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Calcium dependent chloride transport in the rectal gland of the dogfish shark squalous acanthias

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CALCIUM DEPENDENT CHLORIDE TRANSPORT IN THE RECTAL GLAND OF THE DOGFISH SHARK SQUALUS ACANTHIAS

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ABSTRACT. The rectal gland of the dogfish shark Squalus acanthias is an extrarenal osmoregulatory organ that secretes a hypertonic sodium chloride solution into the intestine to help the shark maintain salt and water homeostasis in its hypertonic environment. Because calcium has been implicated as a second intracellular messenger of secretion in a number of epithelia, the purpose of this study was to investigate the role of calcium in control of chloride secretion by the isolated perfused rectal gland preparation. Several drugs were used: calcium ionophore A23187 and carbamylcholine, which increase intracellular calcium and would be expected to stimulate calcium dependent processes; calcium channel blockers verapamil and D600, which impede calcium entry into cells, and RMI 12330A, an inhibitor of adenylate cyclase and other calcium mediated events. The latter two drugs would be expected to inhibit calcium dependent processes.

Ionophore A23187 and carbamylcholine (10^{-5} M and 2×10^{-3} M respectively) modestly but significantly enhanced chloride secretion by the isolated perfused rectal gland as compared to baseline secretion (a three-fold increase); further stimulation was achieved with the addition of theophylline (0.25 mM) and dibutyryl cAMP (0.05 mM). L-D600, the isomer of methoxyverapamil that specifically blocks calcium channels, significantly inhibited adenosine stimulated rectal gland secretion throughout the 30 minutes of administration in the presence of an extracellular calcium concentration that was one-fifth of normal. This inhibitory effect was reversed by adenosine when l-D600 was discontinued. In l-D600 and racemic verapamil studies in normal extracellular calcium (2.5 mM), inhibition was less dramatic and more transient, such that a significant difference was seen only during the first 10 minutes of administration. D-D600, the isomer believed to specifically block sodium channels, was found to enhance adenosine stimulated rectal gland secretion after a brief inhibitory response that was not statistically significant. Finally, RMI 12330A significantly inhibited theophylline stimulated secretion in a partially reversible manner.

These data demonstrate the involvement of calcium in mediating chloride secretion in the rectal gland, and are consistant with an hypothesis involving both cAMP and calcium in control of this transport process.

REVIEW OF THE LITERATURE

The rectal gland of the dogfish shark, Squalus acanthias, and other elasmobranchs is a digitiform intestinal appendage capable of secreting a concentrated NaCl solution that is hypertonic to shark plasma and isosmotic with the shark's seawater habitat. This important extra-renal organ enables the fish to regulate the salt composition of its body fluids and to eliminate excess salt ingested from the hypertonic environment. Because of its accessibility and clearly defined anatomy, the rectal gland has been extensively studied by both in vivo catheterization and in vitro perfusion. These studies have demonstrated that the gland is a convenient experimental tissue for the investigation of hormone-mediated NaCl secretion.

The focus of the following literature review is fourfold: to describe general principles of marine osmoregulation, to review the details of rectal gland physiology, to explore different molecular models for epithelial chloride secretion, and to discuss the possible role of intracellular calcium in regulating this secretory process. This study in particular addressed the issue of calcium dependent regulation of rectal gland secretion by employing calcium ionophores, calcium channel blockers, and other drugs affecting calcium transport such as carbamylcholine and RMI 12330A.

The Problem of Osmoregulation in Marine Organisms

Marine organisms are faced with the homeostatic task of maintaining a constant cell volume and plasma tonicity in the face of a tendency to lose water and gain salt. Several options for achieving this balance can be envisioned. On one hand, a fish could ingest sufficient salt water to meet its fluid needs (0.3 to 1.5% of body weight per hour) and secrete the excess salt by its kidneys, gills, or special secretory cells; this "hypoosmotic regulation" is the option chosen by the teleost. On the other hand, a fish could ingest minimal fluid by protecting itself against water loss by "hyperosmotic regulation", maintaining an osmolality equal to or slightly greater than that of sea water. This is the case with elasmobranchs such as the dogfish shark. To illustrate these patterns of osmoregulation more clearly, three distinct groups will be compared: stenohaline teleosts, eurhaline teleosts, and elasmobranchs.

Marine teleosts can be divided into two groups, the stenohaline fish, which are restricted to waters of a particular salinity, and the eurhaline fish, which can adapt to waters of varying salinity. The first group is mainly concerned with water retention and divalent ion elimination, particularly magnesium and sulfate. Since the latter is accomplished by tubular secretion, many of these species, such as the goosefish, Lophius americanus, have poorly developed glomeruli. Urea is freely excreted, and sodium, chloride, and potassium are significantly reabsorbed by the kidney (85%-90%). These

monovalent ions are excreted by specific transport cells in the gill and buccal region which appear to operate by active chloride transport. (77)

Eurhaline teleosts, on the other hand, can adapt to a wide range of salinity by altering their osmoregulatory processes. Their glomeruli, like those of fresh water fish, are generally well developed. When these fish are introduced to a relatively hypertonic environment, their glomerular filtration rate (GFR) and consequently, urine volume, drop transiently. Thus, water loss is minimized. Gradually the permeability of their tubule epithelium increases and water is conserved without the need for reduced filtration, and GFR returns to normal. When placed in a more dilute environment, these fish react to conserve salt by decreasing their tubular salt secretion. Decreased tubule water permeability follows gradually, and the fish once again return to a steady state. (77)

Studies in the dogfish shark Squalus acanthias by Burger and Hess (19) showed that elasmobranch plasma has an osmolality of approximately 1000 mOsm/L, while that of seawater is about 930 mOsm/L. However, as table i indicates, the concentrations of the most abundant electrolytes in shark plasma, sodium and chloride (250 mM each) account for only half of the total osmotic activity- urea and trimethylamine oxide (TMAO) constitute most of the remainder. Thus by achieving a tonicity close to that of the marine environment, sharks are able to maintain a fluid balance with minimal metabolic

cost. Salt balance, however, is a more involved problem because the shark plasma sodium and chloride concentrations, approximately half of that of seawater, permit a NaCl influx across the gills and gastrointestinal tract. This excess salt must be disposed of by the kidneys or other means.

The elasmobranch kidney is a glomerular organ with a GFR of 4 ml/kg/hr, which is higher than most marine teleosts and is similar to fresh water fish. The glomeruli are large, and it is felt that the GFR capacity is variably underutilized- in fact, sharks tend to decrease their GFR during captivity while teleosts undergo diuresis. The kidney tubules regulate ion reabsorption (sodium and chloride are reabsorbed with a urine to plasma ratio of 1) but more importantly, the tubules reabsorb 95% of the filtered urea and other nitrogenous products which ultimately contribute to the high osmolality of the shark plasma (77). Urea reabsorption seems to vary directly with plasma urea concentration with no clear T_m (96). The resulting urine is 50 to 200 mOsm/L hypoosmotic to blood, and cannot therefore accomodate the excretion of a large salt load in excess of water. To augment its salt loss, the shark utilizes a specialized salt secreting organ, the rectal gland, which produces a NaCl solution about twice as concentrated as plasma and equal to the NaCl concentration in seawater. Like the nasal salt gland of marine birds, which also secretes a hypertonic saline solution (149), the rectal gland produces one of the most concentrated extra-renal fluids yet described (22).

The Anatomy of the Rectal Gland

As shown in figure i, the rectal gland is a finger shaped structure, 1 to 3 g in weight, 4 to 7 cm in length, that lies in the dorsal mesentary between the intestine and dorsal body wall and communicates with the intestinal lumen posterior to the spiral valve. It is supplied by a branch of the dorsal aorta which courses beneath the superficial fibrous capsule of the gland before branching into anterior and posterior rami, circumferential branches, and finally, sinusoids; approximately 10% of glands have a double arterial supply. The central venous system drains the parenchymal sinusoids into a large ventral vein (17). The parenchyma of the gland consists of secretory tubules and sinusoids in varying proportions. At the periphery, tubules are small (5 microns in diameter), numerous, and packed closely into clusters separated by 10 micron sinusoids. They converge toward the central excretory duct in a roughly radial fashion such that there is about one central tubule per eight peripheral tubules; central tubules are larger (1.5 mm), widely separated, and surrounded by confluent sinusoids (37). Thus the volume of secretory ducts increases up to five fold en route to the central canal, and blood flow and fluid secretion proceed in the same direction (periphery to center) so that a counter-current exchange mechanism is not involved (17).

The rectal gland tubule cells, like many other secretory epithelia, have a characteristic ultrastructure consisting

of many basally located mitochondria and extensive basolateral plasma membrane interdigitations, presumably to increase membrane surface area. Cell membrane structures are believed to have a significant role in secretory processes, and recent research indicates that the extent of membrane modifications may correlate with secretory activity. For example, Karnaky, Kinter et al (91) documented a four fold increase in Na^+-K^+ ATPase and basolateral membrane area in the chloride cells of Fundulus adapted to hypertonic seawater. Wendelaar and Veenhuis (185) found similar changes in the kidney of other eurhaline teleosts. Ernst and Ellis (44) and Holmes (80) saw the same effect in the avian salt gland, and Kashgarian (93) found that both rat kidney and the colon increase their basolateral surface area in response to potassium loading, glucocorticoids, or endogenous aldosterone. Wade, O'Neill et al (182) reaffirmed these findings in rabbit collecting tubules treated with DOCA. Thus the basolateral membrane and associated enzyme, Na^+-K^+ ATPase, appear to be functionally important in ion transport in these tissues. The details of membrane permeability changes are still unclear, but recent work by Wade et al (180) provides new information about the structural basis of membrane transport. Freeze-fracture preparations of toad bladders stimulated with ADH showed linear aggregates of intramembrane particles in the luminal membrane of granular cells; these aggregates were fewer in unstimulated cells and correlated in number and arrangement with changes in transport function. The particles are believed to be contained within

cytoplasmic vacuoles in unstimulated cells (181) and reach the membrane surface by vacuolar fusion after ADH stimulation ("shuttle hypothesis") (183), perhaps directed by microtubules (172). It would be presumptive to draw many parallels between ADH stimulated water reabsorption and chloride transport, but the prospect of identifying a structural "chloride channel" in secretory epithelia like the rectal gland is indeed intriguing.

The nature of intercellular junctions and their function in the rectal gland has been the subject of some disagreement in the past. Bulger (17), in her early and extensive description of microscopic rectal gland anatomy, found tight junctions and desmosomes between the apical poles of the tubule cells, presumably restricting the free paracellular passage of ions and water. However, Van Lennep (179) refuted this finding because glands he immersed in lanthanum hydroxide had lanthanum penetrate into both intercellular spaces and lumen. Recent studies by Forrest, Boyer, Wade et al (58) reaffirmed Bulger's original work. In isolated glands perfused with lanthanum chloride, lanthanum was restricted to the intercellular spaces and did not enter the lumen- this perfusion technique was considered to be more physiologic than Van Lennep's immersion method, and may account for the different observations. In addition, in the studies by Forrest et al (58), glands examined by freeze fracture had extensive and heterogeneous membrane "strands" suggestive of complex tight junctions at the apical ands of the intercellular space. These results suggest that there is indeed a barrier to the paracellular passage of small molecules, such as urea, into rectal gland fluid, and that the

rectal gland is a less "leaky" epithelium than other secreting organs such as the proximal tubule of rabbit kidney, frog urinary bladder, and avian salt gland (140), all of which have simpler (fewer stranded) tight junctions on freeze fracture.

The Physiology of the Rectal Gland: Characteristics of Secretion

In Vivo Studies of Secretion

Although the rectal gland was the object of Homer Smith's interest as early as 1931 (162), the function of the gland was not understood until Burger and Hess conducted their first *in vivo* experiments in 1960 (19). They reported that the rectal gland is a variably but persistently active organ, with flow rates ranging from minimal to 1.9 ml/kg/hr, both in different fish and in the same fish at different times. However, the chloride concentration of the secreted solution was always constant at 480 to 500 mM, so the absolute amount of salt secreted depended on the fluid flow rate (19). An arteriovenous chloride difference of up to 32% indicated that the fractional excretion of chloride was high (21), so only a small amount of fluid (less than 2 ml/kg/hr) needed to be excreted to decrease the plasma NaCl concentration from 250 to 240 mM (22). Over long term observation, the volume of rectal gland fluid and urine were approximately equal, but the urine contained significant magnesium whereas the rectal gland fluid did not (19).

To better define the homeostatic significance of the rectal gland, Burger (19) extirpated the organ from a number of sharks and monitored the sharks' salt balance for up to

21 days. He reported that the plasma composition did not change, nor did urinary chloride rise. When NaCl was injected into these fish he noted that plasma chloride concentration did not return to normal as quickly as in control fish, and in dilute seawater he found that plasma osmolality and chloride concentration were slightly higher than controls, though urinary chloride was always less than plasma chloride. From these data he concluded that without a rectal gland, sharks can lose salt gradually but adequately by increasing urinary output. Because he saw no change in plasma electrolytes, he felt that the rectal gland was not an essential organ to survival. However, when similar extirpation studies were repeated in 1973, Forrest, Silva et al (56) documented a rapid and sustained increase in plasma sodium concentration that did not return to normal during the 8 day observation period. Similarly, when Evans and Mansberger (49) removed the rectal glands from dogfish "pups" (prematurely delivered young with yolk sac still attatched), plasma sodium and chloride increased approximately 22 mM over sham operated controls and approximately 40 mM over unoperated controls. The net sodium influx was 28.9 umol/100g/hr, a value very close to the rate of sodium efflux documented earlier for net cloacal (mostly rectal gland) secretion in these animals. Thus, the renal compensation for excreting a salt load appears to be incomplete in the dogfish shark and the rectal gland appears to be necessary for the maintenance of normal extracellular composition.

Additional <u>in vivo</u> studies addressed the question of

the physiologic control of rectal gland activity. In 1962, Burger (20) found that an intravenous salt load slowly increased in vivo rectal gland activity in a dose-dependent manner; other osmotic agents such as sucrose, urea, and KCl were only slightly less effective. Acetylcholine, epinephrine, pilocarpine and pitressin were not stimulatory, and piperocaine was not inhibitory. Burger therefore interpreted this as evidence that salt and osmotic increases were effective secretory stimuli, but that neural control of the gland was unlikely.

In 1965, Burger monitored rectal gland secretion in sharks kept in dilute (80%) seawater (21). He found that not only did the urine volume increase under this condition, but the rectal gland also remained very active. Although the plasma osmolality and chloride concentration decreased and plasma volume increased by up to 16%, the plasma retained its hypertonicity to the environment by readjusting to a lower level. Thus in addition to salinity and osmolality, Burger concluded that volume was a strong stimulus to rectal gland secretion, capable of overriding dilution of the extracellular fluids (22).

Finally, Stoff, Hallac et al (167) noted that an oral saline load evoked an immediate rectal gland response while an intravenous bolus such as employed by Burger was effective only after a thirty minute lag. This suggested that the initial secretory stimulus may actually be change in volume or tonicity in the gut.

In Vitro Studies of Secretion

Further understanding of the secretory process and control followed the development of a reproducible technique for *in vitro* study of the rectal gland, pioneered by Palmer in 1966 (125). This method involves excising the rectal gland from a freshly killed shark by severing the single extracapsular artery, vein, and duct. Each of these is then catheterized with PE 90 tubing and the gland is perfused with an oxygenated Ringer's solution resembling shark plasma in composition. Secreted fluid and venous effluent are collected, measured, and analyzed for chloride content and osmolality.

The earliest perfusion results reported by Palmer (125) indicated a secretory flow rate of approximately 17 ul/min/g (calculated from the reported data, assuming an average gland weight of 2 g) with a chloride concentration ranging from 250 to 500 mM, but remaining constant within a given preparation. This flow value correlates with Burger's earlier value of 11 ul/min/g (as recalculated by Siegel, Silva, Epstein et al (157)) but was attained by perfusing at 6 to 25 ml/min, a higher than physiologic rate. Hayslett, Schon et al (75) improved the isolated perfusion technique in 1974 and documented a rate of 2.2 ul/min/g with a constant chloride concentration of 450 mM; the perfusion rate in these experiments was 2 to 4 ml/min, 4 to 8 times the estimated *in vivo* rate, but was the minimal rate needed to maintain a secretory flow near the documented *in vivo* rate. No decrease in secretion with time was noted. In 1975, Siegel et al (157) reaffirmed an *in vitro* secretory rate of 3.6 ul/min/g with a chloride concentration

of 496 mM, but no time-dependent secretory decrease was observed. However, a correlation was made in this study between a suddenly increasing secretory rate, decreasing chloride concentration and increasing urea content of rectal gland fluid, indicating that the secretory epithelium had become damaged in some manner and passive diffusion was occurring, driven by hydrostatic perfusion pressure (157).

Finally, in the following year Siegel, Schon and Hayslett (158) reported that rectal gland secretion declined gradually with time, from 2.6 ul/min/g to 1.8 ul/min/g in 70 minutes. This indicated that either the original endogenous secretory stimulus was an evanescent phenomenon or that some metabolic constituent was depleted by long-term perfusion. In 1977 Stoff, Silva, Field, Forrest et al (159, 170) observed that the addition of exogenous cyclic AMP dramatically increased the rate of rectal gland secretion by ten to twenty fold, indicating that cAMP was the second messenger of chloride secretion in this tissue. Though the basal secretory flows in these studies were 40 to 50% below Burger's early observation (3.6 ul/min/g (170) and 9.5 ul/min/g (159)), they were consistent with Siegel's data in absolute value and time dependency. Thus the isolated perfused preparation is well documented as a physiologically accurate system enabling the investigation of many aspects of rectal gland secretion. A summary of the most salient observations follows.

1. The gland has specific ionic requirements for secretion. Silva, Stoff et al (159) showed that chloride output is directly proportional to the sodium concentration of the perfusate, such that when sodium is replaced by choline, chloride secretion is reversibly inhibited. A similar effect is seen when perfusate chloride is replaced by acetate or sulfate (164).

2. Secretion proceeds against an unfavorable electrochemical gradient for chloride. Measurements of the ionic concentrations and electrical potential difference (PD) between perfusate and duct fluid have yielded data on the electrochemical gradients in the rectal gland, which ultimately determine the driving forces on the ions involved. Hayslett et al (75) first noted that the PD was lumen-negative during in vitro chloride secretion. Stoff et al (170) measured a PD of -7 mv in the resting gland, which increased to -19.7 mv after stimulation, both lumen negative. In similar studies, Silva et al (159) measured a basal PD of -6.8 mv and a stimulated PD of -15 mv (lumen negative) and further estimated that the intracellular potential was approximately -70 mv when the extracellular fluid was 0 and the secreted fluid, -15 mv. Thus the overall gradient for chloride secretion from extracellular space to lumen is negative, the greatest electrical barrier being across the basolateral membrane of the tubule cells. When considered with the calculation that intracellular chloride concentration is 2 to 4 times greater than that predicted by the Nernst equation for passive equilibration (159), it becomes clear that chloride secretion proceeds against a large electro-

chemical gradient and that some form of active transport must be operative.

3. Na^+-K^+ ATPase is involved in the secretory process, as shown by several lines of evidence. First, the activity of the enzyme in the rectal gland was found to be higher than in any other known tissue (1.32 M/kg) (11, 47). Second, ouabain at 10^{-4} M, an inhibitor of Na^+-K^+ ATPase, decreased oxygen consumption by 25% and abolished secretion in an unstimulated gland; removal of potassium from the extracellular fluid also inhibited secretion (159). These findings suggested that Na^+-K^+ ATPase is involved in generating the ionic gradients for rectal gland transport. However, cytochemical studies localizing this enzyme to the basolateral rather than apical membrane of the cell initially posed a paradox (67). Since the rectal gland tubule cells secrete NaCl into a central lumen, the ATPase might logically be predicted to occur on the luminal membrane, transporting sodium outward. However, the same basolateral ATPase location was found in the secretory epithelia of the avian salt gland (45) and the teleost chloride cell (91). Presumably, then, sodium ions must be extruded from the tubule cells into the interstitial fluid and must reach the tubule lumen by an intercellular route (44). This arrangement is consistent, as well, with the maintenance of a high transmembrane PD across the basolateral membrane, and the maintenance of a low intracellular sodium concentration, providing a driving force for the entry of sodium (possibly coupled to chloride) into the cell.

4. Secretion is sensitive to cyclic AMP. As previously mentioned, the secretory rate of the isolated perfused rectal gland tends to be lower than the rate in vivo, and declines gradually with time, but a high secretory rate can be restored and maintained in vitro by the addition of cAMP to the perfusate. In experiments by Stoff et al (169), glands receiving Ringer's solution alone dropped from a variably high initial chloride secretion rate to a rate of less than 50 mEq/hr/g in thirty minutes, but glands then perfused with dibutyryl cAMP (a more stable cAMP derivative) showed a remarkable rebound to as much as 4000 mEq/hr/g over the next thirty minutes. Chloride concentration increased only at the highest level of secretion. Glands treated with theophylline alone after thirty minutes of perfusion showed a similar, but less impressive, stimulatory effect. The theophylline response was dose-dependent from 0.01 mM (maximum 150 mEq/hr/g) to 5 mM (maximum 1800 mEq/hr/g) with some decrease after twenty minutes of administration. Ouabain-inhibitable oxygen consumption also increased six fold after theophylline exposure (160). Since theophylline is an inhibitor of phosphodiesterase, its stimulatory effect may be due in part to decreased breakdown of endogenous cAMP, however Stoff et al (169) found no significant increment in rectal gland fluid cAMP levels after theophylline stimulation. Finally, perfusion with both theophylline (0.25 mM) and dibutyryl cAMP (0.05 mM) produced a synergistic effect which resulted in stable and sustained secretion at approximately 1400 mEq/hr/g.

These experiments clearly show that cAMP is an intra-

cellular messenger mediating the rectal gland secretory response. The association of cAMP with secretion, particularly hormone-mediated secretion, was first described by Orloff and Handler with ADH in the toad bladder (71, 121). The concept has since been applied to the insect salivary gland (131), the intestine (152, 155) and the rabbit colon (54); in these latter tissues, cAMP both reverses the normal pattern of chloride absorption and elicits chloride secretion. The exact mechanism by which cAMP regulates secretion is unknown, but presumably is initiated by hormone stimulation of adenylate cyclase. CAMP may then either exert its secretory effects by activating a protein kinase cascade, ultimately resulting in some permeability change, or may act on the luminal membrane directly to alter permeability, as suggested by experiments on insect salivary glands by Prince and Berridge (3,5,6).

The discovery of cAMP mediated secretion in the rectal gland prompted further search for the hormone or first messenger that triggers the gland to secrete in vivo. Stoff, Hallac et al (168) tested a number of substances which failed to alter secretion rate, including ADH, oxytocin, norepinephrine, epinephrine, 5-HT, substance P and calcitonin. Since the gland is actually an intestinal appendage, attention was then turned to several structurally similar gastrointestinal hormones, such as pentagastrin, glucagon, secretin, and vasoactive intestinal peptide (VIP). Only VIP consistently stimulated rectal gland secretion. In the isolated gland, VIP (10^{-6} M) increased flow rates more than 500% (600 ul/hr/g to 5000

ul/hr/g) and raised oxygen consumption by 265% (168). Chloride concentration of rectal gland fluid increased, as did lumen electronegativity and intracellular cAMP levels (10 pg/mg protein to greater than 30 pg/mg protein). These effects were potentiated by theophylline, suggesting that VIP operates via cAMP, and were inhibited by somatostatin (170), which inhibits VIP release by the intestine (28).

In vivo studies by this same group (168) showed that when hypertonic saline was infused by vein, plasma osmolarity and sodium concentration rapidly rose to 1381 mOsm/L and 358 mM, respectively, but that plasma VIP levels fell 45%- the opposite of what might be expected if VIP governs rectal gland secretory activity. However, when saline was given orally, osmolarity and sodium concentration rose slightly but VIP levels increased 71% in 30 minutes. These results suggest that intestinal hypertonicity, rather than extracellular volume expansion, affect the endogenous VIP modulation of rectal gland activity, but to date the significance of VIP as an in vivo physiologic regulator has not been fully appreciated.

5. The role of carbonic anhydrase is puzzling. The rectal gland has a higher concentration of carbonic anhydrase than any other elasmobranch tissue (113), but the function of this enzyme in the shark remains entirely unclear. A role in secretion was questioned by Silva et al (159), because carbonic anhydrase inhibitors like ethoxolamide and acetazol-

amide had no effect on fluid or salt output. More recent studies by Maren (114) showed that methazolamide, another carbonic anhydrase inhibitor known to be freely permeable across the rectal gland epithelium (13), also failed to reduce secretion. However, Maren also reported preliminary studies showing that cytoplasmic and membrane associated carbonic anhydrase in the rectal gland was highly resistant to inhibition by chloride, while the enzyme in shark erythrocytes was more sensitive to high chloride concentrations (114). He interpreted this difference to mean that carbonic anhydrase has some role in saline secretion, perhaps by maintaining an alkaline environment within the gland, and suggested that perhaps the true role of the enzyme is not fully appreciated because of the relatively low absolute sodium turnover rates in the rectal gland as compared with rates in other tissues sensitive to carbonic anhydrase inhibition (114).

6. Secretion is inhibited by furosemide and thiocyanate. Loop diuretics, including furosemide and ethacrynic acid, have been reported to inhibit a number of transport processes in both epithelial and non-epithelial tissues, including frog cornea (26), squid giant axon (144), mammalian intestine (84), Erlich ascites tumor cells (76), and fish gill (157). The pharmacologic effect of these compounds seems to be an inhibition of active chloride transport processes, as originally shown in the thick ascending loop of Henle, the site of furosemide action in the mammalian kidney (18). Recent work by

Palfrey and Greengard (123, 124) has provided some information about the molecular mechanisms of furosemide action. This group studied the effects of a series of chemically related furosemide derivatives on a particular Na^+-K^+ cotransport system in turkey erythrocytes. This transport system, which is activated by catecholamines and cyclic AMP, requires extracellular chloride, and is insensitive to ouabain, was found to be inhibited by furosemide and related compounds, such as bumetamide. Since the degree of transport inhibition correlated with the intensity of natriuresis induced in dogs, Palfrey suggested that a similar enzyme system may be the active site for these drugs in other furosemide sensitive tissues, as well. The cellular site of action of these loop diuretics may well be the basolateral membrane, since furosemide inhibited chloride transport in rectal gland vesicles composed primarily of basolateral membranes (50).

In the rectal gland, Silva et al (159) showed that furosemide (10^{-4} M) reversibly inhibited output to 40% of control rates without changing the electrolyte composition of the fluid; thus total chloride secretion fell. Removal of furosemide restored the secretory rate by 60%, mainly by decreasing the volume of secretion, but this effect was less reversible than the furosemide inhibition. Solomon, Silva et al (163) and Epstein, Silva et al (41) also demonstrated thiocyanate inhibitable chloride secretion in the teleost gill, possibly associated with a ouabain insensitive cation independent pump. The significance and mechanism of this thiocyanate

inhibition was not entirely clear, but Epstein et al (41) suggested that due to a similarity in charge or size of hydrated shell, thiocyanate displaces chloride from an enzyme or carrier with an affinity for both ions.

Further investigation into the effects of loop diuretics on the rectal gland has followed the development of an even more specific *in vitro* design for studying rectal gland membrane physiology. This system, as developed by Eveloff, Kinne et al (50), utilized isolated plasma membrane vesicles obtained by homogenizing extirpated rectal glands that had been preperfused with theophylline and cAMP. The isolated membrane fractions had a 4.5 fold enrichment of Na^+-K^+ ATPase activity as compared with intact tissue, indicating that the vesicles were primarily composed of basolateral membrane, and electron microscopy showed that the membrane fractions were pure.

These investigators reported that the uptake of $^{22}Na^+$ required the presence of a chloride gradient directed inward, much as NaCl excretion by the intact rectal gland requires chloride in the perfusing medium. A nitrate or gluconate gradient was ineffective, and prior incubation of vesicles with KNO_3 or KCl eliminated any contribution of electrical coupling in causing chloride flux. Sodium uptake ultimately became saturated in the presence of chloride and was inhibited by furosemide. These results not only reconfirmed the furosemide sensitivity of rectal gland ion transport, but focused attention primarily on basolateral transmembrane processes, eliminating variables inherent in the isolated perfused gland preparation, such as vasoconstriction, mucous plugging of ducts, twists in

the arterial line, and unrecognized trauma. This vesicle technique is not without its own limitations, however: membranes were predominantly basolateral, an artificial situation if both luminal and basolateral membranes are involved in the transport process, and chloride uptake was not measured per se (presumably because the specific activity of the available $^{36}Cl^-$ was low)- this should no doubt be studied specifically in a tissue believed to transport chloride actively. Nevertheless, this work does provide further evidence for the coupling of sodium and chloride fluxes via a cotransport system in which chloride transport may be secondarily active (50).

In summary, the preceding studies suggest that the rectal gland secretory mechanism is a VIP stimulated, cAMP mediated system involving some form of sodium-chloride coupled transport, either primary active chloride transport, as suggested by potential difference and chloride chemical gradient data, or active chloride transport secondary to sodium gradients established by Na^+-K^+ ATPase (secondary active transport). The following section will discuss a model for chloride secretion that has been proposed to account for these diverse findings.

A Model of Active Chloride Secretion

The study of membrane transport phenomena involves the integration of two subsets of information: electrophysiological data (transepithelial potential differences, short-circuit transmembrane electrical currents, and unidirectional ion fluxes), which inddcate the direction and magnitude of

ion transport, and biochemical data (requirements for specific ions, sensitivity to inhibitors), which define the metabolic characteristics of the system. The earliest models of active membrane transport focused primarily on sodium, but with improvement in electrophysiological and isotopic techniques, active chloride transport, originally proposed by Krogh as an osmoregulatory mechanism (101), was again appreciated. In recent years, active chloride transport has been identified in many secretory epithelia such as frog cornea, teleost chloride cell, intestine, and dogfish rectal gland.

Frizzell, Field and Schultz (63) have defined several electrophysiologically distinct modes of transepithelial chloride transport: passive, coupled, non-neutral coupled and electrogenic. The former three, which involve either low or favorable potential differences, are not applicable to the rectal gland, so will be described only briefly.

In strictly passive diffusion, as in the toad bladder and frog skin (102), chloride movement depends entirely on its electrochemical gradient and on the PD generated by active sodium transport. Chloride is thus electrically coupled to sodium and if sodium transport ceases, so does chloride transport.

Neutral NaCl coupled transport was first suggested by Diamond (35), who noted that sodium and chloride were absorbed in the rabbit gallbladder without the development of a significant PD, and that replacement of either by a nonabsorbable

ion abolished all transport. Later studies of unidirectional ion fluxes by Frizzell et al (61) established that there was one-for-one coupling between the two ions. This finding was strengthened by the report of Duffy, Turnheim et al (38) that the chloride content of gall bladder cells was two to three times greater than the predicted Nernst value in the presence of sodium, but not in its absence. Thus, there appears to be a tightly coupled mechanism for NaCl entry at the mucosal membrane of the gall bladder cell, in which sodium diffuses inward along its concentration gradient, thereby energizing uphill chloride entry. Chloride then exits passively with sodium at the serosal membrane. This kind of transport is associated with "leaky epithelia" whose low-resistance paracellular pathways permit rapid isotonic NaCl absorption against minimal potential differences (63). Such a neutral, coupled transport model is thus not appropriate for the "tight" epithelium of the rectal gland.

A variant of this neutral coupled transport was found by Frizzel, Smith, et al (65) in the intestine of the flounder, Pseudopleuronectes americanus. This tissue resembles the leaky epithlium discussed above except for a small (5 mv) serosa negative potential difference. This PD is believed to be generated by NaCl accumulation in intercellular spaces and subsequent diffusion of the two ions in opposite directions due to permeability differences (110). Again, the increase in PD following cAMP in the rectal gland strongly argues against this as a possible secretory mechanism.

Electrogenic chloride secretion, in which a potential difference is generated, was first demonstrated in frog gastric mucosa by Hogben in 1955 (78). Since that time, the process has been described in such tissues as rabbit colon and ileum (61), frog cornea (190), and killifish operculum (40, 85), and several similarities have been recognized. A description of these tissues will illustrate that these similarities exist in the rectal gland as well.

The frog corneal epithelium is a highly resistant tissue that controls the hydration and opacity of the stroma beneath it by transporting ions from the aqueous to tear side (191). Most of this ionic current is due to chloride flux, which like in the rectal gland, is affected by ouabain, furosemide, cAMP, and sodium replacement on its aqueous side (24). Similarly, the chloride cells of the teleost operculum generate ionic current by chloride secretion from blood to seawater via a furosemide and ouabain sensitive process. Like the rectal gland, these cells have a very high concentration of Na^+-K^+ ATPase on the basolateral membrane (191).

Finally, ion transport in the colon is more complex because both absorption and secretion occur. The normal function of the intestine is electrogenic sodium absorption and electrically silent chloride absorption coupled with bicarbonate secretion. The two processes are unrelated, because the diuretic amiloride abolishes the sodium flux without altering the chloride flux (61). Cyclic AMP, which increases in response to prostaglandin or cholera toxin stimulation, increases short circuit current and conductance by reversing chloride flux to

a secretory direction, but sodium flux is not affected. This chloride secretion is inhibited by ouabain, furosemide, or sodium replacement in the serosal fluid (97). The intestine, therefore, has the potential for cAMP-induced chloride secretion superimposed upon a separate chloride-bicarbonate exchange, and the sodium pump is the means by which metabolic energy for chloride transport is harnessed (166).

In short, parallels between intestinal, corneal, opercular, and rectal gland chloride secretion include contraluminal ouabain and furosemide sensitivity, dependence on serosal sodium and chloride, stimulation by cAMP, and stimulation by calcium ionophores, which increase the cytosolic calcium concentraion. This latter effect has been described in rabbit ileum (9), colon (25), frog cornea (62), and was investigated in the present study. The secretory model that has been proposed by Frizzell et al (63) to account for these observations has basolateral NaCl transport as a central feature. A furosemide sensitive mechanism at the basolateral cell membrane mediates chloride entry against its electrochemical gradient by coupling it with the downhill entry of sodium. The favorable sodium gradient is maintained because sodium is extruded by basolateral ouabain sensitive Na^{+}—K^{+} ATPase, which actually transduces metabolic energy into the driving force for uphill chloride entry. Intracellular chloride then moves passively across the apical membrane into the lumen, accompanied by sodium (153). It has been suggested that secretagogues like cAMP increase the permeability of the

luminal membrane to this movement, and that the cells accumulate chloride continuously in preparation for secretagogue stimulation. In support of this, Duffy and Silva (64, unpublished) observed that the resting chloride concentration of the rectal gland cells was seven to nine times the passive predicted value, and that the onset of secretion was accompanied by a fall in cell chloride concentration (159). Other investigators have reported that in the insect salivary gland (6) and frog cornea (99), transepithelial resistance decreased and conductance increased during stimulation of secretion. While this presumably reflects the greater ionic flux during stimulation, it must be remembered that the transepithelial PD is a composite of the potential difference across basolateral, luminal, and intracellular membranes and may reflect increased ion passage through intercellular junctions, as well.

According to this model, chloride transport is not directly coupled to the consumption of energy and is instead "secondarily" active, energized by the basolateral Na^+-K^+ ATPase pump. However, further investigation is needed in several areas. First, brush border cotransport studies, like those of Eveloff and Kinne (50) should be expanded to include specific measurements of chloride flux. Second, bidirectional fluxes of sodium and chloride can be measured directly in flat mounts of rectal gland tissue, as pioneered by Forrest and Zadunaisky (personal communication). Third, intracellular chloride activities should be assessed during rest and secretion in other representative tissues. Finally, the mechanism of

stimulation of secretion by cAMP and other cellular mediators such as calcium should be defined. This topic will be addressed in the final section of this review.

Calcium and Secretion

Two of the three major steps in rectal gland secretion, exogenous hormonal stimulation and the ionic events of transport, are relatively well understood, but many questions remain about the intervening process by which intracellular mediators translate the hormonal message into cellular response. Three classes of "second messengers" have been recognized in biological systems thus far, cyclic nucleotides, calcium, and prostaglandins, and it is assumed that all three interact in many processes like contraction, secretion, and catabolism to provide precise stimulus-response coupling. There is abundant evidence that cAMP has a role in modulating rectal gland secretion, and because the rectal gland resembles other epithelia in which calcium sensitivity has been identified, one can predict a regulatory role for calcium in the rectal gland as well. In this section the evidence for calcium-mediated secretion by various epithelia will be presented and the possible steps for calcium intervention will be discussed.

Several aspects of cellular calcium metabolism, as outlined by Rasmussen (136), are pertinent to the regulatory role of intracellular calcium in the cell. Ionized calcium is unevenly distributed across the cell membrane, as the extracellular concentration is 10^{-3} M and the intracellular

concentration is 10^{-5} to 10^{-7} M. This asymmetric distribution is maintained by factors such as cell charge, pH, phosphate concentration and sodium distribution, and the great driving force for calcium entry is directly counteracted by calcium extrusion (either by calcium-sensitive ATPase or a calcium-sodium exchange mechanism), sequestration by organelles, and formation of exchangeable, nonreactive phosphate salts (which account for much of the total intracellular calcium concentration, 2 to 10 x 10^{-3} M) (137). However, during cell activation, ionized calcium may transiently increase two to five fold, then be quickly removed by the mechanisms outlined above, thus providing a sensitive regulatory control (137).

The source of calcium for this abrupt signal "pulse" may be the extracellular fluid, involving a rapid calcium influx perhaps through membrane calcium channels, or from intracellular stores. The latter is supported by the flux of $^{45}Ca^{+2}$ from insect salivary glands (131) and intestine (62) in response to a secretory agonist. In any case, if calcium is involved in regulation of secretion, increased secretory activity should correlate at least transiently with an increased cytosolic calcium level. Unfortunately, this cannot be documented directly because calcium electrodes for small cells are not generally available, and flourescent calcium indicators such as aequorin are difficult to quantify and may be cytotoxic (141). Measurement of $^{45}Ca^{+2}$ efflux in response to stimulation can be done, but may not reflect true changes in cytosolic calcium activity. Alternatively, changes in cell activity in

response to pharmacologically induced alterations in cell calcium concentrations can be studied easily by the use of calcium ionophores and calcium channel blockers.

Calcium ionophores are compounds that enhance the passage of calcium across cell membranes by forming a neutral lipid-soluble complex that is either itself a carrier or contributes to the formation of calcium channels (139). The most widely used ionophore is A23187, a product of Streptomyces chartreusiensis which combines with divalent metal ions such as calcium or magnesium but is most specific for calcium. There is considerable evidence that A23187 does increase transmembrane calcium passage (for example, sperm incubated in A23187 in high calcium medium showed greater $^{45}Ca^{+2}$ efflux than controls (1)), and that in the presence of extracellular calcium, A23187 will initiate known calcium-dependent events, such as skeletal muscle contraction, lymphocyte activation, platelet aggregation, and neutrophil chemotaxis (143). It is not clear, however, whether the ionophore effect is restricted to the plasma membrane or whether intracellular membranes are also involved, because in some tissues, such as smooth muscle and frog eggs, an ionophore response was seen in the absence of external calcium (143), and Reed and Lardy (139) reported ionophore induced calcium release from isolated mitochondria. Therefore if calcium is an important mediator of secretion in the rectal gland, addition of ionophore to the isolated perfused gland might be expected to raise cytosolic calcium and stimulate secretion.

Calcium ionophores have been used in many tissues in which a calcium sensitive process has been suspected. A23187 has been shown to enhance histamine release by mast cells (55), catecholamine release by cat adrenals (66), glucose production by isolated renal cortical tubules (116) and tear production by the rabbit lacrimal gland *in vivo* (128). A23187 mimicked cholinergic and adrenergic agonists in the rat parotid, stimulating ion and amylase secretion, respectively, but was ineffective when external calcium was chelated (23).

A23187 was also used by Prince and Berridge to investigate the role of calcium in the secretory process of the salivary gland of the blowfly *Calliphora erythrocephala*. This organ secretes an isosmotic fluid in response to two mediators, 5-HT and cAMP (3, 6, 131, 132). Electrophysiologic and ionic studies indicated that the cAMP effect, which hyperpolarizes the apical cell membrane and is believed to initiate cation transport, is actually a second messenger for part of the total 5-HT response, which also involves an earlier apical membrane depolarization associated with increased anion permeability (5). The possibility that this earlier "anion" response could be calcium mediated was suggested by the fact that 5-HT enhanced cellular calcium influx and that 5-HT depolarization was greatly reduced in calcium-free medium, while that cAMP hyperpolarization was only slightly affected (132). As expected, when A23187 was added in the presence of external calcium, the 5-HT response was duplicated (133).

The isolated frog cornea is interesting because its

response to A23187- enhanced active chloride secretion and reduced electrical resistance (27)- is much more marked than the responses seen in most other tissues studied. The minimum effective concentration for ionophore was 10^{-7} M in the cornea, whereas 10^{-6} M was needed in the insect salivary gland and 0.2×10^{-6} M was needed in the rabbit colon. Furthermore, there was a shorter lag time with A23187 than with other corneal secretagogues. These findings suggest that some usual physiologic steps were bypassed in ionophore-induced corneal chloride transport (25)

Finally, ionophore-induced secretion has been documented in the gut, both large and small intestine. In the rabbit ileum, which normally has an absorptive function, Bolton and Field (9) showed that the addition of A23187 to both mucosal and serosal bathing solutions caused a rapid increase in transepithelial PD which persisted for approximately twenty minutes. Chloride secretion increased due to an increase in serosal to mucosal chloride flux with a concomitant decrease in mucosal to serosal sodium and chloride flux. These effects were dose-dependent and were qualitatively identical to those produced by cAMP and theophylline. In contrast to theophylline stimulated secretion, however, intracellular cAMP levels were unchanged. The ionophore response was markedly diminished when calcium was removed from the serosal bathing medium (9). These findings are consistant with the hypothesis that intestinal secretion is a calcium mediated process.

Similarly, in the rat colon, Frizzell (60) showed that A23187 elicited secretion, increased short-circuit current, and

raised tissue conductance 40%. These effects were quantitatively similar to those caused by cAMP. Again, extracellular calcium was required and cellular cAMP did not change. However, response to cAMP was not affected by low calcium medium and, in fact, cAMP was associated with a reversible efflux of $^{45}Ca^{2+}$ from preincubated tissue. Thus, in addition to associating calcium and colonic secretion, this study suggested that cAMP may act in part by mobilizing intracellular calcium stores (60).

Calcium channel blockers include verapamil and D600 (methoxyverapamil), as well as a structurally different compound, nifedipine. These drugs exist as racemic mixtures as well as pure d and l isomer forms. In early studies with verapamil in mammalian cardiac preparations, the l isomer, and to a lesser extent, the racemic mixture (10^{-6} to 10^{-8} M) selectively antagonized the inward slow calcium current with minimal effects on the fast sodium current (143), so were considered specific calcium channel blockers. As such they have been found to have antiarrythmic, antianginal and vasodilatory properties *in vivo* (2). Alternatively, the d isomer had less negative inotropic effect on the heart than the l isomer (94) so was considered to have greater affinity for sodium channels (2). This difference in the activity of isomers of verapamil was extended to the gut by Donowitz, Asarkof and Pike (36), who found that d-verapamil failed to alter 5-HT induced NaCl flux in rabbit ileum, while l-verapamil inhibited 5-HT induced secretion. A similar disparity in isomer activity was observed in the present study as well, since l-D600 inhibited rectal gland

secretion while d-D600 actually enhanced it.

As suggested above, calcium channel blockers, like ionophores, have been widely used in the study of calcium dependent cellular processes. In theory, if a calcium channel blocker limits the availability of calcium to the cell, either by preventing extracellular influx or intracellular translocation (55), a cellular response dependent on the presence of increased calcium, such as secretion, might be blunted. For example, Malaisse et al (111) found that verapamil suppressed glucose-stimulated insulin release by pancreatic beta cells and decreased $^{45}Ca^{2+}$ uptake. Somers (165) reported that this verapamil effect was not seen in beta cells treated with theophylline, so either glucose-stimulated insulin release has a different mechanism in the presence of theophylline or theophylline permits the translocation of extracellular calcium and is therefore protective when external calcium is limited. In the pituitary, Eto (48) described an inhibition of potassium and ouabain stimulated ACTH, GH, and TSH release, thereby implicating calcium in that secretory process, and in the adrenal, D600 was shown to inhibit ACTH and potassium induced catecholamine secretion (129).

Although calcium blockers have been somewhat less extensively studied in the intestine, Hubel and Callanan (83) found that verapamil reduced the increment in serosal to mucosal chloride flux usually seen after electric field stimulation of rat ileum, but did not alter basal short circuit current. They did note that nifedipine had a basal effect, and attributed

this to its structural difference from verapamil. Finally, as previously mentioned, Donowitz et al (36) reported that racemic verapamil inhibited 5-HT induced increases in short circuit current and ion flux when applied to the serosa of rabbit ileum. No basal effects were reported.

The preceding pharmacologic studies all implicate calcium as a mediator of secretion, but the specific steps that are calcium sensitive have not been entirely defined. Since secretion in the frog cornea, salivary gland, or intestine is sensitive to both calcium and cAMP, it is possible that the two mediators are integrated, either sequentially or by a feedback loop, but they may have independent effects, as well. Starting peripherally, calcium may act directly on the cell membrane by altering the permselectivity of membrane channels, as in the insect salivary gland (6), by modifying receptor sensitivity to hormone stimulation (18), or by changing the size and permeability of intercellular junctions, as in the Chironomous salt gland (142). In such cases, hormonal or cAMP stimulation should be sensitive to changes in intracellular calcium.

Moving inward, calcium may affect membrane bound enzymes such as Na^+-K^+ ATPase (studied by Lelievre in plasmocytoma tissue culture (103)), or may affect those controlling cAMP metabolism: adenylate cyclase and phosphodiesterase. A calcium dependent adenylate cyclase has been found in rat cerebral cortex (15), and phosphodiesterase activation in bovine heart and rat brain (89) has been shown to require calcium as well as a heat

stable protein (calmodulin). This suggests that calcium may tightly control cAMP levels by activating enzymes for cAMP formation and breakdown (5). If calcium levels were insufficient, hormonal stimulation would not elicit a cAMP rise, but exogenous cAMP would effectively stimulate secretion. Calcium may also be necessary for the intracellular action of the cAMP-protein kinase cascade, which may phosphorylate proteins involved in permeability or metabolism. Here, if calcium was unavailable, neither hormone nor cAMP would be effective because the calcium requirement is distal to both in the metabolic chain.

Alternatively, because calcium ionophore elicited secretion without changing cAMP levels, perhaps in certain tissues calcium, rather than cAMP, is the final secretory mediator, and the cAMP cascade is actually a mechanism for rapidly and reversibly altering the cytosolic calcium concentration. As pointed out by Rasmussen (135), cAMP has been shown to mobilize intracellular calcium in toad bladder and liver, to bind to microsomes from adrenal cortex (146), and to enhance calcium turnover in isolated sarcoplasmic reticulum of dog heart (39). Methylxanthines such as theophylline also affect intracellular calcium flux in this way (147). Thus cAMP may be instrumental in causing the transient increase in calcium concentration associated with calcium dependent events, and because it pools calcium from intracellular stores, is effective even when external calcium is unavailable.

Calcium may also interact with other putative cell

messengers, such as cGMP and prostaglandins. Calcium is required for activity of phospholipase A2, which generates arachidonic acid precursors, and prostaglandins themselves may have ionophoretic properties or alter membrane permeability to calcium directly (144).

Admittedly, these theories are still speculative and the presumed multiple calcium sensitive sites cannot always be identified. Recently, a specific protein has been isolated and recognized as a primary intracellular calcium receptor and an essential component of calcium mediated enzymatic regulation. This protein, calmodulin (calcium dependent regulatory protein, calcium binding protein) was independently discovered as a calcium dependent activator of rat brain phosphodiesterase by Kakiuchi (89), Weiss (184), and Cheung (29). It was then associated with adenylate cyclase (14, 16, 178) and Ca^{2+}-Mg^{2+} dependent ATPase (10). Calmodulin has since been isolated from a variety of tissues including smooth muscle (68), liver, adrenal, lung, spleen, vas deferens, and heart (72), and has been characterized as a heat stable acidic protein with a molecular weight of 16,700. It is not tissue specific, and shares significant sequence homology with troponin (30)-like troponin, it contains four calcium binding sites (115). The protein undergoes a conformational change when calcium is bound, becoming more helical and compact (188). Only this conformation, achieved by the calcium-calmodulin complex, binds to the apoenzyme (adenylate cyclase, phosphodiesterase, etc.) to activate it (150). Thus, calmodulin captures the

cytosolic calcium "pulse" that results from some stimulatory event and transmits it to the susceptible enzyme system. Such systems now include phospholipase A2, myosin light chain kinase, phosphorylase kinase, NAD kinase, and calcium dependent protein kinase from brain (30), as well as those mentioned above.

Concomitant with the discovery of calmodulin's effect on enzymatic activity, other investigators found that phenothiazines, specifically trifluoroperazine, inhibited hormone sensitive adenylate cyclase (187), phosphodiesterase (177), and Na^+-K^+ ATPase (34). In 1976, Levin and Weiss (104) noted that to be inhibitory, this compound had to bind both an activator protein and calcium. The activator was subsequently recognized as calmodulin (105), and the inhibitory effect of trifluoroperazine was extrapolated to all the calmodulin sensitive enzymes. The direct interaction of calmodulin and trifluoroperazine on enzyme action was recently demonstrated by Levin and Weiss (107), who showed that rat erythrocyte ATPase could be activated by the addition of calcium-calmodulin and then inhibited by the phenothiazine.

The role of calmodulin activation in the control of intestinal secretion has been investigated by Ilundain and Naftalin (86), who showed that in the presence of trifluoroperazine, secretagogues such as cholera toxin and theophylline did not increase cAMP levels and did not enhance secretion as usual by decreasing mucosal to serosal chloride flux relative to serosal to mucosal flux. In addition, the binding of ^3H-trifluoroperazine increased markedly when secretory stimuli

raised the level of intracellular free calcium. Thus calmodulin and calcium are involved in intestinal secretion as well, perhaps at the level of adenylate cyclase.

In the present studies, we examined the role of calcium in the regulation of dogfish rectal gland fluid secretion by employing drugs that alter calcium transport and availability to the cell: calcium ionophore A23187; calcium channel blockers, racemic verapamil and both d and l isomers of D600; carbamylcholine, an intestinal secretagogue; and RMI 12330A, a new compound whose effects include inhibition of adenylate cyclase. The data presented are generally consistent with the association of calcium with secretion in this tissue, so hormone dependent rectal gland secretion may soon be added to the growing list of calcium and cAMP sensitive cellular processes.

METHODS

Dogfish of either sex were taken by hook and line from Frenchman's Bay, Maine, for study at the Mount Desert Island Biological Laboratory. Fish weighing between 3 to 6 kg were kept in marine livecars for up to three days, until killed by segmental transection of the spinal cord. The rectal gland was removed immediately through an abdominal incision, and its artery, vein, and secretory duct were cannulated within five minutes with PE 90 tubing. The gland was then placed in a plexiglass perfusion chamber cooled to 15°C with running seawater, and perfused by gravity flow from an oxygenated reservoir at a pressure of 3.5 to 4 mm Hg and an approximate flow rate of 4 to 9 ml/min. The shark Ringer perfusion solution, modified from Silva et al (159), contained the following (in mM): sodium 280; potassium 5.5; chloride 283; bicarbonate 8; magnesium 1.2; phosphate 1; sulfate 0.5; urea 350; glucose 5; and calcium either 2.5 or 0.5.

Samples of rectal gland fluid and venous effluent were collected in 10 minute periods throughout the experiments. The chloride concentration of each sample was determined by amperometric titration by a Buchler-Cotlove Chloridometer after dilution 1:4 with distilled water. After perfusion, all glands were weighed on an analytic balance. Chloride secretion was expressed as μEq/hr/g wet tissue weight.

Studies with Ionophore A23187

In this group of experiments, glands were perfused with shark Ringer's containing 2.5 mM Ca^{2+} for 30 to 40 minutes to document basal secretion (period 1), then ionophore A23187 (Eli Lilly, Inc.) prepared as a stock solution of 5×10^{-3} M in ethanol, was added to the perfusate to a final concentration of 10^{-5} to 10^{-6} M. Continuous stirring of the perfusate with a magnetic stirrer was necessary to prevent precipitation of ionophore. Following the basal period, glands were perfused with ionophore for 60 minutes (period 2); finally the perfusion medium was changed to 0.25 mM theophylline (Merck) and 0.05 mM dibutyryl cAMP (Calbiochem) in the absence of ionophore to determine maximum chloride secretion (period 3). Rectal gland fluid and venous effluent were collected and analyzed as outlined previously.

Studies with Carbachol

Carbachol, a cholinergic ester and intestinal secretagogue that mediates secretion in a calcium dependent manner, independent of cAMP, was investigated in five glands. After basal perfusion in 2.5 mM Ca^{2+} Ringer's for 30 minutes (period 1), carbachol 2×10^{-3} M (Sigma) was administered for 50 minutes (period 2), followed by 30 minutes of theophylline 0.25 mM and dibutyryl cAMP 0.05 mM (period 3).

Studies with RMI 12330A

This specific inhibitor of adenylate cyclase and iono-

phore effects in certain tissues was studied in four glands. Glands were given theophylline, 0.25 mM, and dibutyryl cAMP, 0.05 mM, in 2.5 mM Ca^{2+} Ringer's for 20 to 40 minutes (period 1). RMI, 5 x 10^{-4} M (Merrel National Laboratories, gift of Dr. Norbert Wiech) was then added for 30 minutes (period 2). Finally, glands were perfused with theophylline-cAMP Ringer's in the absence of RMI (period 3).

Studies with Calcium-Free Ringer's Solution

The effect of the absence of calcium from the shark Ringer's was studied in six glands. In four experiments, glands were perfused in a calcium-free Ringer's solution throughout the experiment, and measurements were made at baseline for 30 minutes (period 1) and during theophylline stimulation (0.25 mM) for 60 minutes (period 2). The remaining two glands received theophylline 0.25 mM and dibutyryl cAMP 0.05 mM throughout, and were perfused first in 2.5 mM Ca^{2+} Ringer's for 40 minutes (period 1), then with a Ca^{2+}, Mg^{2+} free Ringer's for 40 minutes (period 2), and finally were returned to 2.5 mM Ca^{2+} Ringer's for the last 30 minutes (period 3). Rectal gland fluid samples were collected and studied as previously described.

Studies with Calcium Channel Blockers: Verapamil and D600

The effect of calcium channel blockers on adenosine stimulated rectal gland secretion was studied with two compounds, verapamil and D600 (methoxyverapamil) (a gift of Dr. David Goodman, originally from Knoll Chemische Fabriken Ludwigschafen

am Rhein). We obtained verapamil in racemic form and D600 as both the l-isomer (T200 HCl, L-Isoptin) and the d-isomer (T201 HCl, D-Isoptin). These drugs were stored in dessicated powder form at 0 C when not in use. Studies were performed at two concentrations of calcium in the perfusate, 0.5 mM and 2.5 mM, but the calcium concentration was constant within individual experiments.

In experiments with racemic verapamil and l-D600, compounds believed to block calcium channels specifically (143), glands were perfused for 30 minutes of baseline measurements with Ringer's containing either 2.5 mM or 0.5 mM calcium (period 1) and were then stimulated with adenosine (Sigma) 10^{-4} M in Ringer's for 30 minutes (period 2). Verapamil or D600 was then added to the adenosine and perfusate to a final concentration of 10^{-4} M and was administered for another 30 minutes (period 3). The perfusate was switched back to shark Ringer's containing adenosine alone for the final 30 minutes of the experiment (period 4). Control glands were treated identically with 30 minutes of basal perfusion followed by 90 minutes of adenosine stimulation except that calcium channel blockers were not added. Samples were analyzed as described above.

In four glands, verapamil was given in 2.5 mM calcium Ringer's after 30 minutes of basal perfusion with no prior adenosine stimulation (period 1). Theophylline, 0.25 mM, was then added to the verapamil-containing Ringer's for the next 30 minutes (period 2), followed by 30 minutes of perfusion

with theophylline alone and Ringer's (period 3). Controls for these experiments received theophylline for 90 minutes after 30 minutes of basal flow.

Three glands received sodium-specific d-D600, the isomer that is believed to have a greater affinity for sodium channels than calcium channels (2) and has been shown to be ineffective in eliciting intestinal secretion (36). The time course of the experimental maneuvers, which included basal flow, adenosine stimulation, and D600 administration, was identical to the verapamil-D600 experiments outlined above.

Data and Statistics

Results are expressed as rectal gland fluid chloride secretion in uEq, Cl/hr/g. In each group of experiments, the secretion rates for different glands were averaged both within each 10 minute collection interval and within each 30 minute experimental period. These mean values were used to compare changes in secretory rate between experimental periods and between salient individual collection intervals. Statistical analysis of different experimental periods was performed with the non-paired Student's T test, while the paired Student's T test was used in analyzing the 10 minute collection periods for individual glands (33).

RESULTS

In this section, the results of 74 perfusion experiments are presented. No data were excluded from this analysis. While standardization of results to control values (% control) would have diminished the influence of biological variation, we elected to give all data as absolute values without exception.

Perfusion in the Basal State

Our perfusions in the basal state were comparable to those described earlier by Silva et al (159) and Stoff et al (169). The initial secretory rate for 17 glands after 10 minutes of perfusion with 2.5 mM Ca^{2+} Ringer's was 221 ± 45 µEq Cl/hr/g; values ranged from 48 to 669 µEq Cl/hr/g. The chloride concentration of this rectal gland fluid was 442 ± 16 mM. The secretion rate always decreased after the first 10 minute period and fell continuously during unstimulated perfusion. By 30 to 40 minutes, secretion reached a baseline rate of 60 ± 12 µEq Cl/hr/g (ranging from 14 to 153 µEq Cl/hr/g) regardless of the initial rate of secretion, so that experimental maneuvers begun at this time started with comparable glands. Chloride concentration of this basal fluid, 430 ± 15 mM, was not markedly different from the initial chloride content. Venous effluent rates varied with arterial flow and ranged between 2 and 4 ml/min. Venous flow rates did not change in a consistent way during rectal gland stimulation. Chloride concentration of the venous effluent fluid was between

270 and 290 mM (perfusate [Cl^-], 280 mM).

Studies with Ionophore A23187

To determine the purity of the ionophore A23187 used in these experiments, an ultraviolet spectrophotometric scan was performed on A23187 dissolved in ethanol between the wavelengths of 420 and 180 nm. Five absorption peaks were recorded, occurring at 374 nm, 290 and 294 nm (a doublet), 225 nm and 205 nm. This pattern was reproducible on two occasions and is consistent with the known spectrum for this compound.

Some difficulty was encountered initially with precipitation of the ionophore when ionophore-ethanol stock solution was added to the perfusate. Rapid, continuous stirring of the perfusate minimized this problem, but on occasion a faint opalescence was still noted after the ionophore was added.

As shown in table 1, the mean basal chloride secretion rate (period 1) for 9 glands used in the ionophore studies was 76 $\pm$ 17 µEq Cl/hr/g, a value comparable to the mean basal value for all glands studied. The response to the ionophore (period 2) was not dramatic, but after 20 to 30 minutes of ionophore administration the secretion rate rose 2.5 fold to 191 $\pm$ 56 µEq Cl/hr/g, a value significant at $p < 0.02$ compared with the mean basal value. Chloride concentration of the rectal gland fluid did not change between these two periods. Chloride secretion continued to rise with ionophore (275 $\pm$ 96 µEq Cl/hr/g at 80 to 90 minutes) but the chloride concentration fell

markedly from 416 to 316 mM ($p<0.02$). The subsequent response to theophylline and cAMP (period 3) demonstrated a 3.5 fold increase in chloride secretion (13 fold compared with baseline) and a tendency for chloride concentration to rise, as compared with the end ionophore response (316 $\pm$ 21 to 388 $\pm$ 14 mM). These studies indicated a modest but significant effect of A23187 to stimulate chloride secretion.

In seven glands, the calcium concentration of the perfusate was altered during ionophore administration to determine the relationship between external calcium availability and ionophore response. A minimum of 0.5 mM calcium was always used in the perfusate because total absence of calcium and magnesium was associated with continuously falling rectal gland fluid chloride concentration (equal to the perfusate value of 280 mM), suggesting that permselectivity was decreased or epithelial integrity was disrupted.

In three of these experiments, glands were perfused with 10 mM calcium for 30 minutes before ionophore (10^{-5} M) was added. The secretory response to ionophore, 120 $\pm$ 19 µEq Cl/hr/g, was no greater than that of glands that had not been previously calcium loaded. In an additional four experiments, calcium was raised from 0.5 to 5 mM during the course of ionophore administration with no increase in chloride secretion- in fact, chloride secretion and concentration dropped after this maneuver. Thus neither preloading with calcium nor increasing perfusate calcium enhanced the rectal gland response to ionophore.

Studies with Carbachol

The intestinal secretagogue carbachol (2×10^{-3} M) caused an almost three fold stimulation of chloride secretion at 40 minutes, with a gradual decline in secretion by the end of the 80 minute carbachol period (table 2). Both peak and end carbachol values were statistically significant ($p<0.02$ and $p<0.001$, respectively) as compared with the preceding 30 minute basal perfusion (period 1). Chloride concentration of the rectal gland fluid was constant throughout this time. Subsequent stimulation of the carbachol-treated glands with theophylline (0.25 mM) and dibutyryl cAMP (0.05 mM) produced a vigorous secretory response ($p<0.02$ compared with the peak carbachol response) with some increase in chloride concentration as well (430 ± 24 to 462 ± 4 mM). These responses were similar in magnitude to the ionophore response, although the ionophore induced secretory rate did not fall with time. This observation is of interest because carbachol, like ionophore, may operate by a calcium dependent mechanism.

Studies with RMI 12330A

As table 3 indicates, both chloride secretion and chloride concentration were reduced markedly by the addition of 5×10^{-4} M RMI 12330A to glands already stimulated to secrete by theophylline (o.25 mM) and dibutyryl cAMP (0.05 mM). This change was significant at $p<0.001$. Thus RMI 12330A, a compound whose effects include adenylate cyclase inhibition, is a potent inhibitor of rectal gland secretion. This implicates adenylate

cyclase, as well as other calcium dependent RMI sensitive steps, in the secretory process.

Studies with Calcium-Free Ringer's Solution

The effect of perfusion with calcium-free Ringer's in the rectal gland resembled that seen in calcium-free studies in other tissues: there was a loss of selective permeability and epithelial integrity (74, 154). When calcium-free medium was used throughout four experiments, basal secretion rates were low (33 ± 7 μEq Cl/hr/g), theophylline stimulation was minimal (65 ± 38 μEq Cl/hr/g), and the chloride concentration of the rectal gland fluid produced was equal to perfusate [Cl] (290 mM). The osmolality of this fluid would have been expected to be high, however, due to the presumed leak of urea from the damaged gland. Similarly, in two other experiments, when calcium was removed during theophylline stimulation, chloride secretion dropped from 1193 ± 59 μEq Cl/hr/g to 523 μEq Cl/hr/g with a decrease in chloride concentration from 473 ± 8 to 399 ± 3 mM. Restoration of 2.5 mM calcium caused a slight rise in chloride secretion (714 μEq Cl/hr/g) with further fall in chloride concentration (368 mM). Thus, total removal of calcium was clearly detrimental to gland integrity and function. Although the minimum calcium concentration for gland viability was not specifically determined, the value of 0.05 mM (one-fifth of the usual Ringer's calcium concentration) was subsequently chosen for incorporation into low calcium perfusate. This low calcium Ringer's was used extensively in the calcium channel blocker experiments which follow.

Studies with Calcium Channel Blockers: Verapamil and D600

Our work with calcium channel blockers included several subsets of experiments: studies with racemic verapamil, calcium specific l-D600, and sodium spcific d-D600. The studies with racemic verapamil and calcium specific l-D600 were done at two calcium concentrations, 2.5 mM (normal shark Ringer's) and 0.5 mM (low calcium shark Ringer's); calcium concentration was kept constant within an individual experiment, however. Except where indicated otherwise, all glands were stimulated with adenosine. Control experiments using adenosine alone were performed at both calcium concentrations as well.

Adenosine Control Experiments

As shown in table 4, the mean rate of chloride secretion in adenosine stimulated glands perfused with 0.5 mM calcium Ringer's was 500 $\pm$ 57 µEq Cl/hr/g, a ten-fold increase over the mean basal secretory rate, and chloride concentration of the rectal gland fluid rose from 385 $\pm$ 37 mM to 471 $\pm$ 3 mM. These values were maintained throughout the stimulation period. In adenosine stimulated glands perfused with 2.5 mM calcium Ringer's, the basal secretory rate was somewhat higher than in the low calcium glands (109 $\pm$ 12 vs 46 $\pm$ 9 µEq Cl/hr/g), but the response to adenosine was comparable. Since secretion rates were fairly constant throughout adenosine administration, when a significant drop in stimulated secretion was seen in the presence of calcium channel blockers, the decrease was attributed to the inhibitory actions of the drug.

Experiments with Verapamil

Table 5 shows the effect of racemic verapamil on adenosine stimulated glands. In contrast to the adenosine control experiments discussed above, in this group, glands perfused with low calcium Ringer's had a much greater secretory response to adenosine than glands perfused with normal calcium Ringer's. In the low calcium group, verapamil caused an abrupt drop in secretion by more than one half (58%) within 10 minutes ($p<0.025$ compared with the last collection interval for adenosine). Because the secretion rate gradually increased over the next 20 minutes, the difference in mean chloride secretion between period 2 (adenosine) and period 3 (adenosine and verapamil) was not statistically significant. Nevertheless, the clearly inhibitory trend of verapamil can still be appreciated in figure 1. Chloride concentration remained constant throughout the experiments and no secretory rebound was seen in period 4 when verapamil was discontinued.

Similarly, when verapamil was added to adenosine stimulated glands in 2.5 mM calcium Riger's (table 5), chloride secretion fell by 37% during the next 10 minutes ($p<0.01$ compared to the previous two collection intervals). Secretion rose again gradually, however, so that the mean 30 minute decrease in secretion (period 3) was not statistically different from the preceding stimulation period (period 2). Subsequent stimulation with adenosine alone (period 4) was also not statistically significant.

In the four glands that received verapamil and 2.5 mM

calcium in the unstimulated state, mean basal secretion was unusually and unexplainably high (324 ± 32 μEq Cl/hr/g). After addition of verapamil, secretion dropped by more than two thirds (71%) to 94 ± 20 μEq Cl/hr/g ($p<0.01$). This percentage decrease in secretion was actually greater than that seen in either group of adenosine stimulated glands treated with verapamil. However, no comparable control glands were perfused in the basal state for 60 minutes to see whether a similar decrease also occurred in the absence of verapamil. The addition of theophylline (0.25 mM) to the verapamil inhibited glands resulted in significantly stimulated secretion (347 ± 68 μEq Cl/hr/g, p 0.02), which increased further (595 ± 176 μEq Cl/hr/g) when verapamil was discontinued. Thus verapamil appears to partially and reversibly block the stimulatory effect of theophylline.

Experiments with l-D600

The experiments with l-D600 (calcium specific) in 0.5 mM calcium Finger's provided our most dramatic evidence of inhibition by calcium channel blockers. The chloride secretion rate and venous effluent flow rate for a representative experiment in this series are plotted in figure 2. The venous effluent flow remained fairly constant throughout adenosine stimulation and D600 inhibition, suggesting that vasoconstriction was not responsible for the observed changes in secretory activity. As indicated by table 6 and figure 3, l-D600 caused a significant reduction in adenosine stimulated secretion during both the first 10 minutes (58%, $p<0.0125$)

and the entire 30 minutes (37%, $p<0.01$) of l-D600 administration. Secretion recovered after l-D600 had been discontinued for 20 minutes, and this increase was statistically significant ($p<0.05$) compared with the last 10 minutes of l-D600 inhibition.

Similarly, glands treated with l-D600 in 2.5 mM calcium Ringer's (table 6) showed a significant inhibition of adenosine stimulated secretion during the first 10 minutes of l-D600 ($p<0.025$), but the percentage decrease (46%) was less than in the low calcium group, suggesting that as expected, the lower perfusate calcium enhanced the inhibitory effect of the calcium channel blocker. Recovery of secretion with discontinuation of l-D600 in this 2.5 mM calcium group, however, was not statistically significant.

A comparison of the mean adenosine-stimulated secretion rates (period 2 in all calcium blocker experiments) at the two concentrations of perfusate calcium illustrated the marked variation in secretory response that exists among different rectal glands. In the adenosine control experiments, glands perfused with 2.5 mM calcium had generally higher adenosine stimulated secretion rates than glands perfused with 0.5 mM calcium (table 4). However, the opposite was true for the verapamil and D600 experiments: adenosine stimulation was greater with 0.5 mM calcium than with 2.5 mM calcium (tables 5 and 6). This cannot be explained by observer bias alone, because glands were assigned to experimental or control groups before the perfusion was begun, and it is not caused by the calcium blocking drugs because they were not administered until later.

The most likely explanation therefore is the normal biologic variation of rectal gland activity.

Experiments with d-D600

In the group of glands treated with sodium specific d-D600 in 0.5 mM calcium (table 7), the adenosine stimulated secretion rate dropped abruptly during the first 10 minutes of D600 administration (although this difference was not statistically significant). However, as d-D600 was continued, the secretion rate recovered and actually surpassed the maximum rate attained with adenosine during the previous period. Chloride concentration remained constant. When d-D600 was discontinued in the presence of adenosine, the secretory rate decreased significantly ($p<0.05$). These results suggest that the sodium specific d-D600 isomer is stimulatory, rather than inhibitory, to rectal gland secretion, and may be synergistic with adenosine. In addition, these experiments can be considered as controls for the l-D600 experiments described above. A striking but transient decrease in secretion was seen immediately after either isomer was given, which raises the question of whether the early secretory inhibition observed with these drugs was not due to a nonspecific calcium-independent event.

In summary, we observed that ionophore A23187 stimulated rectal gland secretion presumably by raising intracellular calcium, while calcium channel blockers inhibited rectal gland secretion, presumably by impeding an increase in intracellular calcium. (l-D600 was the most potent and specific antagonist

in this regard.) We also documented that rectal gland secretion was sensitive to carbachol and RMI 12330A, drugs which may alter intracellular calcium activity as well. These results suggest that chloride secretion by the rectal gland of Squalus acanthias occurs by a calcium dependent mechanism.

DISCUSSION

The In Vitro Perfused Rectal Gland: General Considerations and Limitations

The isolated perfused rectal gland is an easily prepared and maintained *in vitro* experimental model for chloride secreting epithelia but there are some problems inherent in the system that should be recognized. First, several technical difficulties exist that could contribute important but unquantified variables. These include the sensitivity of secretory flow to the hydrostatic pressure of perfusion (as described by Hayslett (75)), which can vary between different perfusions and during a single perfusion as perfusate solutions, bottles and tubing are switched, the occasional formation of mucous plugs in the secretory ducts, and the possible introduction of air microemboli into the gland when perfusion solutions are changed or tubing stopcocks are manipulated. In general, these variables can be controlled by particular attention to the constancy of arterial and venous flow rates, careful removal of all air bubbles from the perfusion system before starting the arterial flow, and gentle suction applied to the secretory duct catheter when sudden flow stoppage occurs.

A second problem that is less easy to control is the great biological variation in endogenous rectal gland activity at the time of removal from the shark. Since most sharks had been fasted for at least 24 hours before use, their rectal

gland secretory rates might be expected to be comparable at sacrifice, but in actuality they varied from below mean basal (14 μEq/hr/g) to moderately stimulated (320 μEq/hr/g). This variation could have been entirely hormonal in origin (i.e., the shark was killed shortly after a VIP or other hormonal stimulus), or could have been caused by a traumatic excision or a delay in initiating perfusion. To minimize the potential for anoxic damage, perfusion was begun as quickly as possible, usually within 5 minutes. Nevertheless, there remained substantial variation not only in basal secretion but in response to stimulatory and inhibitory agents as well. Similar variation has been noted by others (159, 169) but has not been addressed directly. This unavoidable biologic "noise" may have had two consequences in this in vitro study. First, it may have been a factor in the differences in secretion rates between different groups of rectal glands, particularly with the small sample size of certain experiments. Second, it may have contributed to the rather large variation (standard error of the mean) of individual collection periods. Because of this variability, means were first calculated for each 10 minute secretory interval and were subsequently averaged to obtain the mean for the entire experimental period (usually 30 minutes).

The rate of chloride secretion shown by our 17 unstimulated glands correlated in magnitude and duration with that reported by Silva et al in 1977 (159). The chloride concentration of the secreted fluid was also comparable, indicating that

our perfusion technique was accurate, reproducible, and satisfactory for carrying out the intended experiments.

Limitations in the Study of Calcium Dependent Callular Processes

Before addressing the specific experiments in this study, it is first appropriate to consider some of the limitations encountered in investigating calcium mediated processes. First and foremost is the present limited ability to measure changes in intracellular calcium activity. That information would not only provide direct evidence for the involvement of calcium in physiologic responses, but could also lend credibility to pharmacological studies such as performed here, in which the intracellular calcium concentration or activity is presumed to change but is not directly measured. A second limitation is the potentially great variation between individual glands in response to the calcium-altering drugs, particularly in light of the biological "noise" already present. Third, both calcium ionophores and channel blockers work by altering calcium transport across the plasma (and probably intracellular) membranes, and their efficacy could depend on the extent of initial calcium stores in the cytosol or organelles. This quantity of stored calcium may vary at different levels of endogenous activity, thus affecting the glands' apparent secretory response to these agents.

An additional difficulty encountered in studying calcium mediated processes is the inability to correlate changes in the extracellular calcium concentration with precise changes

in intracellular physiologic activity. This ambiguity exists because the extracellular calcium, perhaps through its equilibrium with intracellular calcium, can affect a number of cell processes and structures. For example, Hays et al (74) reported that when calcium was removed from the bathing medium of toad bladders, there was an increase in permeability to inulin, chloride, water and thiourea along with a decrease in resistance and short circuit current- this was believed to be the result of disruption of intercellular junctional complexes and detatchment of epithelial cells. Similar observations were made by Sedar and Forte in gastric epithelium (154).

Effect of Calcium-Free Perfusions on Basal and Stimulated Secretion

Our studies with calcium-free perfusate agreed with the above observations in that when calcium was omitted, normal rectal gland function was compromised. When calcium was omitted from the perfusate entirely, secretion was minimal and chloride concentration was very low. Presumably, calcium was necessary for the maintainence of intercellular tight junctions, and when these junctions were disrupted, the normal permeability restriction to urea, glucose, and water was lost. This increased permeability would explain why the chloride concentration fell to perfusate levels, but might also be expected to result in increased fluid passage rather than decreased fluid flow. The observed decrease in fluid production might have been a consequence of calcium depletion on other cell processes or structures (enzymes, microtubules, microfilaments, etc.).

On the other hand, the effect of calcium omission after rectal gland stimulation had been established is less clear cut. The secretory rate and chloride concentration fell considerably, but not to the low level seen in the first group of unstimulated glands. A relatively high rate of secretion (953 ± 48 µEq Cl/hr/g) may have persisted during the first 10 minutes of calcium depletion because the gland was already stimulated and had sufficient stored calcium to meet its secretory needs. In the next 30 minutes, as secretion dropped to 500 µEq Cl/hr/g, cellular damage was probably occurring. Had calcium-free perfusion continued, secretion may have continued to fall, but instead, calcium was replaced and theophylline-dibutyryl cAMP was added. Secretion gradually returned to the initial stimulated values, but chloride concentration did not. This suggests that although flow rate was recoverable, changes in transepithelial transport of chloride were not reversible. Thus, investigating the role of calcium in cell function by removing external calcium is not possible in the rectal gland and other tissues (74, 154) because of the apparent ubiquitous role of calcium in cell function.

Experiments with Ionophore A23187 and Carbachol

The experiments with calcium ionophore provided the first indication that chloride secretion in the rectal gland is calcium dependent. This conclusion was based on the observation that ionophore increased secretory activity and

presumably cytosolic calcium at the same time. However, in contrast to other chloride secreting epithelia, such as the cornea (27), the increment in secretion was modest. A number of reasons for this modest response to stimulation by ionophore can be postulated. First, the marginal solubility of the ionophore in perfusate may have resulted in blockage of the gland's microcirculation and poor distribution of the drug. Second, the increase in cytosolic calcium activity induced by ionophore may have been insufficient to cause maximum secretion. Third, the ionophore may have had other unrecognized pharmacologic effects. Fourth and most intriguing, perhaps both calcium and cyclic AMP act together to stimulate secretion: when ionophore was given alone, secretion would have been limited by the endogenous level of cAMP in the cells, but when theophylline and cAMP were also added, secretion was able to increase markedly. The fact that our glands ultimately responded to theophylline, dibutyryl cAMP and ionophore in a range comparable to that seen by other investigators (159, 169) using theophylline and cAMP alone has several implications. Most important, it indicates that ionophore did not seriously injure the tissue. It also suggests that if both calcium and cAMP are required for secretion, calcium is not usually the limiting factor. Our theophylline stimulated rates were actually slightly lower than those reported by Stoff et al (169) (1010 µEq Cl/hr/g vs 1245-1879 µEq Cl/hr/g), which may be a result of the longer duration of perfusion in our study.

It is interesting that increasing perfusate calcium

in the presence of ionophore did not enhance rectal gland secretion, and that the ionophore seemed to be as effective in 0.5 mM calcium as in 2.5 mM calcium. This indicates that either the ionophore or the calcium dependent secretory steps may be maximally active at or below the physiologic concentration of extracellular calcium (2.5 mM) (another mediator, such as cAMP, may be the rate limiting factor) and suggests that the optimal calcium concentrations for rectal gland activity are not fully appreciated. The significance of experiments done in varying calcium concentrations, including the calcium channel blocker studies to be discussed subsequently, should be interpreted in this light.

In short, the ionophore studies suggest that in the rectal gland, like other tissues in which A23187 elicits secretion, calcium is involved in the secretory process, perhaps in cooperation with cAMP, a well-known stimulant of rectal gland secretion. Further support for this hypothesis is generated by data with carbachol and calcium channel blockers, verapamil and D600.

Carbamylcholine is a cholinergic derivative shown to be an intestinal secretagogue that does not increase cellular cAMP (169). Our interest in this compound stemmed mainly from consideration of the rectal gland as an intestinal appendage which may respond to similar secretory stimuli as the gut, but carbachol has been implicated in alteration of calcium dependent processes, as well. For example, Putney and Parod (134) demonstrated that the action of carbachol to increase sodium uptake and potassium output from rat parotid involved increasing calcium

permeability and influx. Kondo and Schulz (100) showed that in isolated exocrine pancreas cell, both carbachol and A23187 stimulated $^{45}Ca^{2+}$ uptake, implying that both the ionophore and the cholinergic secretagogue enhance calcium entry. This effect was paritally inhibited by the calcium channel blocker D600. Similarly, Bolton and Field (9) reported that in rabbit intestine, the change in PD seen with both ionophore and carbachol was reduced in the absence of external calcium, but that the action of carbachol alone was restored by the addition of strontium to the external medium. Finally, Jafferji and Michell (87) suggested that carbachol stimulation of intestinal secretion may be mediated by increasing cell permeability to calcium. It is interesting, therefore, that in our studies both carbachol and A23187 caused a modest but significant increase in chloride secretion that was transient (as seen by decreasing chloride concentration in the ionophore experiments and a falling flow rate with carbachol). Thus, it is possible that both carbachol and ionophore stimulate rectal gland secretion by increasing cellular calcium uptake, and these results may be considered as an additional indication that calcium mediation is involved.

Studies with Calcium Channel Blockers: Verapamil and D600

Because our ionophore responses were relatively small compared with those seen in other tissues, the calcium blocker experiments were designed to optimize the expected secretory inhibition in two ways. First, some of the experiments were conducted with low (0.5 mM) calcium perfusate instead of the

2.5 mM calcium usually used. If calcium channel blockers work by decreasing calcium entry, this should make calcium even less available to the cells and further decrease calcium dependent secretion. Second, the channel blockers were administered to highly stimulated glands, under the assumption that a drop in secretion would be more apparent in a maximally secreting gland, provided that the secretory stimulus occurred before the calcium-dependent step(s) being studied. A similar assumption was made by Donowitz et al (36) in studying verapamil inhibition of intestinal responses to serotonin, and by Hubel and Callanan (83) in showing that verapamil reduced chloride flux in the gut after electric field stimulation, but did not affect baseline short circuit current. Therefore, because adenosine had been found to have an intense stimulatory effect in the rectal gland (43), adenosine was chosen as the initial stimulatory agent in these experiments. Preliminary research now indicates that the rectal gland has specific membrane receptors for adenosine (59). The results and implications of these two maneuvers, adenosine stimulation and relative calcium deprivation, will be discussed in greater detail.

The relationship between adenosine and cyclic nucleotides was first studied by Sattin and Rall in 1970 (148), and later by Huang et al (82) and Schultz and Daly (151) in guinea pig cerebral cortical slices. These investigators discovered that adenosine increased adenylate cyclase activity and cAMP production, and that theophylline blocked this effect. Substitutions on the purine ring of adenosine had no effect, but modification of the ribose moiety greatly reduced the stimulation

produced. Subsequently, adenosine was found to stimulate adenylate cyclase in mouse neuroblastoma cells (8), isolated bone cells (127), and human astrocytoma cells (32). This latter group also studied papaverine, a specific phosphodiesterase inhibitor, and found that it lacked the adenosine antagonism seen with theophylline; they therefore concluded that adenosine and theophylline share an active site, one which is distinct from the phosphodiesterase inhibitory site. These effects were also observed in skin (85), isolated fibroblasts (171), platelets (73) and isolated frog cornea (27).

At the same time, other investigators were finding that adenosine had an inhibitory effect on adenylate cyclase in certain cell systems. Moriwaki and Foa noted this in rat liver (117) and reported that the adenosine inhibited activity of adenylate cyclase could be reversed by stimulation with flouride. Adenosine inhibition was found as well in fat cell ghosts (51), smooth muscle (119), and intestine (192) (specifically, inhibition of cholera toxin-sensitive adenylate cyclase).

In short, these numerous studies suggested that adenosine may modify adenylate cyclase at a membrane receptor distinct from the catalytic site- a separate regulatory subunit- to produce noncompetitive and reversible inhibition or stimulation (119). Because the effects of adenosine were often similar to those of catecholamines, Tolkovsky and Levitzki (176) concluded that both agents have distinct binding sites but are coupled to adenylate cyclase by a single GTP regulatory unit (108). Recent studies with purine and ribose modified adenosine analogues led Londos and Wolff (109, 189) to conclude that

the adenosine receptor itself is composed of two subunits, a "ribose sensitive site" which requires an unsubstituted ribose ring and causes adenylate cyclase stimulation, and a "purine sensitive site" which requires an intact purine ring and causes adenylate cyclase inhibition. This theory may explain the varying and biphasic adenosine effects of different tissues.

Therefore, adenosine's vigorous stimulation of the rectal gland was similar to that seen in other organs and appeared to be originating at the cell membrane at a site distinct from the usual hormone sensitive adenylate cyclase receptor. As such it was believed to influence an early step in secretion and permit other agents, such as verapamil, to act further along in the secretory sequence. Since it was not our original purpose during these experiments to investigate the mechanisms of adenosine stimulation on the rectal gland, further studies with theophylline inhibition or adenosine analogues were not performed, but these questions have since been addressed. Forrest et al (59) have recently characterized a ribose specific receptor in the rectal gland similar to that described above. It is particularly interesting that theophylline, a compound best known for its phosphodiesterase inhibition, may exert a major effect as well at the cell membrane through modification of adenylate cyclase, and future studies should be designed to investigate this possibility.

The most marked inhibition seen with calcium channel blockers occurred in 0.5 mM calcium Ringer's with l-D600, in which secretion dropped significantly during the entire 30 minutes of D600 administration and later recovered. Both

racemic verapamil and l-D600 in 2.5 mM calcium Ringer's caused a significant drop in secretion during the first 10 minutes of administration, but not over the entire 30 minute inhibitory period. These results have two implications. First, because of its greater activity and reversibility, l-D600 seems to be the more specific of the two calcium channel blockers. This may be because the D600 compound used was the pure calcium specific l-isomer while the verapamil was a racemic mixture of both calcium and sodium specific forms which probably decreased its blocking activity somewhat. Alternatively, the additional methoxy group of D600 may make the molecule a better "fit" for the calcium channel. However, when Kaumann and Uchitel (95) compared the action of D600 and verapamil on skeletal muscle fibers from frog, they found no remarkable difference.

The second implication is that as expected, reducing extracellular calcium enhanced the inhibitory effect of the calcium channel blockers. However, it is not clear why secretion rates tended to be greater overall in 0.5 mM calcium Ringer's than in 2.5 mM calcium Ringer's (with the exception of the adenosine control groups). Perhaps particularly vigorous glands were selected by chance for the low calcium groups. If variation between sharks could be better controlled, it might be interesting to determine the dose-response relationships of rectal gland secretion, extracellular calcium, and calcium channel blockers in future studies.

The ramifications of biological variation may apply as

well to the group of four glands that received verapamil in the basal state. In these glands, basal secretion dropped significantly in response to racemic verapamil and recovered with the addition of theophylline. However, the mean unstimulated basal secretion rate for this group was unusually high (326 $\pm$ 32 uEq Cl/hr/g). Therefore these glands seemed to be endogenously stimulated and perhaps were not physiologically very different from the adenosine stimulated glands used in the other studies.

The experiments with sodium-specific D600 were intended not only to compare the secretory response of the two D600 isomers but also to determine whether D600 had any pharmacologic effects separate from its antagonism of calcium. As previously explained, the d-D600 isomer is believed to have preference for fast sodium channels, at least in heart muscle (94). In the gut, Donowitz et al (36) found that sodium-specific verapamil had no effect on serotonin induced changes in ion flux. However, we found sodium-specific D600 to be stimulatory and even synergistic with adenosine in the rectal gland. The significance of this was not understood, because blocking sodium channels would favor the maintainence of the basolateral sodium gradient on which active chloride transport depends, but would inhibit the coupled basolateral NaCl entry that brings chloride into the tubule cell.

Because the initial response to both sodium and calcium specific D600 was a transient (10 minute) relative decrease in secretory activity, it is possible that the drugs induced

temporary vasospasm in addition to or instead of affecting calcium. Again, this question cannot be settled definitively without measuring changes in intracellular calcium, but in experiments where careful consecutive records of arterial and venous flow were kept (figure 2), no dramatic decrement in venous output relative to arterial input was noted when verapamil or D600 were added. Thus, the contribution of vasospasm can be excluded.

In summary, both racemic verapamil and l-D600 significantly decreased adenosine-stimulated rectal gland secretion immediately, but only the l-D600 effect in low calcium was statistically significant throughout the entire 30 minutes. Neither total inhibition nor complete recovery were seen. These results suggest that calcium may be dynamically involved in rectal gland secretion. In interpreting the calcium blocker data, however, it must be reemphasized that chloride secretion is probably not the only calcium dependent process in the rectal gland cell, and that cell activity as gauged by secretion may incorporate the effects of inhibition on any or all of these processes.

Studies with RMI 12330A

The experiments with RMI 12330A were performed because this agent inhibits both adenylate cyclase and calcium ionophore mediated effects in some tissues (86). RMI is an organic cycloalkyl lactamimide that was believed to be a specific adenylate cyclase inhibitor on the basis of its antagonism of gastric secretion, choleragen induced intestinal secretion (186), and liver plasma membrane cAMP production (70). In vivo,

it was found to have hypoglycemic, diuretic, and platelet aggregating effects, and in early animal studies it caused severe cardiac decompensation similar to digitalis intoxication (Dr. Norbert Wiech, personal communication). Later studies indicated that the adenylate cyclase inhibition was less specific than originally believed, and that in high doses the compound inhibited Na^+-K^+ ATPase, mitochondrial oxidative phosphorylation, and had other nonspecific membrane effects (69). Thus, the significant and dramatic reduction in rectal gland secretion seen with RMI may have been the result of several of these actions.

Two recent studies raise the question of whether RMI also inhibits calcium dependent processes. Work by Grupp et al (69) with isolated heart muscle showed that RMI seriously compromised cardiac dynamics to the point of failure, but this effect was completely reversed by increasing extracellular calcium above 2.5 mM. If RMI is indeed a calcium antagonist as these results suggest, perhaps the RMI effect we observed in the rectal gland could have been reversed by increasing extracellular calcium as well.

Ilundain and Naftalin (86) recently classified intestinal secretagogues into two groups, those which increase cAMP (choleragen, theophylline) and those which do not (carbachol, A23187), and noted that secretion stimulated by any of these is inhibited by either the calmodulin antagonist, trifluoroperazine, or the adenylate cyclase inhibitor, RMI. In addition, they recognized that both trifluoroperazine and RMI compete for

the calcium binding site on calmodulin extracted from rabbit small intestine. Although not explicitly stated, a possible inference is that both of these compounds can be considered calmodulin antagonists. If one of the mechanisms of RMI action is calmodulin inactivation, then the enzymes found to be RMI sensitive, such as adenylate cyclase and Na^+-K^+ ATPase, may be calmodulin-calcium dependent. Our RMI experiments in the rectal gland, therefore, may be in some ways equivalent to trifluoroperazine studies in the rat erythorcyte (108), rat brain (104), and recently, human platelets (98). The correlation may then be made that because secretion was RMI sensitive, it is a calcium-calmodulin dependent process. This conclusion requires that both trifluoroperazine and RMI be specific binders of calmodulin, an assumption that is questionable for both drugs. Phenothiazines have well-known membrane effects (112), and may have other effects independent of the calcium-calmodulin system; these and other as yet unidentified properties could be responsible for the inhibitory effects seen. Similarly, the RMI data are insufficiently firm, particularly because the various effects of the drug may not be fully recognized. A clearer understanding of the pharmacology of trifluoroperazine and RMI in the future will help put this data into perspective.

In conclusion, this work with the isolated perfused rectal gland of the dogfish shark approached the issue of calcium dependent active chloride secretion in three ways:

by increasing intracellular calcium levels with ionophore and perhaps carbachol; by decreasing intracellular calcium levels with calcium channel blockers; and possibly by inactivating calmodulin, the calcium binding protein through which all calcium-enzyme interactions are mediated, with RMI. Despite the coexistence of biological variation and responses of lesser magnitude than seen in other systems, the data provided by these studies are consistent with the hypothesis that secretion in the rectal gland is calcium dependent. Therefore, the rectal gland of Squalus acanthias should be included among the other epithelia in which the transport of chloride is regulated at least in part by calcium sensitive mechanisms, such as the rat intestine, insect salivary gland, and frog cornea.

The site(s) at which calcium acts in this secretory process have not been clearly defined, but probably involve multiple calmodulin-dependent events including the activation of adenylate cyclase (possibly by interaction with the GTP regulatory unit), the activation of other enzymes in the cAMP-initiated cascade, and ultimate alteration of chloride permeability at the apical (luminal) membrane. Further investigation into this subject of calcium control should therefore address 1)the elucidation of the precise molecular mechanisms and effects of pharmacological agents, such as ionophores, calcium channel blockers, and calmodulin antagonists; 2) the perfection of means for measuring the instantaneous changes in cellular

calcium activity and correlating them with the effects of secretory agonists and antagonists; and 3) the development of a better understanding of the enzymatic and molecular steps involved in initiating and executing chloride secretion.

Table i: The ionic composition of seawater, plasma, and urine in elasmobranchs and teleosts*

	Elasmobranch		Teleost		Seawater
	plasma (mM)	urine (mM)	plasma (mM)	urine (mM)	(mM)
Na^{+}	250	240	147	17.1	440
K^{+}	4	2	3	1.4	9
Ca^{+2}	3.5	3	2.7	19.3	10
Mg^{+2}	1.2	40	1.1	133	50
Cl^{-}	250	240	147	121	490
SO_4^{-2}	0.5	70	0.2	69	25
PO_4^{-2}	0.9	33	2.7	9.6	0
Urea	350	100	-	-	0
Osm.	1000	800	319	304	930

*Hickman C and Trump B. "The Kidney". In: Hoar and Randall, eds. Fish Physiology. NY: Academic Press, 1969.

Figure 1: The intact rectal gland of the dogfish shark exposed through an abdominal incision. The single rectal gland artery is shown entering the rectal gland capsule at the upper left. The intestine is opened on the right and forceps indicate the opening of the rectal gland secretory duct.

Table 1: Effect of calcium ionophore A23187 (10^{-5} to 10^{-6} M) on chloride secretion by the isolated rectal gland perfused with 2.5 mM calcium shark Ringer's solution (n=9)

Period	Time (min)	Cl^- Secr. µEq Cl/hr/g	$[Cl^-]$ RGF*
(1) Basal	10-30	76 ± 17	416 ± 14
(2) Peak Ionoph.	40-60	191 ± 56[a]	416 ± 17
(3) End Ionoph.	80-90	275 ± 96	316 ± 21[b]
(4) Peak Theo. and cAMP	110-120	1010 ± 179[c]	388 ± 14

* RGF = rectal gland fluid

[a] $p < 0.02$ compared with basal collection period

[b] $p < 0.02$ compared to peak ionophore collection period

[c] $p < 0.001$ compared with end ionophore collection period

Table 2: Effect of carbachol (2×10^{-3} M) on chloride secretion by the isolated rectal gland perfused with 2.5 mM calcium shark Ringer's solution (n=5)

Period	Time (min)	Cl^- secr. µEq Cl^-/hr/g	$[Cl^-]$ RGF*
(1) Basal	30-40	78 ± 29	441 ± 6
(2) Peak carbach.	40-60	211 ± 35[d]	444 ± 12
(3) End carbach.	70-80	188 ± 15[b]	430 ± 24
(4) Peak theo. and cAMP	100-110	870 ± 223[e]	462 ± 5

* RGF = rectal gland fluid

[a] $p<0.02$ compared with basal collection period

[b] $p<0.02$ compared with peak ionophore collection period

[c] $p<0.001$ compared with end ionophore collection period

[d] $p<0.001$ compared with basal collection period

[e] $p<0.02$ compared with peak carbachol collection period

[f] $p<0.02$ compared with end carbachol collection period

Table 3: Effect of RMI 12330A (5×10^{-4} M) on chloride secretion by the isolated rectal gland stimulated with theophylline (0.25 mM) and dibutyryl cAMP (0.05 mM) perfused with 2.5 mM calcium shark Ringer's solution (n=4)

Period	Time (min)	Cl^- secr. μEq Cl^-/hr/g	$[Cl^-]$ RGF*
(1) theo. cAMP	20-40	1374 ± 95	490 ± 21
(2) theo. cAMP RMI 12330A	60-90	179 ± 63[a]	409 ± 54
(3) theo. cAMP	110-130	499 ± 97[a]	393 ± 70

* RGF = rectal gland fluid

[a] $p<0.001$ compared with the previous period

Table 4: Effect of Adenosine (10^{-4} M) on chloride secretion by isolated rectal glands perfused with 0.5 mM or 2.5 mM calcium shark Ringer's solution

Period	Time (min)	0.5 mM Ca^{2+} (n=6)			2.5 mM Ca^{2+} (n=16)		
		10' Cl^- secr µEq Cl/hr/g	30' Cl secr µEq Cl/hr/g	[Cl^-] RGF*	10' Cl secr µEq Cl/hr/g	30' Cl secr µEq Cl/hr/g	[Cl^-] RGF*
(1) Basal	10	56 ± 18			169 ± 38		
	20	35 ± 5	46 ± 9	385 ± 37	100 ± 35	109 ± 12	467 ± 12
	30	33 ± 10			31 ± 15		
(2) Adenosine	40	430 ± 123			599 ± 116		
	50	568 ± 99	500 ±57	447 ± 13	752 ± 160	716 ± 96	480 ± 96
	60	703 ± 81			692 ± 157		
(3) Adenosine	70	655 ± 156			791 ± 267		
	80	779 ± 190	709 ± 78	471 ± 3	608 ± 232	674 ± 149	475 ± 6
	90	667 ± 202			712 ± 200		
(4) Adenosine	100	700 ± 244			1285 ± 144		
	110	631 ± 174	666 ± 108	468 ± 10		931 ± 153	449 ± 13

* RGF = rectal gland fluid

Table 5: Effect of verapamil (10^{-4} M) on adenosine stimulated chloride secretion by the isolated rectal gland perfused with 0.5 mM or 2.5 mM calcium shark Ringer's solution

Period	Time (min)	0.5 mM Ca^{2+} (n=5)			2.5 mM Ca^{2+} (n=4)		
		10' Cl^- secr µEq Cl/hr/g	30' Cl secr µEq Cl/hr/g	$[Cl^-]$ RGF*	10' Cl secr µEq Cl/hr/g	30' Cl secr µEq Cl/hr/g	$[Cl^-]$ RGF*
(1) Basal	10	-			111 ± 17		
	20	62 ± 3	50 ± 12	320 ± 4	51 ± 6	50 ± 18	445 ± 4
	30	26 ± 1			17 ± 0.3		
(2) Adenosine	40	813 ± 275			253 ± 114		
	50	1135 ± 303	1047 ± 153	433 ± 37	738 ± 152	527 ± 229	478 ± 1
	60	1225 ± 252			589 ± 151		
(3) Adenosine Verapamil	70	512 ± 135[a,c]			372 ± 108[d,e]		
	80	643 ± 127[b]	602 ± 69[f]	419 ± 32	410 ± 163	446 ± 80[f]	488 ± 7
	90	648 ± 99[a]			525 ± 190		
(4) Adenosine	100	647 ± 138			402 ± 138[f]		
	110	768 ± 329	688 ± 124[f]	441 ± 37	467 ± 175	414 ± 74[f]	485 ± 5
	120	575 ± 82			375 ± 129		

* RGF = rectal gland fluid

a $p<0.01$ compared with the 60' interval

b $p<0.0125$ compared with the 60' interval

c $p<0.01$ compared with the 50' interval

d $p<0.025$ compared to the 60' interval

e $p<0.01$ compared with the 50' interval

f insignificant compared with previous period

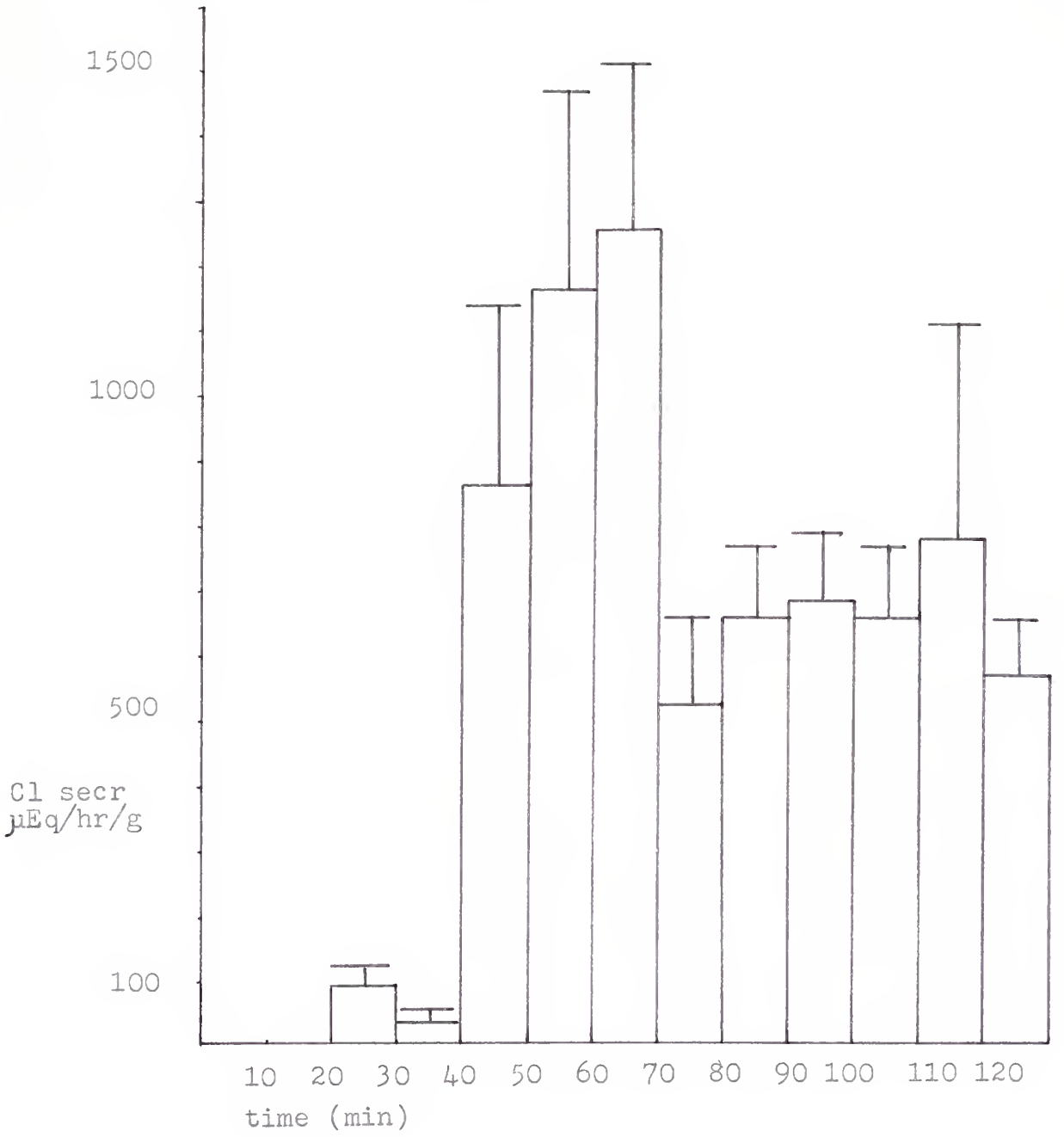

Figure 1: Effect of verapamil (10^{-4} M) on adenosine stimulated chloride secretion by the isolated rectal gland perfused with 0.5 mM calcium shark Ringer's solution

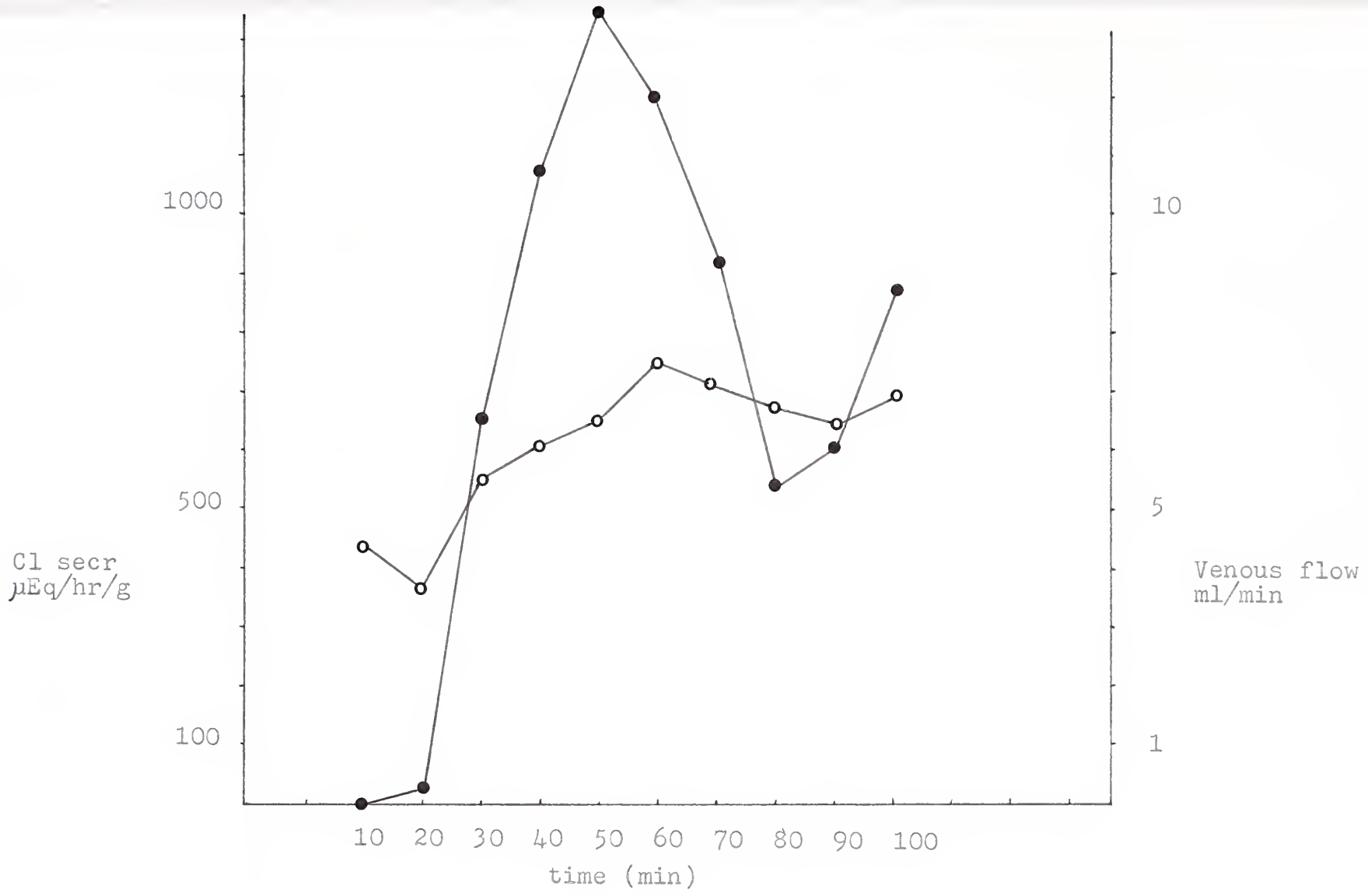

Figure 2: Chloride secretion (●–●–●) and venous effluent flow (o–o–o) during adenosine stimulation and 1-D600 inhibition in an isolated rectal gland perfused with 0.5 mM Ca shark Ringer's solution

Table 6: Effect of l-D600 (10^{-4} M) on adenosine stimulated chloride secretion by the isolated rectal gland perfused with 0.5 mM or 2.5 mM Calcium shark Ringer's solution

Period	Time (min)	0.5 mM Ca^{2+} (n=6)			2.5 mM Ca^{2+} (n=4)		
		10' Cl^- secr µEq Cl/hr/g	30' Cl secr µEq Cl/hr/g	$[Cl^-]$ RGF*	10' Cl secr µEq Cl/hr/g	30' Cl secr µEq Cl/hr/g	$[Cl^-]$ RGF*
(1) Basal	10	138 ± 70			319 ± 120		
	20	60 ± 27	90 ± 37	417 ± 38	95 ± 39	163 ± 62	478 ± 7
	30	29 ± 11			23 ± 10		
(2) Adenosine	40	952 ± 150			484 ± 138		
	50	1458 ± 274	1347 ± 145[f]	473 ± 16	625 ± 186	757 ± 91[f]	481 ± 8
	60	1630 ± 209			911 ± 107		
(3) Adenosine l-D600	70	692 ± 215[a]			492 ± 115[g]		
	80	969 ± 192[b]	844 ± 94[e]	468 ± 6	567 ± 177	528 ± 74[h]	468 ± 5
	90	628 ± 108[c]			523 ± 127		
(4) Adenosine	100	609 ± 49			594 ± 112		
	110	908 ± 149[d]	762 ± 78[h]	466 ± 6	754 ± 140	698 ± 66[h]	483 ± 4
	120	1105 ± 227			781 ± 109		

* RGF = rectal gland fluid

a p<0.0125 compared with the 60' period

b p<0.005 compared with the 60' period

c p<0.05 compared with the 60' period

d p<0.05 compared with the 90' period

e p<0.01 compared to the previous per.

f p<0.001 compared to the previous per.

g p<0.025 compared to the 60' period

h insignificant compared to the previous period

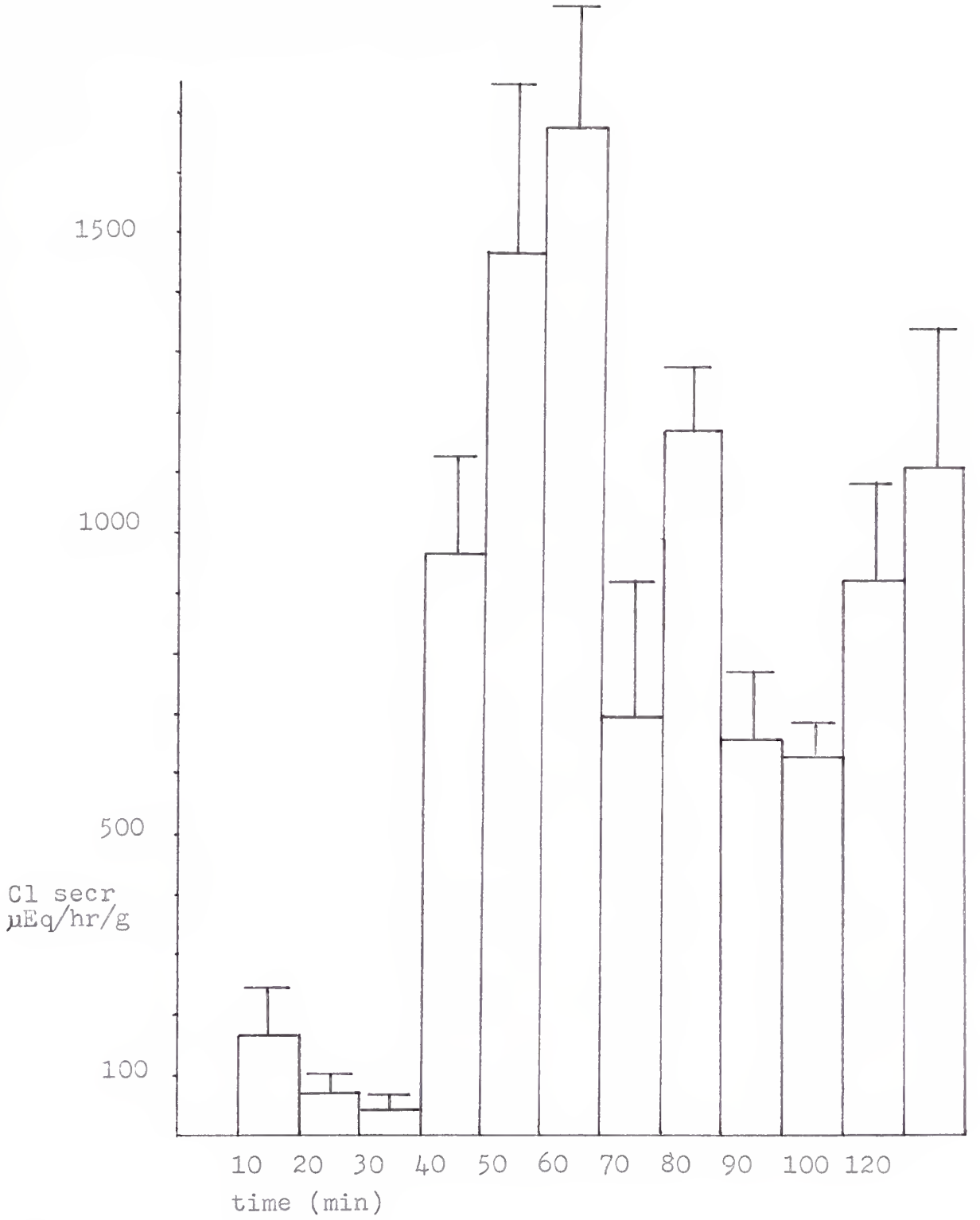

Figure 3: Effect of l-D600 (10^{-4} M) on adenosine stimulated chloride secretion by the isolated rectal gland perfused with 0.5 mM calcium shark Ringer's solution

Table 7: Effect of d-D600 (10^{-4} M) on adenosine stimulated chloride secretion by the isolated rectal gland perfused with 0.5 mM calcium shark Ringer's solution (n=3)

Period	Time (min)	10' Cl secr µEq Cl/hr/g	30' Cl secr µEq Cl/hr/g	$[Cl^-]$ RGF*
(1) Basal	10	88 ± 42		
	20	30 ± 6	55 ± 18	382 ± 60
	30	13 ± 5		
(2) Adenosine	40	558 ± 239		
	50	1092 ± 242	948 ± 153	471 ± 10
	60	1193 ± 234		
(3) Adenosine d-D600	70	823 ± 52[b]		
	80	1158 ± 60	1093 ± 109[b]	479 ± 7
	90	1259 ± 284		
(4) Adenosine	100	788 ± 162[a]		
	110	875 ± 275[a]	815 ± 153[b]	475 ± 3
	120	780 ± 433		

* RGF = rectal gland fluid

[a] $p < 0.05$ compared to the 90' collection period

[b] not significant compared to previous period

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