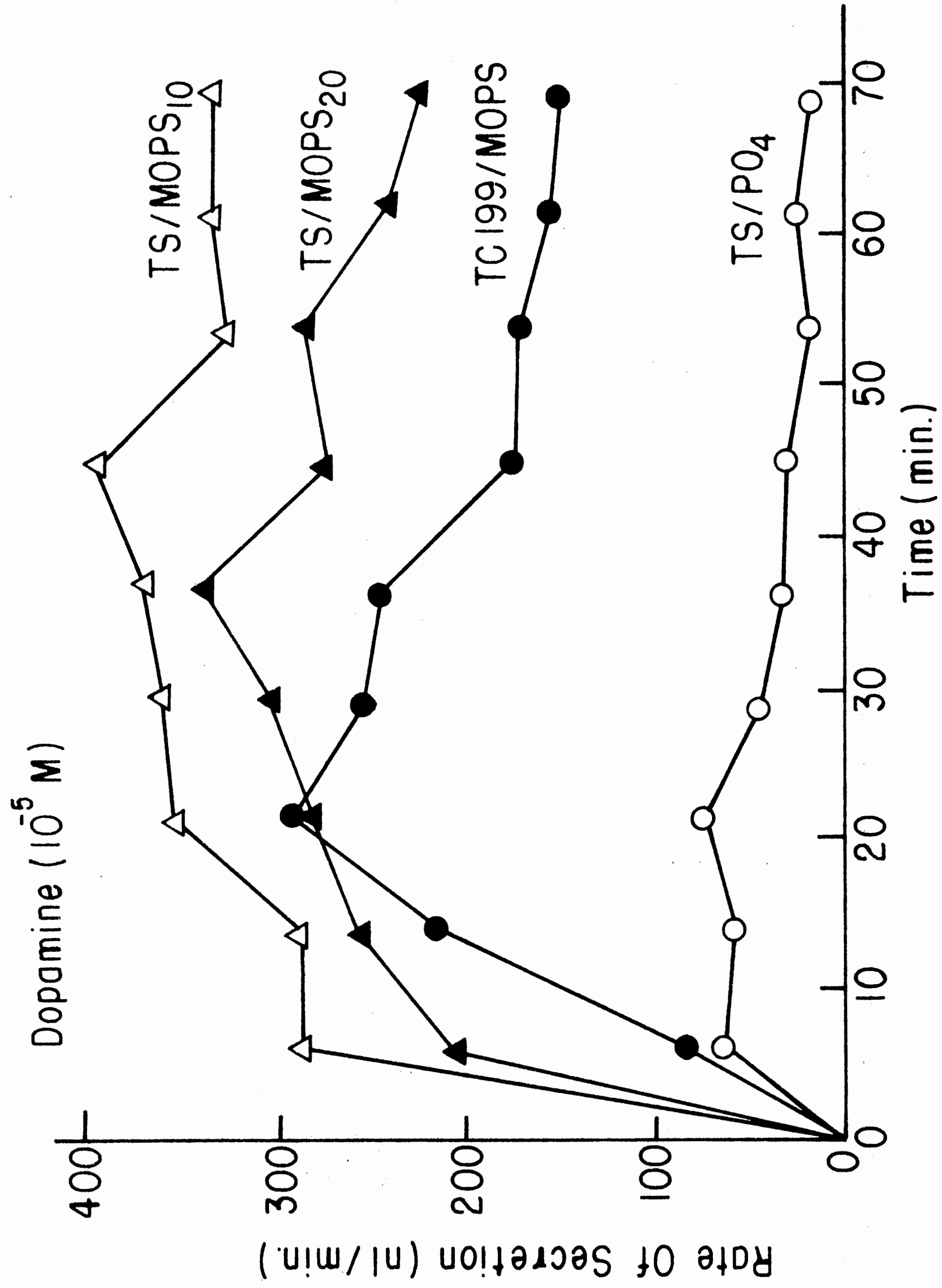
[2] Modified tissue culture medium 199 with Hank's balanced salt solution and 10 mM MOPS buffer (Kaufman, 1976).

[3] Tick saline containing 20 mM MOPS.

[4] Tick saline containing 10 mM MOPS; alkalinization occurred in TS/MOPS after oxygenation in the presence of HCO_3^-.

[5] The average fluid secretory response includes 9 measurements/gland over 69 min.; (n) equals the number of points included in the mean.

Figure 4. Comparison of the Abilities of Four Support Media to Maintain *In Vitro* Fluid Secretion Stimulated by Dopamine (Δ, n=6); (o, n=4); (▲, n=6), (o, n=6). TS/PO_4 contains μM calcium while the other three media have mM calcium


Dopamine (10^{-5} M)
$TS/MOPS_{10}$
$TS/MOPS_{20}$
TC199/MOPS
TS/PO_4
Rate Of Secretion (nl/min.)
Time (min.)
0
100
200
300
400
0
10
20
30
40
50
60
70

supported by these support media. The TS/PO_4 medium may support slower secretion due to a lower level of cAMP ($4 \times 10^{-3}M$).

Further comparison of the above show that $TS/MOPS_{10}$ supports a more rapid initial rate of secretion when compared to TC199/MOPS ($0.05 < P < 0.025$; Figure 4). $TS/MOPS_{10}$ supports a higher maximum rate of secretion that TC199/MOPS ($0.025 < P < 0.01$; Table I). The abilities of $TS/MOPS_{10}$ and $TS/MOPS_{20}$ are not significantly different in this regard. However, the average responses for all four bathing media were significantly different from each other ($P < 0.005$, Table I).

The initial sensitivity of glands to NA and DA in different support media are compared. The average initial rate of secretion (first 5 minutes) by glands stimulated with DA in $TS/MOPS_{10}$ is twice that observed when NA stimulated the glands. The same phenomenon is not observed when glands are bathed in other support media. The maximum rates of secretion by glands bathed in $TS/MOPS_{10}$ and stimulated by these two catecholamines are not significantly different (DA, 487 nl/minute; NA, 435 nl/minute). The overall average rates of secretion by glands stimulated by DA and NA in $TS/MOPS_{10}$ are 372 and 325 nl/minute, respectively and not significantly different.

Gland Calcium

One important criterion for establishing calcium as a potential intracellular effector of cell activity is to demonstrate a calcium influx and/or efflux from the stimulated tissue at rates greater than those observed in tissue at rest. Data of this kind provide information about the importance of intracellular calcium as a source for elevating the cytosolic concentration during stimulation. After exposing isolated

Figure 5. Comparison of the Abilities of Three Support Media to Maintain _In Vitro_ Fluid Secretion Stimulated by cAMP and Theophylline. cAMP concentration is 10^{-2} M in TS/MOPS and TC199/MOPS, 4×10^{-3} in TS/PO_4; theophylline (10^{-2} M); (Δ, n=9), (⊙, n=5), (●, n=5)

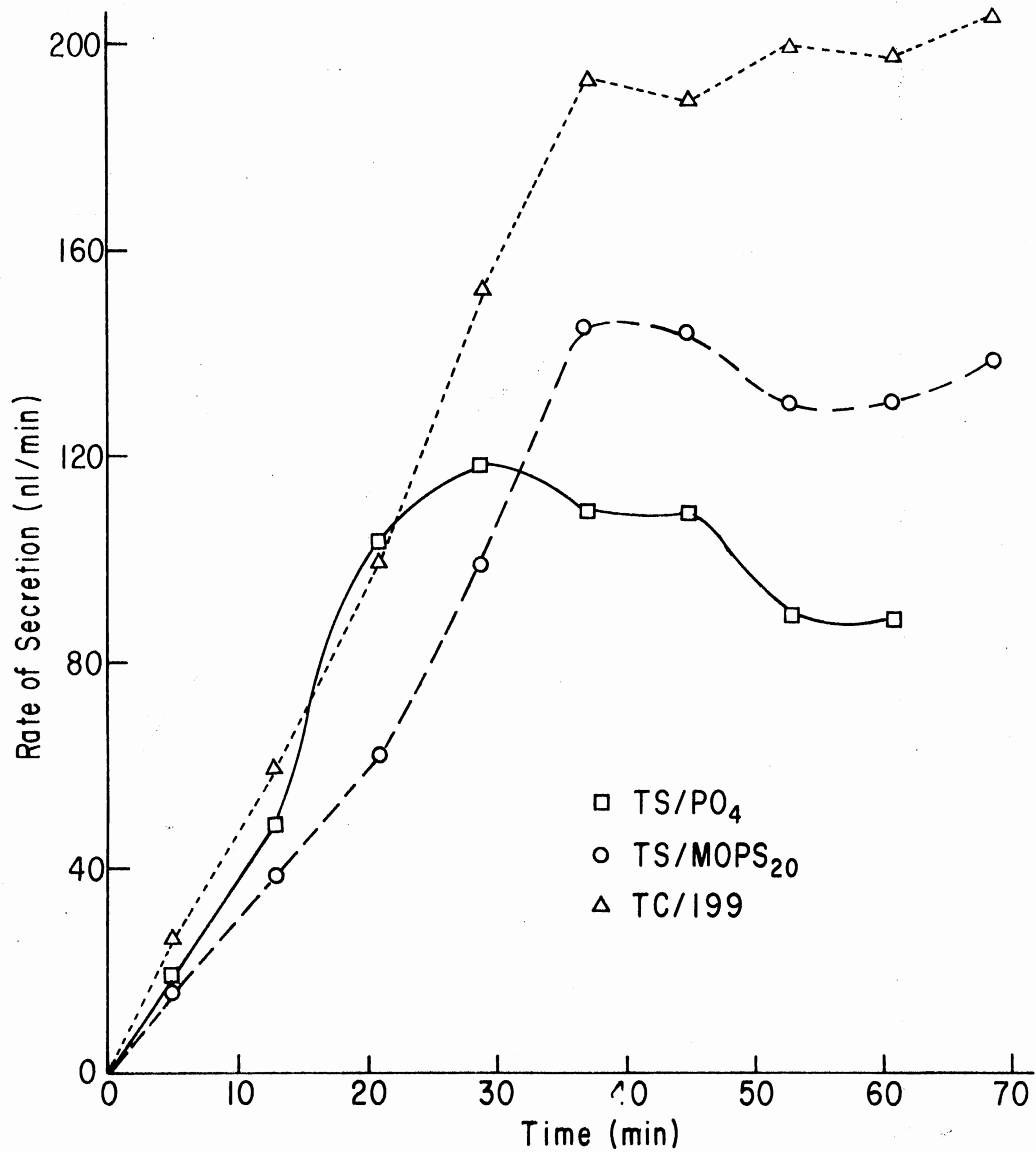
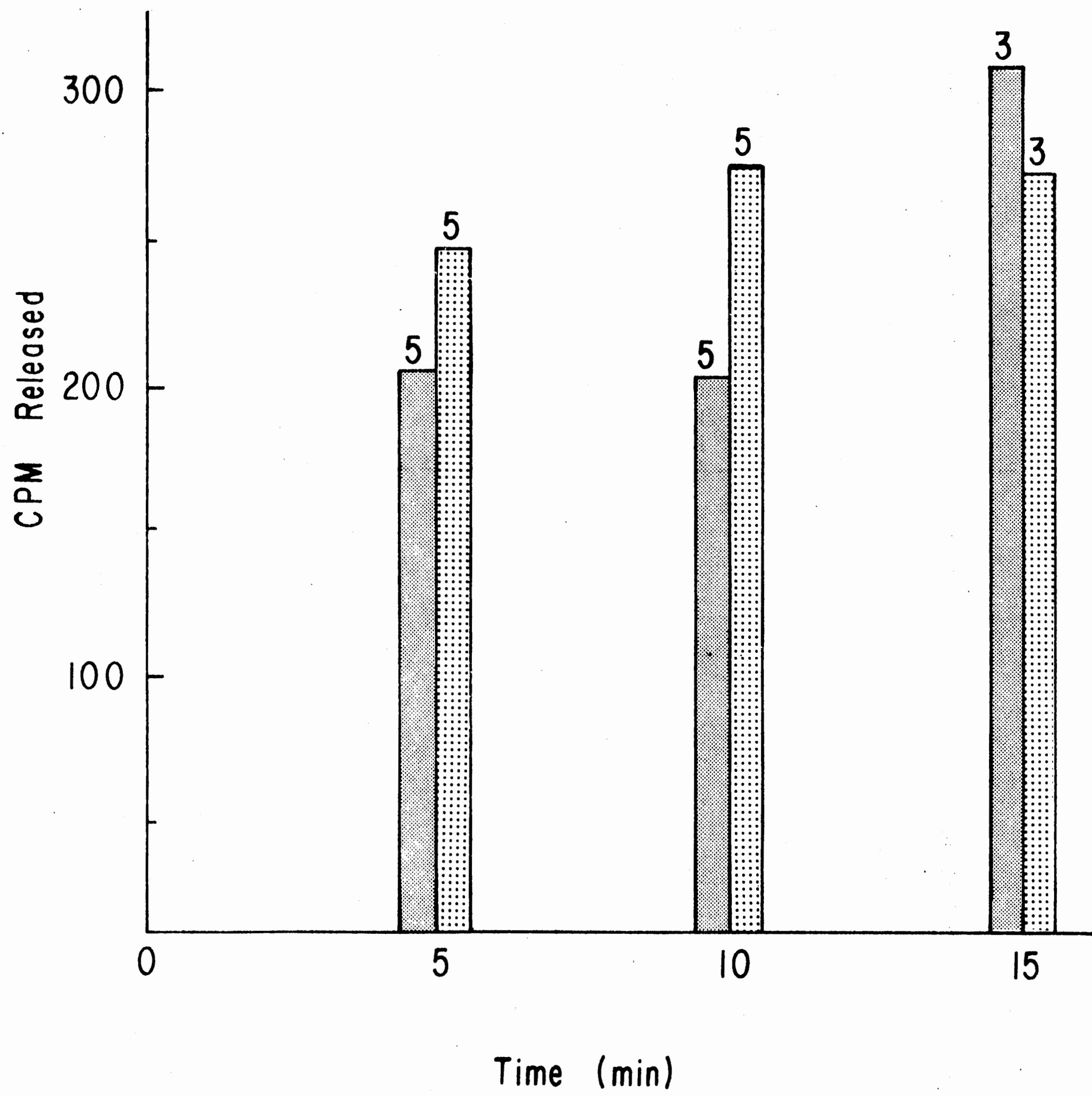

200
160
120
80
40
0
Rate of Secretion (nl/min)
0
10
20
30
40
50
60
70
Time (min)
TS/PO4
TS/MOPS20
TC/199

Figure 6. Effect of Noradrenaline on Efflux of ^{45}Ca by Isolated Salivary Glands. Solid bars indicate radioactivity released from control TC 199/MOPS only; stippled bars represent radioactivity released from glands bathed in TC 199/MOPS and 10^{-5}M noradrenaline. Numbers at the top of bars indicate replications and values are CPM/10 μl bathing medium solution. Noradrenaline stimulated efflux is significantly different from controls at 5 and 10 min. but not at 15.



300
200
100
CPM Released
5
5
5
5
3
3
0
5
10
15
Time (min)

glands to NA (10^{-5}M) for 15 minutes there is a substantial loss of total gland calcium from stimulated glands as compared to controls (Table II).

Radiocalcium Efflux

Paired glands from the same tick were prelabeled with trace amounts of $^{45}Ca^{++}$ and placed in TC 199/MOPS with or without NA (10^{-5}M) for variable amounts of time (Figure 7). The presence of the catecholamine causes a greater efflux of the isotope into the bathing medium at 5 and 10 minutes. However, after 15 minutes there is no significant difference in the amount of radioactivity present in the two media, possibly because of the minimal radioactivity originally present in the glands, a large bathing medium volume (200 μl) to gland weight (2-6 mg) ratio and equilibration of gland $^{45}Ca^{++}$ with "cold" calcium in control experiments.

Radiocalcium Uptake

To help determine if extracellular calcium contributes to elevating cytosolic calcium during cell activation, isolated gland pairs were incubated in TS/PO_4 or TS/PO_4-DA (10^{-5}M) and small amounts of $^{45}Ca^{++}$ variable amounts of time (Table III). Interestingly, control glands contained more $^{45}Ca^{++}$ after 1, 5 and 10 min. Attempts to measure uptake at 45, 30 and 15 seconds were inconsistent and too variable and are not shown. Equilibrium of bathing medium $^{45}Ca^{++}$ with gland $^{45}Ca^{++}$ was evident after 15 min in both controls and experiments.

Verapamil Antagonism of Fluid Secretion

Because of verapamil's (VA) known ability to block calcium movement

TABLE II

CALCIUM CONCENTRATION IN SALIVARY GLANDS AFTER INCUBATING GLANDS WITH 10^{-5}M NORADRENALINE IN TS/PO_4 FOR 15 MINUTES

Gland treatment	mM (S.E.M.)	P*
Control-saline	10.1 ± 1.2 (24)**	
Experimental-noradrenaline (10^{-5}M)	5.7 ± 0.9 (13)	$P < 0.025$

*t-test.

**Numerals in parentheses indicate number of glands measured.

TABLE III

DETERMINATION OF THE EFFECT OF DOPAMINE (10^{-5}M) ON ^{45}Ca UPTAKE BY ISOLATED SALIVARY GLANDS

1 min.			5 min.			10 min.			15 min.		
C*	D**	Δ%***	C*	D**	Δ%***	C*	D**	Δ%***	C*	D**	Δ%***
7753	3703	-52	4409	1373	-69	3787	1755	-54	2761	2286	-17
3411	3698	+ 8	1886	683	-64	2337	1275	-45	455	527	+16
3592	624	-83	3317	269	-92	5320	4700	-12	4653	5595	+20
429	92	-79									
822	1219	+48									
		$\bar{X}$ -32			$\bar{X}$ -75			$\bar{X}$ -37			$\bar{X}$ +6

*C = CPM/mg wet weight, control, non-stimulated gland.

**D = CPM/mg wet weight, dopamine (10^{-5}M) stimulated gland.

***$\Delta\% = 100 - \left[\frac{(D)}{(C)} \times 100\right]$.

across the plasma membrane of many cells, it was added to isolated glands along with DA (Figure 8). VA at the highest effective concentration of 10^{-3}M almost completely blocks secretion initially (0-21 min; Figure 7). When VA is removed from the bathing medium, recovery is immediate and reversible (24-37 min.). Upon the readdition of VA (40-53; 80-93 min.) inhibition of secretion is again apparent. Because this effect on fluid secretion appears to be even more dramatic than deleting calcium from the bathing medium (Figures 2 and 3) a calcium-free (5 mM EGTA) solution was tested to confirm this observation (104-133 min.). Interestingly, VA limits the recovery of glands with calcium in the support medium (compare to recovery seen in Figure 3, 48-53 min.).

At lower concentrations of VA one sees less inhibition of DA stimulated secretion (Figure 8) and the threshold inhibitory concentration for this drug appears to be in the range of 10 μM for glands stimulated by 10^{-5} M DA.

Cyclic AMP/theophylline stimulated secretion is blocked by VA (10^{-4}, 10^{-3}M; Figure 9) with recovery to control values being depressed upon the removal of VA. At the 0-29 min. interval, 10^{-4}M VA treatment is different from both the control and 10^{-3}M VA incubation ($0.01 < P < 0.025$), while 10^{-3}M VA was even more effective than 10^{-4}M VA ($P < 0.0005$). Comparisons of each treatment after 29 min. show that even though VA was not added to the bathing solution, there is a reduction in the glands ability to respond to cAMP/theophylline ($P < 0.0005$). Glands treated with 10^{-4} VA are affected less than glands exposed to 10^{-3}M VA ($0.025 < P < 0.05$).

Figure 7. Effect of Verapamil (10^{-3}M) on Dopamine Stimulated Fluid Secretion, (n=5).

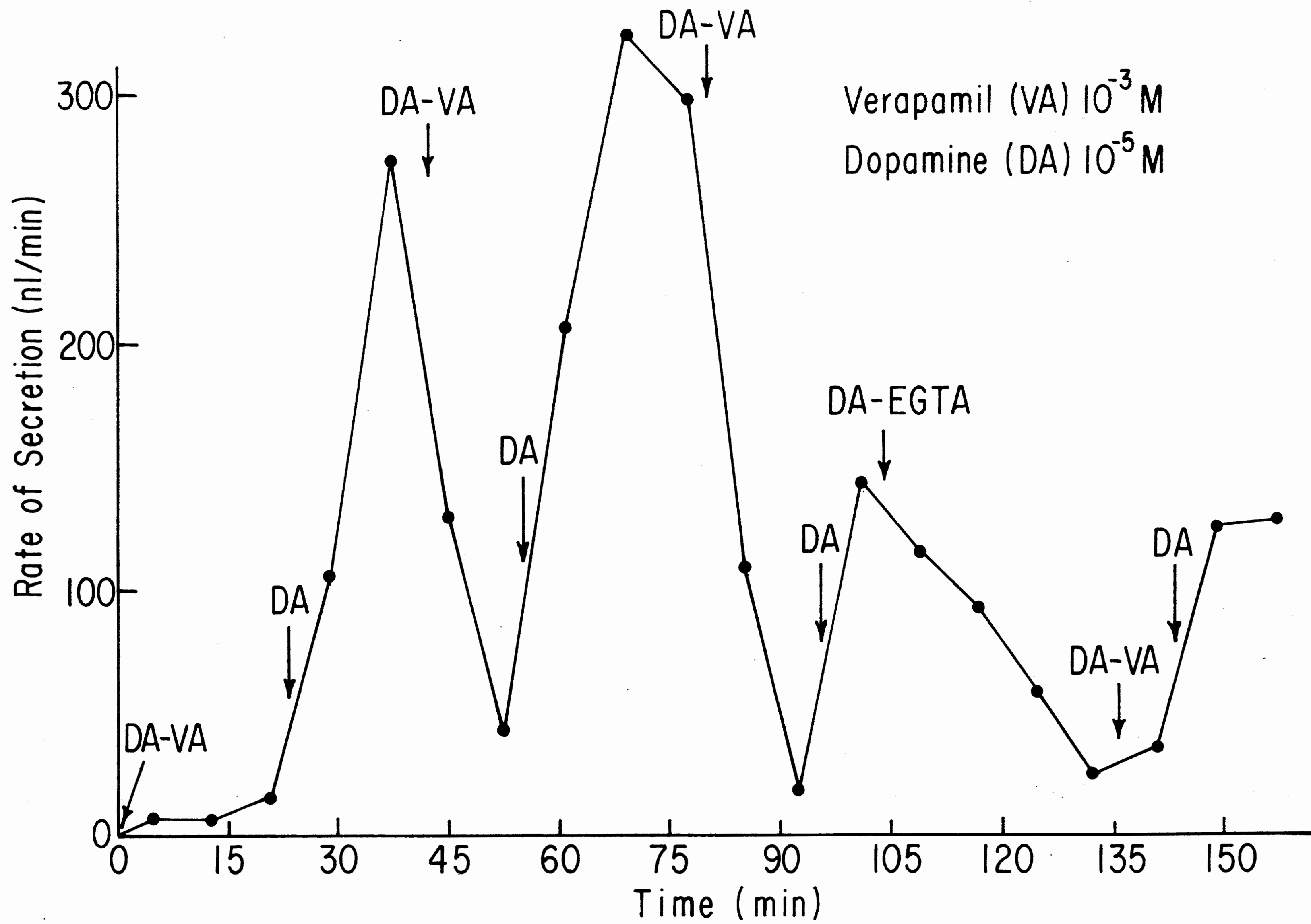
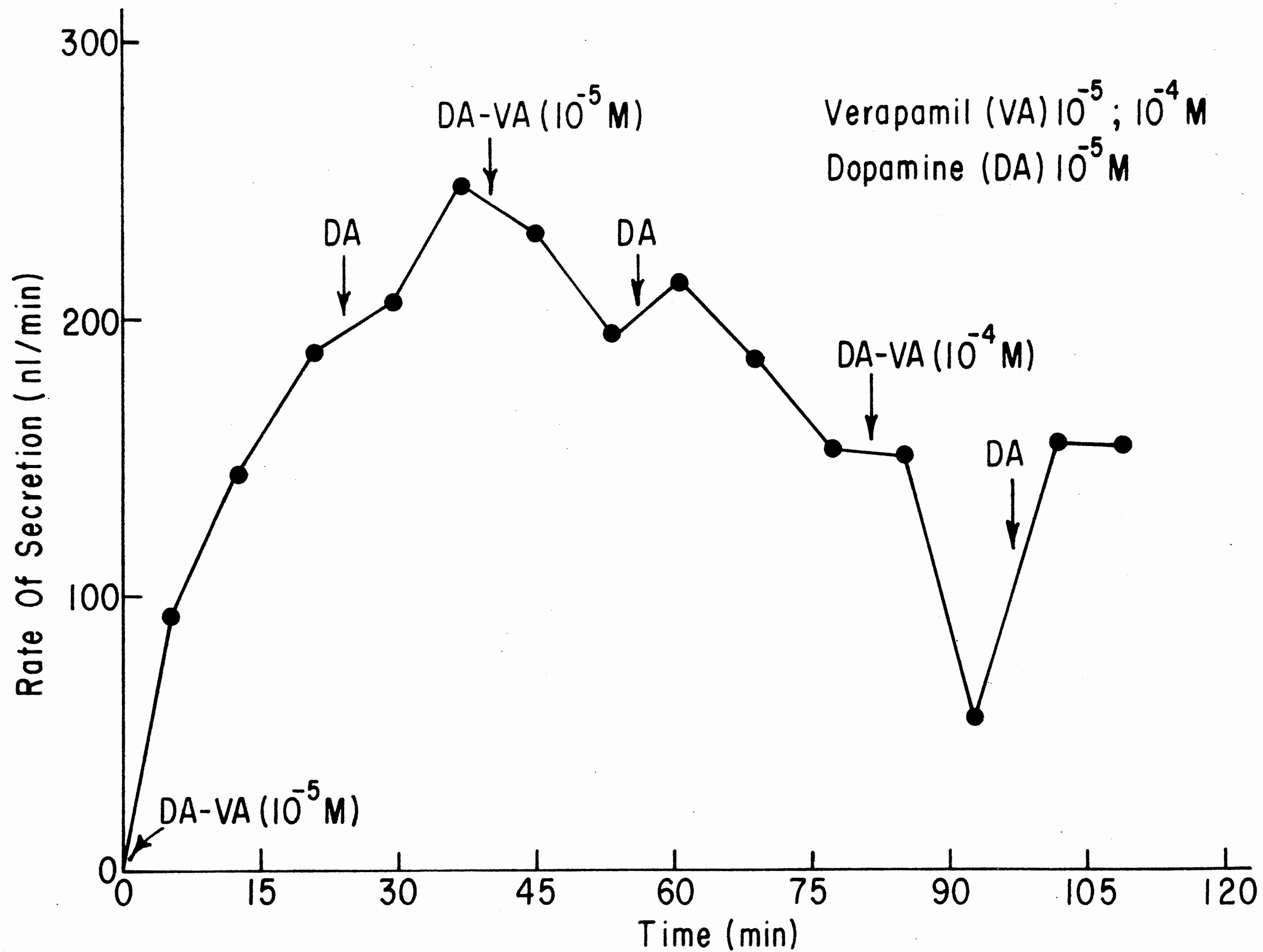
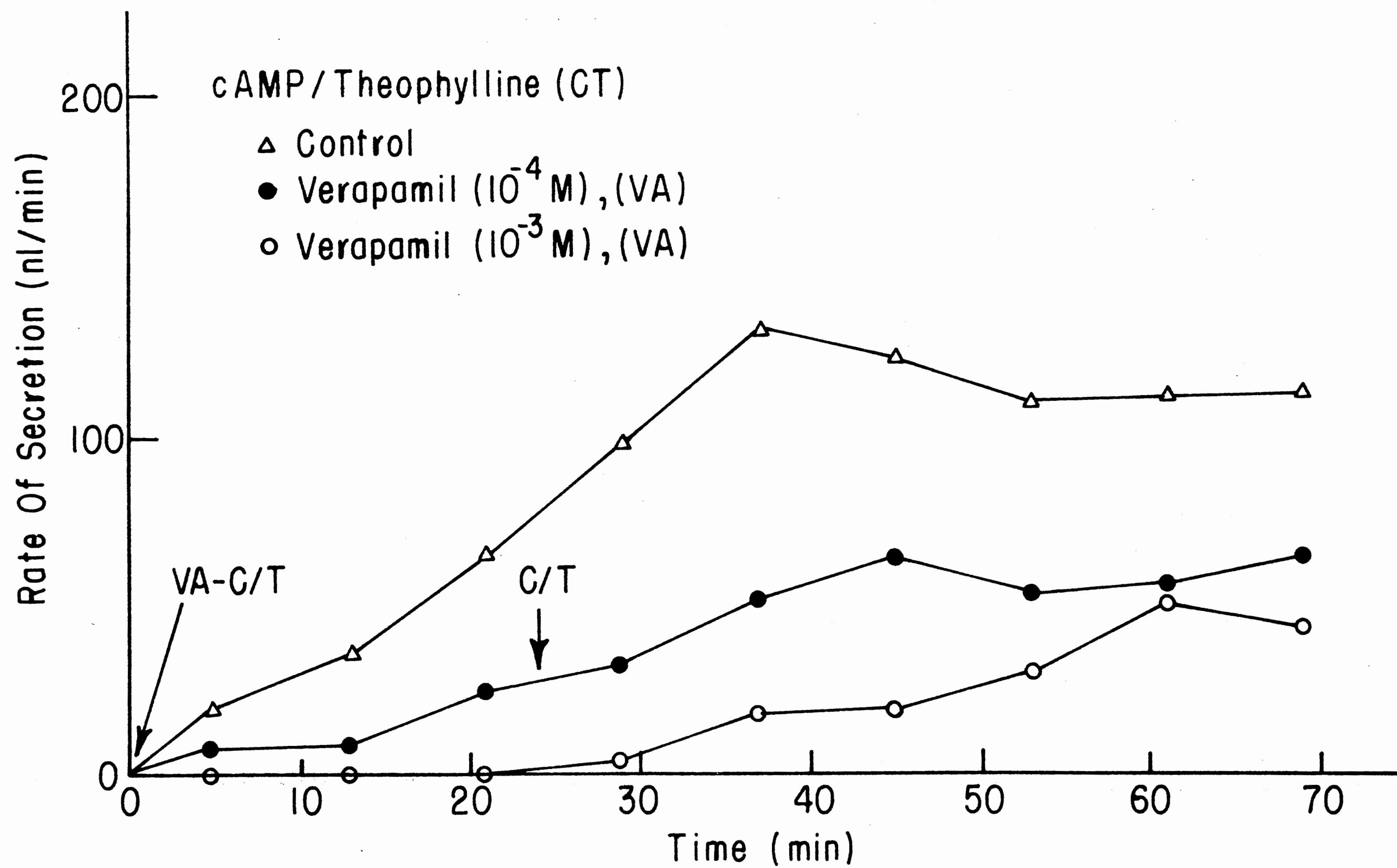

DA-VA
DA-VA
DA-VA
DA
DA
DA
DA-EGTA
DA-VA
DA
Verapamil (VA) 10^{-3} M
Dopamine (DA) 10^{-5} M
Rate of Secretion (nl/min)
0
100
200
300
0
15
30
45
60
75
90
105
120
135
150
Time (min)

Figure 8. Effect of Two Concentrations of Verapamil (10^{-4}, 10^{-5}M) on Dopamine Stimulated Fluid Secretion, (n=4)



Verapamil (VA) 10^{-5}; 10^{-4} M
Dopamine (DA) 10^{-5} M
DA-VA (10^{-5} M)
DA
DA
DA-VA (10^{-4} M)
DA
DA-VA (10^{-5} M)
Rate Of Secretion (nl/min)
300
200
100
0
0
15
30
45
60
75
90
105
120
Time (min)

Figure 9. Effect of Two Concentrations of Verapamil (10^{-4}, 10^{-3}M) on cAMP/theophylline Stimulated Fluid Secretion (Δ n=5, ● n=3, o n=4)


cAMP/Theophylline (CT)
Control
Verapamil (10^{-4} M), (VA)
Verapamil (10^{-3} M), (VA)
VA-C/T
C/T
Rate Of Secretion (nl/min)
200
100
0
0
10
20
30
40
50
60
70
Time (min)

Calcium Alteration

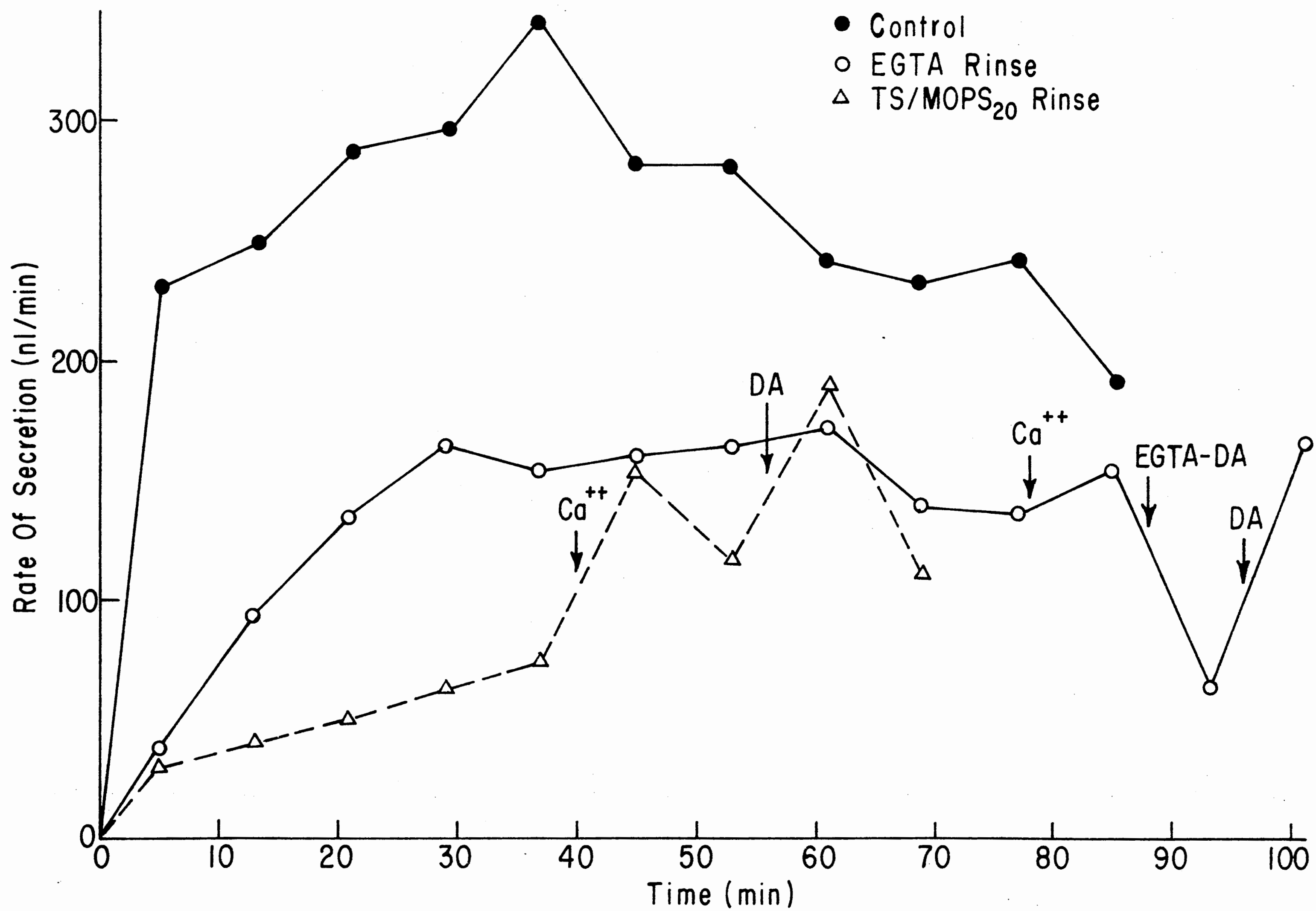
Comparisons were made between glands exposed to TS/$MOPS_{20}$-DA (10^{-5}M) stimulation and Ca-free(EGTA 5mM) rinses; and glands incubated in Ca-free-(EGTA)-DA solutions but rinsed in TS/$MOPS_{20}$ (3.5 mM calcium; Figure 10). The purpose of this experiment was to determine if the primary stimulus is necessary for glands to take up calcium (as indicated by results described above). The Ca-free-(EGTA) rinse appeared to have less of an affect upon fluid secretion and was significantly different than the TS/$MOPS_{20}$ rinse. The secretion that occurred in the latter appeared to be affected by adding calcium to a gland still under the influence of DA because vigorous secretion generally occurred during the rinse but slowed as the DA was rinsed from gland. This is reflected in the ability of calcium alone to potentiate secretion at the 40-53 min. interval (EGTA rinse experiment) and 77-85 min. (TS/$MOPS_{20}$ rinse).

Interestingly, removal of calcium from the EGTA rinse experiment (88-93 min.) reduced secretion to a much greater extent than previous exposures to TS/MOPS-DA indicating more of a need for calcium during the stimulation phase of the experiment. Both rinsing regimens were significantly different from each other and the control ($P < 0.0005$; 0-37 min.).

Effect of Calcium Ionophore A23187 on Fluid Secretion

Ionophore A23187 at concentrations of 0.1 μM to 10 μM (dissolved in DMSO) did not induce fluid secretion by *in vitro* glands; furthermore, DMSO and A23187 (μM) (n=3) did not seem to slow DA stimulated secretion.

Figure 10. Effect of Calcium Alteration Between Stimulation and Rinse Periods on Dopamine Stimulated Fluid Secretion. (o) Dopamine-TS/$MOPS_{20}$, n=6; (o) Dopamine-TS/$MOPS_{20}$ Stimulation and Calcium-free Rinse, n=6; (Δ) Dopamine-TS/$MOPS_{20}$ Calcium-free Stimulation and TS/$MOPS_{20}$ Rinse, n=4


Control
EGTA Rinse
TS/MOPS20 Rinse
Rate Of Secretion (nl/min)
300
200
100
0
DA
Ca++
Ca++
EGTA-DA
DA
0
10
20
30
40
50
60
70
80
90
100
Time (min)

The ability of A23187 (μM) to bring about secretion with glands bathed in a calcium-free (5 mM EGTA) solution yielded the same lack of response and no secretion was observed after the addition of 3.5 mM calcium ($TS/MOPS_{20}$). A stock solution of A23187 (10 mM) dissolved in absolute ethanol yielded essentially the same negative results.

It was thought that the lack of positive results could be explained by the ionophore inducing too much calcium into the cytoplasm so lower levels of calcium were used in combination with the ionophore. Utilizing 0.1 mM and 1.0 mM calcium ($TS/MOPS_{20}$) with 1.0 adn 0.1 μM A23187 I was unable to potentiate *in vitro* salivation in two gland preparations.

In a personal communication with Dr. Robert Hamill of Eli Lilly Co., Indianapolis, Indiana, I described the technique used to solubilize the ionophore and administer it to glands *in vitro* and his only suggestion was that the original sample used may have been a "poor batch" so a new lot of A23187 was incorporated into the last set of experiments described without success. Therefore, I must cautiously conclude that under the present experimental conditions A23187 is incapable of initiating all the events necessary to bring about fluid secretion by *in vitro* salivary gland preparations.

CHAPTER IV

DISCUSSION

Experiments discussed in this thesis were designed under the premise that when a cell is under the influence of the primary stimulus that this message is transduced across the plasma membrane to mobilize some intracellular second messenger(s) which in turn activate(s) the cell to perform its specific activity.

Several recent reviews (Berridge, 1975; Rasmussen, 1970; Rasmussen and Goodman, 1977) have demonstrated the importance of calcium as an intracellular second messenger or cofactor and have hypothesized a mechanism by which this divalent cation interacts with cyclic nucleotides to control the activity of many cell types. The key to calcium acting as an effector of cell activity appears to be its low unbound cytosolic concentration (10^{-7} to 10^{-5}M). At rest, active extrusion mechanisms across the plasma membrane and/or uptake of the ion by mitochondria or microsomes maintain these low levels. When these cells are activated by an external stimulus (i.e. neurotransmitter or hormone) the plasma membrane becomes permeable to extracellular sources or calcium may be released from intracellular stores, or both, to change the cytosolic level of the ion and cause a change in cell activity.

With these basic concepts in mind, the important question with respect to tick salivation concerns the role of calcium in maintaining salivary fluid secretion and the source of free calcium during cell

stimulation. Furthermore, the elucidation of cAMP, calcium and catecholamine interaction to control salivation would provide key insights into the mechanisms of salivation control in this gland.

Evidence for extracellular calcium involvement during cell activation by catecholamines and cAMP is supported by the following results. Removal of calcium from support media reduces or inhibits the ability of glands to respond to catecholamine and cAMP/theophylline stimulation; furthermore, the addition of calcium to the bathing solution allows for complete recovery of catecholamine stimulated glands. Although a calcium chelator (i.e. EGTA) was not added to the bathing medium during these particular calcium deletion experiments, EGTA (5 mM) was included in the bathing medium in subsequent experiments and similar rates of fluid secretion were observed (see Figure 7; 104-133 minutes). Possibly, in situations where some secretion occurs upon the addition of calcium without dopamine (Figure 3), neurotransmitter matierial is released from nerve endings still attached to glands after dissection. It is known that calcium is essential for the release of catecholamines and neurohormones from vertebrate and invertebrate nerve cells (Rubin, 1970; Miledi, 1973; Maddrell and Gee, 1974). The fact that removal of calcium from the bathing medium depresses secretion, but introduction of the ion causes a rapid recovery to rates observed in control glands suggests that external calcium is necessary for the primary stimulus to protentiate maximum secretory rates.

Interestingly, once calcium has been deleted from cAMP/theophylline stimulated glands, recovery can be accomplished only after exposure to dopamine. The requirement of external calcium is evidently quite small, however, because TS/PO_4, which contains only uM free calcium, is able

to maintain *in vitro* fluid secretion (Figure 5). Low calcium may actually enhance secretion in some tissues, this is supported by evidence where calcium-free solutions increased the level of cAMP during simulation by reducing the inhibitory effect calcium has on adenylate cyclase (Nagata and Rasmussen, 1970; Prince et al., 1972). This type result may have occurred when calcium was added to calcium deleted glands (Figure 3; 48-53 minutes). The lack of calcium may have allowed cAMP levels to increase but not to a sufficient concentration to maintain high secretion rates, and with the addition of calcium and dopamine one sees a dramatic overshoot as compared to control glands.

The inability of cAMP to stimulate secretion in a calcium-free support medium is an important point because in other experiments cAMP/theophylline has been able to mimic the effect of the primary stimulus. For example, cAMP induces fluid secretion in the blowfly salivary gland incubated in a calcium-free medium but with a slower reaction time (Prince et al., 1972). One explanation for the result seen in tick salivary glands could be that an absence of calcium changes membrane permeability so that cAMP cannot cross the plasma membrane. It is a well known fact that cAMP is highly impermeable to membranes (Berridge and Prince, 1972). An alternate hypothesis is that cAMP is not able to mobilize intracellular calcium to such an extent that fluid secretion can be initiated. The role of cAMP in changing plasma membrane permeability to external calcium has been discussed by other investigators (Berridge, 1975). Possibly, cAMP affects the plasma membrane rather than intracellular stores, but one would expect the return of calcium

to the bathing medium in calcium deletion experiments to facilitate cAMP/theophylline stimulated secretion, but this does not occur.

In the support of the latter hypothesis is the blockage of cAMP/ theophylline stimulated fluid secretion by the calcium antagonist VA (Figure 9). This allows one to leave calcium in the support medium so that membrane integrity is not altered and might discount the hypothesis where calcium is affecting membrane permeability to cAMP. Glands are able to secrete upon verapamil removal (29-69 minutes) while recovery is not seen when calcium is added to calcium-free glands. Evidently, removal of calcium disrupts cell processes associated with fluid secretion especially when cAMP/theophylline are the stimulating drugs.

Other information implicating external calcium as a source evolves from the comparison of the abilities of four salines to support fluid secretion (Figure 4; Table I). It appears that even though some differences in support abilities of the four media may be caused by differences in the pH, Na^+:K^+ and HCO_3^- of each solution, the factor that correlates best with the ability of each medium to support secretion is it's level of free calcium. Media containing mM free calcium support much faster rates of secretion as compared to TS/PO_4 with uM levels of this cation.

The success of cAMP in the three support media is probably related to the availability of free calcium as well. Interestingly, the TS/PO_4 contains a very low concentration of calcium (uM) compared to $TS/MOPS_{20}$ (mM), yet both support similar responses. Perhaps the difference in support ability of these two salines may be accounted for in the lower concentration of cAMP used in TS/PO_4 (5×10^{-3}M). It appears from these data that external (hemolymph) calcium is necessary for cAMP/

theophylline stimulated secretion, but as mentioned above, the concentration required for stimulation is not high. One explanation for the success of TC 199/MOPS was discussed by Kaufman (1976) where he suggests that some factor in modified TC 199 (Tween 80) may act to permit cAMP to enter the cells more easily. When tested, however, Tween 80 enhanced theophylline but did not affect cAMP stimulated secretion in isolated glands from D. andersoni (Kaufman, 1976). Also of interest is the finding that TC 199/MOPS potentiates cAMP stimulated secretion to a much greater extent than the catecholamines when compared to TS/PO_4 or TS/MOPS (Figures 4 and 5).

With the above information in mind, it should be stressed that any factor which may potentially alter support medium concentrations of unbound calcium could significantly affect that tissue or cell with respect to membrane permeability, cell adhesion, enzyme activity, energy production, etc. Therefore caution should be used in choosing buffers that may immobilize calcium to any appreciable extent (i.e. phosphate, citrate).

Probably the best evidence supporting extracellular calcium movement into cells as a part of cell activation is the successful inhibition of in vitro fluid secretion by the calcium antagonist verapamil (VA) (Isotopin®) (Figure 7). The use of VA to block calcium influx is significant for several reasons. First, it appears that this calcium antagonism is not involved directly or indirectly with enzyme inhibition associated with calcium translocation at VA concentrations of 2×10^{-8} to 2×10^{-4}M (i.e. Ca-ATPase, Na+K-AtPase, adenylate cyclase or phosphodiesterase; Hoeschen, 1977). Secondly, use of VA allows one

to leave calcium in the support medium and avoid the problem of cytotoxic effects from calcium deletion itself and inclusion of a calcium chelator (EGTA), but still block calcium entry. Malaisse et al. (1977) suggest that VA's mode of action at the physiochemical level involves interference with natural calcium-binding ionophores in the plasma membrane. VA's antagonism of dopamine stimulated secretion is much quicker and effective than calcium deletion (Figure 7). Possibly it prevents a re-uptake of calcium extruded during activation because of a more intimate association with the plasma membrane. In comparison, it may require more time for the EGTA to remove released calcium from deep basal and lateral infolds of the water cell (or for calcium to diffuse away) than for VA to reach these sites and antagonize calcium influx. Interestingly, once the gland has been depleted of internal calcium by prolonged exposure to the calcium free medium, VA prevents recovery upon calcium's readdition (Figure 7). Contrast this to the recovery seen when calcium is returned to the support medium in calcium deletion experiments (Figure 2 and 3). This suggests that VA is blocking or stabilizing the plasma membrane of the water cell at normal calcium entry sites during stimulation.

Some caution must be exercised however, in the interpretation of VA data because recent reports confirm that the (+) optical isomer of this Ca^{++} antagonist can affect Na^{+} influx in some tissues (Cardiac cells; Boyer et al. 1975). This could be of particular significance because sodium is the primary cation in the saliva produced by these glands (Hsu and Sauer, 1975). To be certain that the antagonism is involved with Ca^{++} and Na^{+}, one could subject the verapamil antagonized

glands to high Ca^{++} or Na^{++} concentrations and determine which cation restores fluid secretion.

The evidence then seems to indicate a need for extracellular (hemolymph) calcium during stimulation. On the other hand the case for the importance of an influx of calcium into the cytoplasm is complicated by ionophore A23187's inability to stimulate fluid secretion. A23187 is an ionophore that facilitates the specific translocation of divalent cations across membranes (Reed and Lardy, 1972). This ionophore is thought to induce both a mitochondrial release of calcium and an influx across the plasma membrane (Babcock et al., 1976; Prince et al., 1973). If an elevated cytosolic level of calcium is required, then one can facilitate this process with the ionophore and most importantly bypass the primary stimulus. In experiments using ionophore concentrations ranging from 10^{-7} to 10^{-5} M in $TS/MOPS_{20}$ (3.5 mM calcium), isolated tick salivary glands did not respond. Micromolar amounts of this ionophore have been most frequently used (Steinhardt and Epel, 1974; Prince et al., 1973).

The concentration of ionophore used may determine where the ionophore will have its effect (i.e. mitochondria versus plasma membrane) and whether one will see an influx or efflux of calcium (Babcock et al., 1976; Jensen et al., 1977). The ionophore may also concentrate itself in the mitochondria or plasma membrane over a period of time and cause a biphasic movement of calcium, first an efflux then uptake (Rasmussen and Goodman, 1977).

It is therefore possible that the concentration of ionophore used in these salivary gland experiments was too high and flooded the cell

with calcium. If the activity of many enzymes require a very narrow or critical intracellular calcium concentration then inhibition could have resulted. Mennes et al. (1978) report such a phenomenon in isolated renal tubules where high calcium (>1mM) or ionophore (10^{-6}M) concentrations inhibit gluconeogenesis. The effective ionophore and calcium levels were 10^{-8}, 10^{-9} M and 0.1 mM or lower. Calcium (0.1, 1.0 mM) was used in two gland preparations with A23187 at 10^{-6} and 10^{-7}M and no secretion was produced. An interesting observation is that these glands do not swell when exposed to the ionophore as is seen when catecholamines or cAMP/theophylline bathe glands. It appears that the primary stimulus or cAMP/theophylline (with calcium) are necessary for the initiation of salivation and that an influx of calcium alone is not enough in and of itself to stimulate secretion. Calcium flux studies would be required before it could be concluded that the ionophore is inducing calcium flux. Possibly the range for an effective cytosolic calcium concentration may be so narrow that the correct ionophore-calcium ratio has not been used. ^{45}Ca uptake studies may provide some insights for interpretating information discussed to this point (Table III). When gland pairs are incubated in dopamine or saline (control) for variable amounts of time, one sees an apparent higher uptake in control glands at 1, 5, 10 minutes. At first inspection, this result would seem to contradict a hypothesis where activated cells allow for an influx of exogenous calcium which acts to elevate cytosolic levels of this cation. Indeed, what may be occurring is that calcium is taken up by the activated water cell and either moved back into the hemolymph or lost with the secreted fluid (or both). Such a

scheme is strongly supported by ^{45}Ca efflux and gland calcium measurements after exposure of isolated glands to catecholamines. Furthermore, the actual amount of calcium being mobilized during stimulation is probably quite small and localized along the plasma membrane. Thorn (1974) and Berridge (1975) warn that, although ^{45}Ca uptake has been measured during similar kinds of functional processes, interpretation of the data is subject to some criticism because factors other than those associated with the process being studied can influence uptake. This problem could be better studied by looking at calcium movements via x-ray probe ion analysis or injection of calcium sensitive photoproteins during stimulation. One additional explanation of the radiocalcium uptake experiments would be that influx of calcium may not be as important as heretofore described and that intracellular stores of calcium play a more vital role in raising cytosolic calcium sufficient to activate salivation.

The high level of calcium in tick salivary glands (~10 mM) is not unlike that found in other fluid transporting tissues (Rubin, 1974). For example, the medulla and cortex of the mammalian kidney contain 8.9 and 16.9 mM calcium respectfully, and the salivary glands, 7.3 mM, Generally, mitochondria and microsomal fractions are credited with having the greatest facility for storing calcium with mitochondria being the most important (Rubin, 1974). Present experiments reveal a 56% reduction in tissue calcium when salivary glands are incubated with 10^{-5} M NA for 15 minutes. A similar loss of tissue calcium is observed in blowfly salivary glands exposed to 5-HT (serotonin) and EGTA for 60 minutes (Berridge et al., 1975). The results of histochemical tests

and microprobe analysis suggest that mitochondrial reserves are being depleted in blowfly salivary glands during secretion, and the authors suggest that cAMP may be involved in the intracellular release of calcium. However, a possible role for cAMP in causing a release of calcium from mitochondria has been the subject of considerable controversy and a clear-cut picture has not emerged (Borle, 1976; Scarpa et al., 1976). It must be kept in mind also, that an additional cause of reduced calcium could be calcium's involvement in packaging and release of granular material (i.e. cement, cytolysins, etc.) from type II and III alveoli (Meredith and Kaufman, 1973; Megaw, in press) with the catecholamines initiating the process.

This information coupled with ^{45}Ca efflux studies and the observation that glands can continue to secrete _in vitro_ without calcium in the support medium, indicates that intracellular stores may be liberated during cell stimulation. The control of this release is thought to be mediated by cAMP in several cell systems (Rasmussen and Goodman, 1977) but the inability of cAMP/theophylline to initiate salivation in a calcium-free saline casts some doubt on such a scheme in tick salivary glands at this juncture. The picture is clouded further because glands were unable to recover once calcium was restored to the support medium.

The calcium alteration experiment was designed to determine if the primary stimulus is necessary for recovery of calcium lost during cell activation (Figure 10). Because of a carry-over affect due to calcium's presence in the rinse, EGTA-dopamine additions are influenced in a positive manner and the results are rather difficult to analyze.

However, it does appear that glands perform better when dopamine bathes glands in the presence of calcium and the (EGTA) calcium-free rinse does seem to depress secretion somewhat. The EGTA-dopamine incubation with calcium in the rinse follows a pattern indicative of earlier calcium deletion experiments.

It is interesting to compare the different rates of secretion observed with DA and NA stimulate glands during calcium deletion experiments (Figures 2 and 3). DA stimulates a more rapid initial secretory rate than NA in both control and experimental glands. In this situation DA may serve as a precursor for NA formation in nerves terminating on the water cells and induce a more rapid release of NA at that point. Alternately, the plasma membrane receptor may more favorably recognize DA than NA. Kaufman (1977) feels that tick salivary glands contain one or several receptors differing pharmacologically from mammalian α-adrenergic, β-adrenergic and dopaminergic receptors. If gland reserves of calcium are affected and mobilized more rapidly when glands are stimulated by DA, one might expect to see the rapid rise followed by a decline in fluid secretion observed when calcium is missing from the bathing medium (Figure 3). As mentioned above, a depletion of cell calcium might also allow cAMP to accumulate in the cytosol as suggested by reports where adenylate cyclase is affected by calcium, and where calcium-free solutions are known to potentiate high levels of cAMP (Nagata and Rasmussen, 1970; Prince and Berridge, 1973). Thus, an elevated level of cAMP coincident with calcium restoration and DA stimulation may explain the unusually high rate of fluid secretion observed in Figure 3.

Recently, Diehl (P. Diehl, Université de Neuchatel, Institut de Zoologie, 2000 Neuchatel 7, Switzerland) and Megaw (M. Megaw, Department of Zoology, Cambridge University, Cambridge, England personal communications) have shown that membrane infoldings and mitochondria of the "water cells" greatly increase in glands from ixodid female ticks during engorgement. The increased number of mitochondria not only supply energy but could serve to store and release calcium in close proximity to transporting epithelia. Not surprisingly, the development of these morphological features correlates well with the gland's ability to secrete fluid maximally (Kaufman, 1976; Sauer et al., in preparation). Also, McMullen and Sauer (in preparation) have recently shown an inverse relationship between tick salivary gland phosphodiesterase (PDE) activity and maximum secretory ability of glands. An "inhibitory" factor may be present in glands taken from rapidly engorging ticks and cause a substantial decrease in PDE activity in the transition from slow to rapid feeding. In addition, the above authors found that preincubation of glands with DA increases PDE activity. This finding together with the effects of catecholamines in experiments described above, raise the possibility that changed levels of cell calcium could be activating PDE and serve as a convenient feedback mechanism to quickly control levels of cAMP. It is known from other cell systems that the protein activator of phosphodiesterase is affected by calcium (Kakiuchi et al., 1973; Teo and Wang, 1973). Such a scheme would not be inconsistent with the findings reported here, and the apparent importance of calcium during the fluid secretory process.

Thus it appears that calcium is involved in some aspect of controlling salivation in ticks and that hemolymph and intracellular sources contribute to this process.

CHAPTER V

CONCLUSIONS

Salivary glands of ixodid ticks are responsible for ridding this acarine of excess ions and water gained while concentrating its blood-meal. The purpose of this investigation was to determine whether calcium is involved in regulating the fluid secretory process of this tissue. Isolated glands were exposed to various drugs and pharmacological agents that can effect calcium movement across biological membranes. In addition, ^{45}Ca flux and change in gland calcium were determined when exposed to stimulated and non-stimulated conditions. Changes in bathing medium calcium levels were examined for their affect on fluid secretion stimulated by cyclic AMP and catecholamines. The importance and relationship between cyclic AMP and calcium were also studied.

Intracellular and extra-cellular calcium are important in cyclic AMP/theophylline and catecholamine stimulated salivary fluid secretion. This is supported by the finding that catecholamine induced salivation (*in vitro*) is reduced when calcium is missing from the support medium but secretion is quickly restored after adding calcium to the support medium. The calcium antagonist verapamil inhibits dopamine stimulated salivary fluid secretion and upon its removal from the bathing medium, secretion is restored. The availability of free calcium affects fluid secretion because a support medium containing μM calcium supports a

much slower rate of secretion than solutions containing mM calcium. With the techniques described, ionophore A23187 was not able to induce fluid secretion. Calcium flux studies show that the primary gland stimulus initiates a release of intracellular calcium which probably raises the cytosolic level of calcium. Cyclic AMP and calcium are thought act in concert to control secretion partly because cyclic AMP (and theophylline) stimulates fluid secretion with calcium, but without calcium in the support medium this cyclic nucleotide is not effective. Bathing medium levels of calcium also affect the ability of glands to respond to cyclic AMP stimulation. It is concluded that a primary catechalamine stimulus probably activates salivary gland cells to secrete via a second messenger system involving cyclic AMP and calcium and this process probably relies on an external source of calcium to elevate cytosolic calcium during stimulation. It also appears that glands can operate on intracellular stores.

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VITA 2

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