The Journal of Experimental Biology 205, 1889–1897 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
JEB4107

A Ca^{2+}-sensing receptor modulates shark rectal gland function

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Accepted 17 April 2002

Summary

The elasmobranch *Squalus acanthias* controls plasma osmolality and extracellular fluid volume by secreting a hypertonic fluid from its rectal gland. Because we found a correlation between extracellular Ca^{2+} concentration and changes in cytosolic Ca^{2+} ($[Ca^{2+}]_i$), we sought the possible presence of a calcium-sensing receptor in rectal gland artery and tubules. Cytosolic Ca^{2+} of both tissues responded to the addition of external Ca^{2+} (0.8–5.3 mmol l^{-1}) in a linear fashion. Spermine, Gd^{3+} and Ni^{2+}, known agonists of the calcium-sensing receptor, increased $[Ca^{2+}]_i$. To assess the participation of inositol triphosphate (IP_3) generation, sarcoplasmic/endoplasmic reticulum (SR/ER) Ca^{2+} depletion, and activation of store-operated Ca^{2+} entry, we utilized thapsigargin and ryanodine to deplete Ca^{2+} SR/ER stores and the inhibitory reagents TMB-8 and 2-APB to block IP_3 receptors. In each case, these agents inhibited the $[Ca^{2+}]_i$ response to agonist stimulation by approximately 50 %. Blockade of L-channels with nifedipine had no significant effect. Increases in ionic strength are known to inhibit the calcium-sensing receptor. We postulate that the CaSR stimulates Ca^{2+}-mediated constriction of the rectal gland artery and diminishes cyclic AMP-mediated salt secretion in rectal gland tubules during non-feeding conditions. When the shark ingests sea water and fish, an increase in blood and interstitial fluid ionic strength inhibits the activity of the calcium-sensing receptor, relaxing the rectal gland artery and permitting salt secretion by the rectal gland tubules.

Key words: dogfish, *Squalus acanthias*, norepinephrine, procaine, cyclic AMP, ryanodine, rectal gland.

Introduction

The elasmobranch, *Squalus acanthias*, controls plasma osmolality and extracellular fluid volume by secreting a hypertonic fluid from its rectal gland. Much research has been directed at dissecting the cellular mechanisms responsible for NaCl secretion by the tubular cells of the gland. Briefly, a $Na^+/K^+/2Cl^-$ cotransporter, in concert with Na^+/K^+-ATPase on the basolateral membrane and a Cl^- channel (analogous to the mammalian cystic fibrosis transmembrane conductance regulator) on the apical surface, control net salt secretion (Silva et al., 1996; Forrest, 1996; Greger et al., 1999).

Less well studied is the relationship between blood flow to the rectal gland and secretory activity. Blood flow to the artery of the rectal gland (RGA) in conscious sharks varies from less than 1 % in some animals to 2–7 % of the cardiac output in others, suggesting a pattern of intermittent blood flow (Kent and Olson, 1982). Such variability may reflect changes in extracellular fluid volume and the effect of hormonal influence on vascular tone during feeding cycles. Shuttleworth has shown that α-adrenergic catecholamines cause vasoconstriction of the RGA and that agents known to stimulate gland secretion of salt, such as cyclic AMP (cAMP) and vasoactive intestinal peptide, abolished the vasoconstriction in *Squalus acanthias* (Shuttleworth, 1983). Subsequent studies in the shark, *Scyliorhinus canicula* L., perfused at *in vivo* pressures, showed that reducing perfusion to one third of control values caused a marked decline in Na^+ excretion by the gland (Shuttleworth and Thompson, 1986).

To better delineate the relationships between catecholamines and substances that stimulate secretion in the rectal gland tubule (RGT) but potentially dilate the RGA [e.g. vasoactive intestinal peptide, cardiac naturiuretic peptide (CNP)], we undertook an examination of calcium signaling pathways in both tissues. Whereas cytosolic calcium ($[Ca^{2+}]_i$) of mammalian vascular smooth muscle cells is relatively insensitive to changes in extracellular Ca^{2+} levels ($[Ca^{2+}]_e$) (Fellner and Arendshorst, 2000; Champigneulle et al., 1997), we found that both RGA and RGT responded in a concentration-dependent fashion to changes in $[Ca^{2+}]_e$. Our preliminary studies appeared to exclude voltage-gated L-type channels or Na^+/Ca^{2+} exchange operating in the reverse direction as pathways for the dependency of $[Ca^{2+}]_i$ on $[Ca^{2+}]_e$ (Fellner and Parker, 2001a,b).

Prior studies in isolated perfused rectal gland tubules showed that carbachol and agents that stimulate cAMP production increased $[Ca^{2+}]_i$ by both Ca^{2+} mobilization and

entry, by enhancing the basolateral K^+ conductance. Blockade of voltage-gated L-type channels with nifedipine did not inhibit cAMP-mediated Ca^{2+} influx (Warth et al., 1998).

A calcium sensing receptor (CaSR) has been described in mammalian kidney tubules, parathyroid and bone cells, dorsal root ganglion cells and other neural tissues (Bukoski et al., 1997; Brown et al., 2001). Only one example of a CaSR in vascular smooth muscle cells has been reported, in the spiral modiolar artery of the cochlea of the gerbil (Wonneberger et al., 2000). We postulated that a CaSR might be responsible for the calcium sensitivity we observed in RGA and RGT of *Squalus acanthias*.

Materials and Methods

Sharks *Squalus acanthias* L. of both sexes were caught in Frenchman's Bay, ME, USA and kept in seawater tanks until the animals were pithed through the snout and killed. The RGA and gland were isolated, and placed in ice-cold shark Ringer's solution, pH 7.8, containing (in mmol l^{-1}): NaCl, 275; KCl, 4; $MgCl_2$, 3; Na_2SO_4, 0.5; KH_2PO_4, 1.0; $NaHCO_3$, 8; urea, 350; D-glucose, 5; trimethylamine oxide (TMAO), 72; and Hepes, approx. 2.5. The complete buffer contained 2.5 mmol l^{-1} calcium (which is the normal concentration in the shark); no $CaCl_2$ was added to the nominally calcium-free buffer. Unless otherwise specified, the calcium concentration in the buffer was 2.5 mmol l^{-1}. All buffers were shark Ringer as detailed above. RGA was minced into pieces <0.5 mm in length. Thin transverse slices of rectal gland were freed of capsule and peripheral tubules, the tissue was chopped and individual or groups of tubules were isolated.

These protocols were approved by the IACUC at Mount Desert Island Biological Laboratory.

RGA and RGT were loaded with the calcium-sensitive fluorescent dye fura-2AM, and $[Ca^{2+}]_i$ was measured as previously described (Fellner and Arendshorst, 1999, 2000). RGA or RGT were placed in an open static chamber and examined in a small window of the optical field of a ×40 oil-immersion fluorescence objective of an inverted microscope (Olympus IX70). The tissue was excited alternately with light of 340 and 380 nm wavelengths from a dual-excitation wavelength Delta-Scan equipped with dual monochronometers and a chopper [Photon Technology International (PTI), NJ, USA]. After passing signals through a barrier filter (510 nm), fluorescence was detected by a photomultiplier tube. The calibration of $[Ca^{2+}]_i$ was based on the signal ratio at 340/380 nm and known concentrations of calcium (Grynkiewicz et al., 1985).

Reagents

Nifedipine, $GdCl_3$, spermine, 3,4,5-trimethoxybenzoic acid-8-(diethylamino)octyl ester (TMB-8), TMAO and 2-amino-ethoxydiphenyl borane (2-APB) were purchased from Sigma (St Louis, MO, USA), thapsigargin, VIP, CNP and ryanodine from Cal Biochem (La Jolla, CA, USA) and fura-2-AM from Teflab (Austin, TX, USA).

Statistics

The data are presented as means ± S.E.M. Each data set is derived from tissue originating from at least three separate experimental days. Paired data sets were tested with Student's paired *t*-test. Multiple comparisons were analyzed using one-way analysis of variance (ANOVA) for repeated measures followed by Student–Neuman–Keuls *post hoc* test. $P<0.05$ was considered statistically significant.

Results

RGA and norepinephrine

To confirm that RGA responded to norepinephrine (NE) with an increase in $[Ca^{2+}]_i$, we added NE (3×10^{-6} mol l^{-1}) to the calcium-containing Ringer bathing the tissue. There was an immediate increase in $[Ca^{2+}]_i$ from a baseline value of 112±28 nmol l^{-1} to 192±23 nmol l^{-1} ($N=7$) that was sustained for at least 100 s ($P<0.01$). In calcium-free buffer, NE stimulated Ca^{2+} mobilization from the SR, increasing the baseline level of 78±13 nmol l^{-1} to 127±17 nmol l^{-1} ($N=11$, $P<0.05$). The responses to NE in calcium-free (mobilization) and calcium-containing buffer (2.5 mmol l^{-1}) (mobilization and entry) differed ($P<0.05$).

RGA response to extracellular calcium

To test the response of RGA (in nominally calcium-free buffer) to graded concentrations of extracellular Ca^{2+}, we added $CaCl_2$ in shark Ringer's solution to the chamber. The concentration–response curve (Fig. 1) shows that there is a stepwise increase in $[Ca^{2+}]_i$ with each increment of extracellular Ca^{2+} from 0.8 to 5.3 mmol l^{-1}. We chose this range of extracellular Ca^{2+} to be within approximately

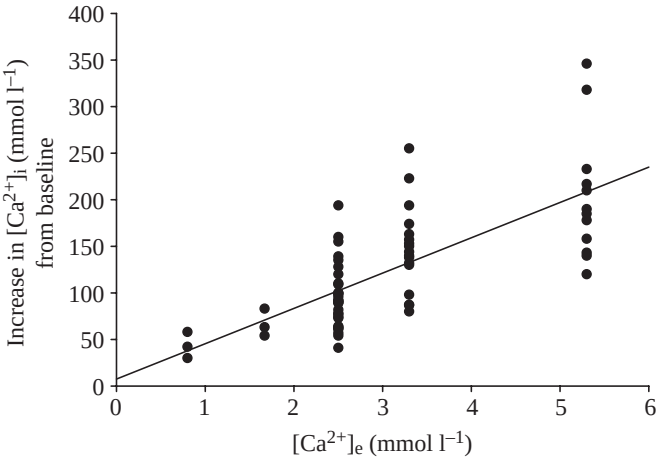

Fig. 1. Cytosolic calcium $[Ca^{2+}]_i$ response of rectal gland artery (RGA) segments to varying concentrations of external calcium $[Ca^{2+}]_e$. Each data point is part of paired observations in which at least two concentrations of calcium were tested with each RGA sample. Between $[Ca^{2+}]_e$ concentrations of 0.8 and 5.3 mmol l^{-1}, there was a linear concentration response between $[Ca^{2+}]_i$ and $[Ca^2]_e$ ($r^2=0.51$, $P<0.01$).

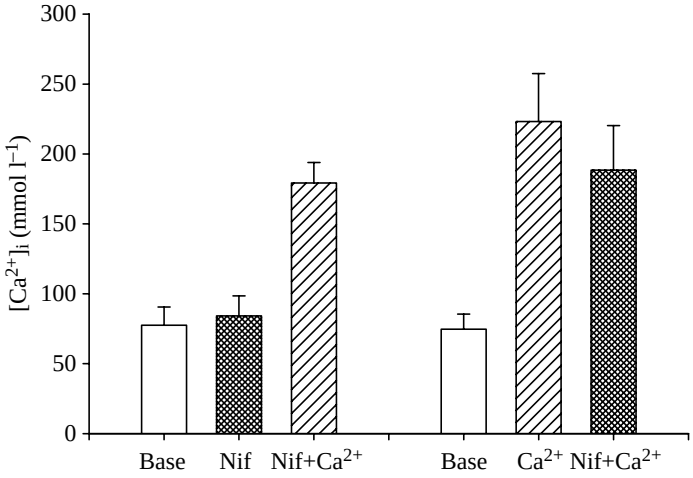

Fig. 2. Effect of L-channel blockade with nifedipine (Nif) on the response of RGA in nominally calcium-free Ringer to the addition of external Ca^{2+}. Values are means ± S.E.M. Nifedipine given pre- (N=8) or post- (N=9) calcium addition did not have any statistically significant effect on the $[Ca^{2+}]_i$ response. Base, baseline value.

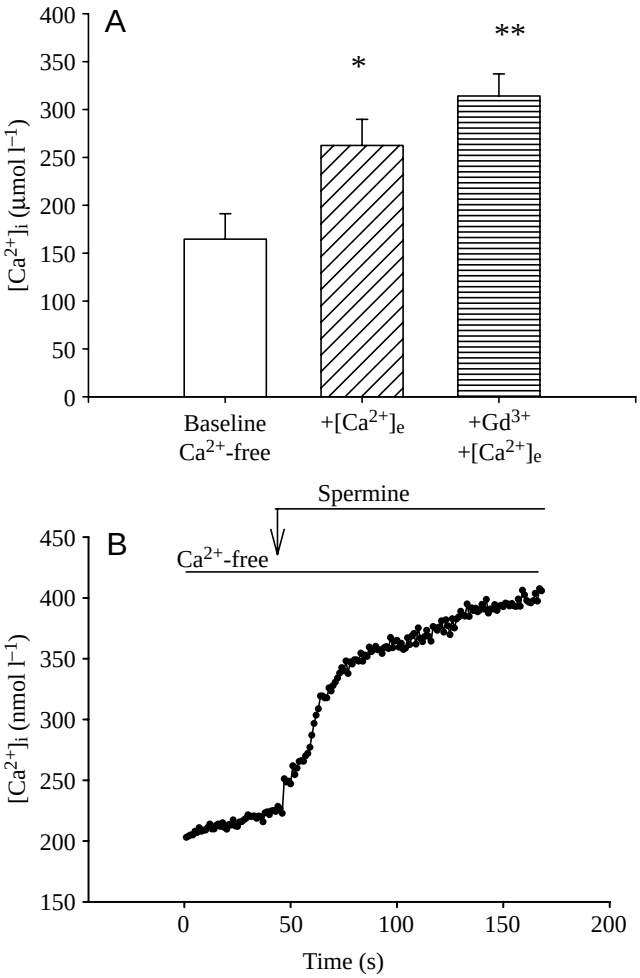

Fig. 3. Cytosolic calcium $[Ca^{2+}]_i$ response of rectal gland artery (RGA) to external Ca^{2+} and Gd^{3+} or to spermine. (A) Change in $[Ca^{2+}]_i$ of RGA maintained in calcium-free shark Ringer following the addition of Ca^{2+} (approx. 3 mmol l^{-1}) and then Gd^{3+} (333 µmol l^{-1}) (values are means ± S.E.M., N=10; *P<0.01 for addition of Ca^{2+} compared to baseline $[Ca^{2+}]_i$ and **P<0.05 for Gd^{3+} compared to Ca^{2+}). (B) Representative example of the increase in $[Ca^{2+}]_i$ of RGA in calcium-free buffer in response to the addition of spermine (333 µmol l^{-1}) at the time indicated by the arrow.

±2 mmol l^{-1} of the physiological $[Ca^{2+}]_e$ of *S. acanthias* (2.5 mmol l^{-1}). Every data point is part of a pair, that is, at least two concentrations of calcium were tested on any one RGA sample. Within this concentration range, there is a linear correlation of $[Ca^{2+}]_i$ with changes in $[Ca^{2+}]_e$ (r^2=0.51, P<0.01).

To assess calcium entry *via* voltage-gated L-type channels, we employed the dihydropyridine, nifedipine. Utilizing concentrations of nifedipine known to block L-channels (Fellner and Arendshorst, 1999), we tested the response of RGA to external calcium (2.5 mmol l^{-1}) either with pre- or post-treatment with nifedipine (3×10^{-6} mol l^{-1}). Fig. 2 shows that L-channel blockade did not have statistically significant effects on the response to the addition of calcium (2.5 mmol l^{-1}) (N=8 pre; N=9, post).

Data to support presence of CaSR in RGA

Agonist stimulation

A variety of cations act as agonists for the CaSR. Gadolinium, which is an inhibitor of store-operated (or capacitative) calcium entry (SOC) at concentrations of 1–3 µmol l^{-1} (Broad et al., 1999), stimulates the mammalian CaSR by 50 % (EC_{50}) at 35 µmol l^{-1} (Brown et al., 1993). We conducted studies of RGA in nominally calcium-free buffer to which we sequentially added Ca^{2+} (3 mmol l^{-1}) and Gd^{3+} (333 µmol l^{-1}). The baseline $[Ca^{2+}]_i$ of 165±26 nmol l^{-1} increased to 263±27 nmol l^{-1} following addition of Ca^{2+} to the buffer (P<0.01) and to 314±23 nmol l^{-1} after subsequent addition of Gd^{3+} (P<0.05) (N=10; Fig. 3A). These data suggest that Gd^{3+} stimulates additional mobilization of Ca^{2+} from the sarcoplasmic reticulum (*vide infra*).

Spermine and other polyamines may also serve as agonists for the CaSR (Quinn et al., 1997). Fig. 3B is a representative response of RGA in Ca^{2+}-free buffer to the addition of spermine (333 µmol l^{-1}). The mean increase in $[Ca^{2+}]_i$ following the addition of spermine was 133±21 nmol l^{-1} (N=5, P<0.01).

Calcium signaling mechanisms

Agonist activation of a CaSR stimulates mobilization of Ca^{2+} from the endoplasmic (ER) or sarcoplasmic reticulum (SR) followed by Ca^{2+} entry through non-voltage-gated calcium channels (Nemeth and Scarpa, 1987). The majority of CaSRs identified currently are linked to G-protein-coupled activation of phospholipase C (Brown et al., 1993; McNeil et al., 1998), with the generation of inositol trisphosphate (IP_3) and diacylglycerol. IP_3 activation of the IP_3 receptor on the ER causes release of Ca^{2+} into the cytosol. The resulting depletion of ER Ca^{2+} then stimulates SOC (Putney, 1990). It follows,

therefore, that any agent which depletes the ER of Ca^{2+} should diminish the response achieved by activation of the CaSR. Accordingly, we chose two pathways of depleting SR/ER calcium that are independent of the IP_3 pathway, namely inhibition of the SR/ER Ca^{2+}-ATPase with thapsigargin and stimulation of the ryanodine-sensitive receptor (Fellner and Arendshorst, 2000).

Stimulation of RGA in Ca^{2+}-free buffer with ryanodine (3 μmol l^{-1}), a concentration known to activate the ryanodine receptor (Fellner and Arendshorst, 2000), mobilized Ca^{2+} from the SR and increased $[Ca^{2+}]_i$ by 64±15 nmol l^{-1} (N=7), demonstrating that the shark RGA has a ryanodine-sensitive receptor that, when stimulated, promotes the release of Ca^{2+} from the SR into the cytosol. Subsequent addition of external calcium (3.3 mmol l^{-1}) further increased $[Ca^{2+}]_i$ by 55±9 nmol l^{-1} (N=8), which is approximately half the response we measured in the absence of ryanodine (N=11, P<0.01). Similar experiments were performed using thapsigargin (10^{-6} mol l^{-1}). Inhibition of Ca^{2+} reentry into the SR with thapsigargin caused an increase in $[Ca^{2+}]_i$ of 69±8 nmol l^{-1}; subsequent addition of calcium to the buffer further increased $[Ca^{2+}]_i$ by 44±8 nmol l^{-1} (N=8, P<0.01 *versus* response to Ca^{2+} without thapsigargin). Thus, using two separate methods of depleting SR Ca^{2+}, the response to external calcium was blunted. That the response to external calcium was not completely obliterated suggests that ryanodine and thapsigargin did not totally empty the calcium stores of RGA SR during the 100 s time period. Fig. 4 compares representative responses of RGA in calcium-free buffer to calcium alone or to calcium in the presence of ryanodine or thapsigargin.

The compound 2-APB has been shown to inhibit the IP_3 receptor and, as well, the SOC channel independent of IP_3 (Missiaen et al., 2001; Ma et al., 2001; Gregory et al., 2001). It has no known effect on Na^{2+}/Ca^{2+} exchange or calcium entry *via* L-channels. Because the CaSR stimulates the generation of IP_3 with subsequent mobilization of Ca^{2+} from the SR/ER and then activation of SOC, 2-APB should inhibit its activity. In experiments in which RGA was stimulated with Ca^{2+} with or without ryanodine, thapsigargin or Gd^{3+}, 2-APB (100 μmol l^{-1}) consistently diminished the response by approximately 50 % (N=8, P<0.01). A representative example of the effect of 2-APB on the response of RGA to the addition of external Ca^{2+} is illustrated in Fig. 5.

RGA and Ni^{2+}

Because Ni^{2+} (1 μmol l^{-1}) constricts ventral aortic rings of the dogfish shark (Evans et al., 1993) and has been shown to be an agonist for the CaSR in some mammalian tissues at concentrations of 0.5–5.0 mmol l–1 or higher (Adebanjo et al., 1998), we did experiments to test the hypothesis that a CaSR sensitive to Ni^{2+} is present in RGA. Ni^{2+} (2–3 mmol l^{-1}) is an inhibitor of SOC and of Na^{2+}/Ca^{2+} exchange. Thus, the choice of the concentration of Ni^{2+} employed is important. RGA in nominally calcium-free medium responded to Ni^{2+} (1.0 mmol l^{-1}), increasing the baseline $[Ca^{2+}]_i$ of

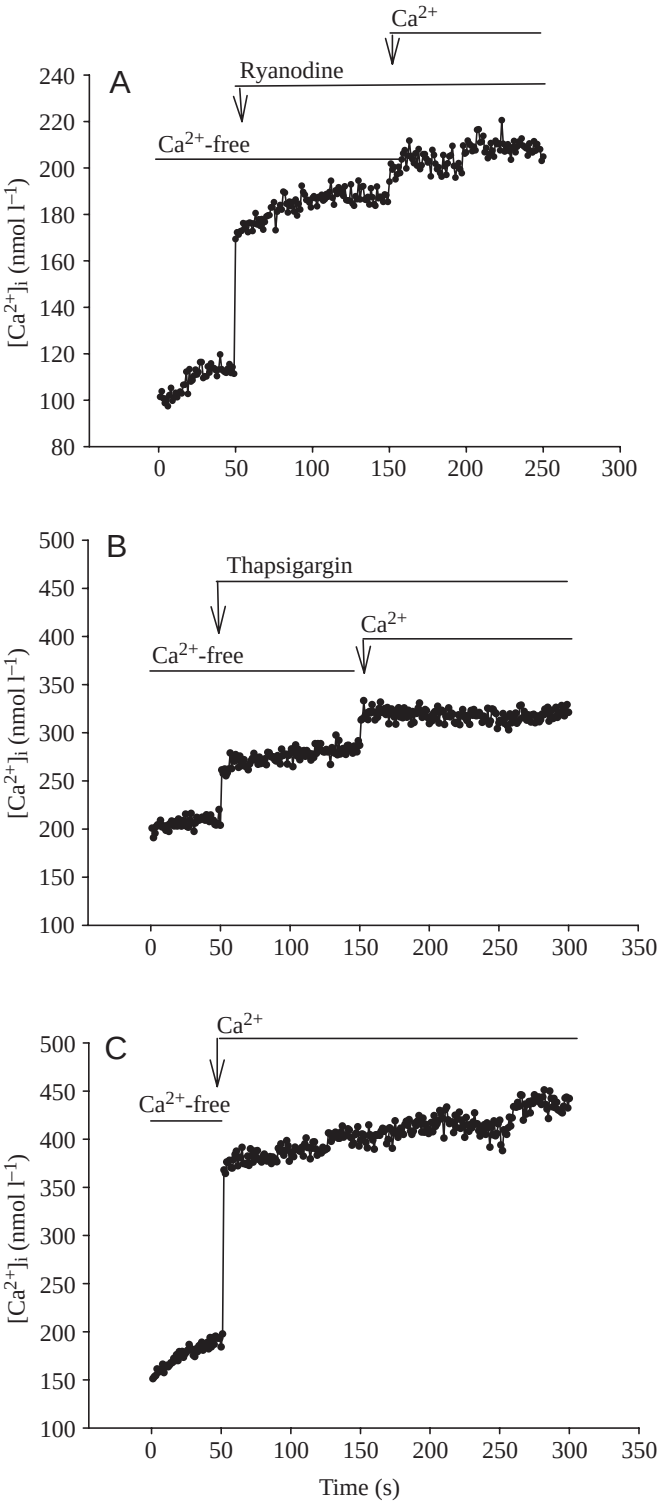

Fig. 4. Representative examples of the cytosolic calcium $[Ca^{2+}]_i$ response of rectal gland artery (RGA) in calcium-free buffer to the addition of external calcium, following treatment with ryanodine (A) or with thapsigargin (TG) (B), compared to the response to external calcium alone (C). Inhibition of sarcoplasmic reticulum Ca^{2+}-ATPase with TG or stimulation of the ryanodine receptor with ryanodine raised $[Ca^{2+}]_i$; subsequent addition of external Ca^{2+} resulted in a smaller increase in $[Ca^{2+}]_i$ compared to that observed in the absence of TG.

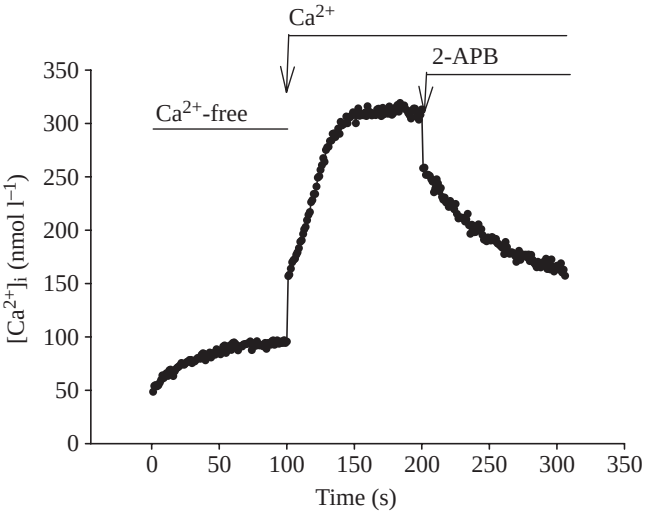

Fig. 5. Representative example of the effect of 2-APB (100 μmol l^{-1}) on cytosolic calcium levels $[Ca^{2+}]_i$ of rectal gland artery (RGA) to the addition of external Ca^{2+} $[Ca^{2+}]_e$. Ca^{2+} and 2-APB were added at the times indicated by the arrows.

83±14 nmol l^{-1} to 109±20 nmol l^{-1}, suggesting that Ni^{2+} promoted mobilization of Ca^{2+} from the SR. Subsequent addition of external calcium (in the presence of 0.7 mmol l^{-1} Ni^{2+}) further elevated $[Ca^{2+}]_i$ to 253±43 nmol l^{-1} (N=6, P<0.01 for both comparisons; Fig. 6A). Ni^{2+} given after Ca^{2+} to RGA previously maintained in calcium-free buffer could either further stimulate Ca^{2+} mobilization or could inhibit SOC. When Ni^{2+} (1.5 mmol l^{-1}) was added after stimulation with calcium, there was a fall in $[Ca^{2+}]_i$, suggesting that inhibition of SOC blunted the calcium entry component of the rise in $[Ca^{2+}]_i$. Baseline $[Ca^{2+}]_i$ of 94±11 nmol l^{-1} rose to 298±29 nmol l^{-1} following the addition of calcium; subsequent addition of Ni^{2+} caused a fall in $[Ca^{2+}]_i$ to 158±14 nmol l^{-1} (N=9, P<0.01) A representative example is illustrated in Fig. 6B.

RGA and procaine

Studies of mesenteric arteries of the rat have suggested that activation of a perivascular sensory nerve CaSR causes Ca^{2+}-induced relaxation of the vessel (Bukoski et al., 1997). Accordingly, we performed experiments in which procaine (5 mmol l^{-1}) was added to the bath in order to inhibit sensory nerve function. There was no difference in the $[Ca^{2+}]_i$ response of RGA in calcium-free Ringer's solution to 2.5 mmol l^{-1} external calcium (136±27 nmol l^{-1}, N=6) in the presence of procaine compared to the response without it (110±13 nmol l^{-1}), suggesting that the CaSR was not located in the perivascular nerve network.

RGT and the CaSR

Agonists

Experiments similar to those conducted with RGA were performed with RGT to investigate the possibility that the rectal gland might respond to agonists of the CaSR as well.

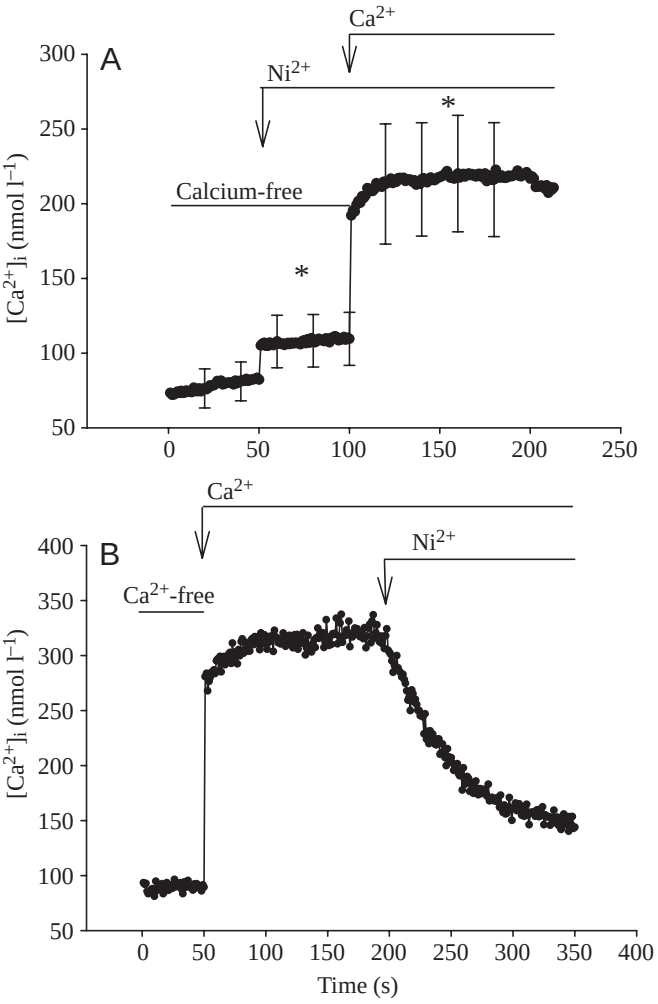

Fig. 6. Temporal responses of grouped recordings of rectal gland artery (RGA) to external Ca^{2+} and Ni^{2+}. (A) In calcium-free medium, Ni^{2+} (1 mmol l^{-1}) promoted Ca^{2+} mobilization; subsequent addition of Ca^{2+} (in the presence of 0.7 mmol l^{-1} Ni^{2+}) further elevated cytosolic Ca^{2+} levels $[Ca^{2+}]_i$ (*P< 0.01 for Ni^{2+} *versus* baseline and Ca^{2+} *versus* Ni^{2+}). Values are means ± S.E.M. (N=6). (B) When Ni^{2+} (1.5 mmol l^{-1}) was added after stimulation of RGA with external Ca^{2+}, an inhibitory effect of Ni^{2+} on Ca^{2+} entry was seen (representative recording). Ca^{2+} and Ni^{2+} were added at the times indicated by the arrows.

Tubules in nominally calcium-free Ringer responded to the addition of calcium (5 mmol l^{-1}) to the medium with a rapid increase in $[Ca^{2+}]_i$ of 250±53 nmol l^{-1} (N=7). Addition of spermine (333 μmol l^{-1}), a known agonist of the CaSR (Quinn et al., 1997), caused a further increase in $[Ca^{2+}]_i$ of 235±94 nmol l^{-1} (P<0.01 for both comparisons, Fig. 7A).

Fig. 7B is a representative example of the effect of spermine added after the response of RGT to external calcium. As shown in Fig. 7C, RGT, like RGA, responded to incremental increases in external Ca^{2+} with a stepwise increase in $[Ca^{2+}]_i$ and responded to Gd^{3+} with a further rise in $[Ca^{2+}]_i$. Group data are illustrated in Fig. 7A. When calcium (5 mmol l^{-1}) was added to tubules in normal calcium Ringer (2.5 mmol l^{-1}),

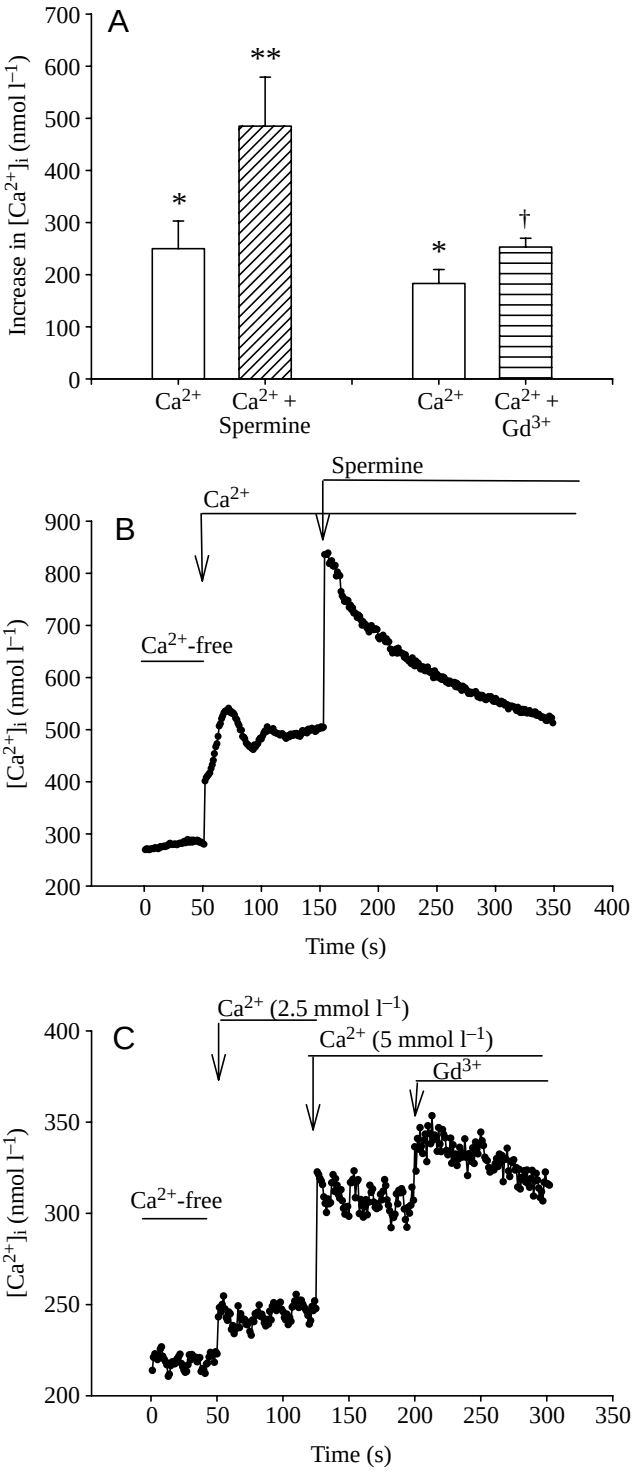

Fig. 7. Changes in $[Ca^{2+}]_i$ of rectal gland tubules (RGTs) in response to calcium, spermine and gadolinium treatment. (A) The stimulatory effect of spermine (N=7) on $[Ca^{2+}]_i$ in RGT was greater than that achieved by Gd^{3+} (N=10) (*P<0.01 compared to baseline, **P<0.01 for spermine *versus* calcium, $^{\dagger}P$<0.05 for Gd^{3+} *versus* Ca^{2+}). Values are means ± S.E.M. (B) Representative example of the response of RGT in calcium-free shark Ringer to $[Ca^{2+}]_e$ followed by spermine. (C) Representative example of RGT showing the stimulatory effect of sequential addition of external Ca^{2+} and Gd^{3+} at the times indicated by the arrows.

$[Ca^{2+}]_i$ increased by 162 nmol l^{-1} (N=10, P<0.01). Gd^{3+} further increased $[Ca^{2+}]_i$ by 60±21 nmol l^{-1} (P<0.05). The increment in $[Ca^{2+}]_i$ produced by the addition of Gd^{3+} is considerably less than that achieved by spermine (235±94 nmol l^{-1}, P<0.01), which may be a consequence of the inhibitory effect of Gd^{3+} on calcium entry *via* SOC.

RGT and TMB-8

To assess the participation of IP_3-mediated calcium release from the ER of RGT, we used the drug TMB-8 (10^{-6} mol l^{-1}), which inhibits the IP_3 receptor (Palade et al., 1989; Salomonsson and Arendshorst, 1999). Tubules were prepared in calcium-free buffer with or without the addition of TMB-8. The increment in $[Ca^{2+}]_i$ when Ca^{2+} (5 mmol l^{-1}) was added was 47±10 nmol l^{-1} (N=7) in the TMB-8 group and 118±24 nmol l^{-1} in the control group (N=18, P<0.01), demonstrating that inhibition of the IP_3 receptor blunted the response of RGT to added external calcium (data not shown).

RGT and thapsigargin

In a manner similar to that performed in RGA, we prepared tubules in calcium-free Ringer's solution, and added thapsigargin (10^{-6} mmol l^{-1}) for 100 s before adding external Ca^{2+} (3.3 mmol l^{-1}). The increment in $[Ca^{2+}]_i$ after thapsigargin was 108±23 (N=5, P<0.01). Fig. 8 illustrates a typical experiment in which calcium was added after thapsigargin. In contrast, in the absence of thapsigargin, the addition of Ca^{2+} to cells in calcium-free buffer increased $[Ca^{2+}]_i$ by 203±10 nmol l^{-1} (N=17) (not shown). This nearly 50 % reduction in response is similar to our findings in RGA with thapsigargin and ryanodine. Because cyclic ADP-ribose, which activates ryanodine receptors, has not been found in renal tubules (Chini et al., 1997), we chose not to study ryanodine in RGT.

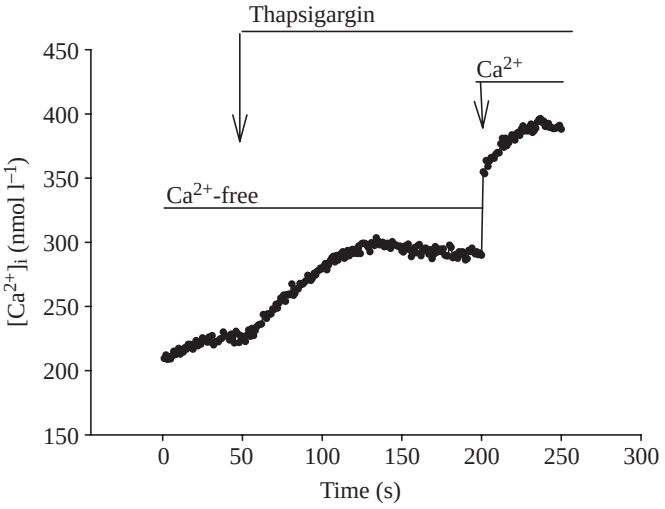

Fig. 8. Representative example of the $[Ca^{2+}]_i$ responses of rectal gland tubules (RGT) to thapsigargin (10^{-6} mmol l^{-1}) and to the subsequent addition of external calcium (3.3 mmol l^{-1}) added at the times indicated by the arrows.

Discussion

The elasmobranch *Squalus acanthias* swims in a sea where the ionic strength is much greater than that of the shark's blood, the sodium chloride concentration of sea water being nearly double that of shark extracellular fluid. Because the shark kidney has a limited ability to elaborate a hypertonic urine, the salt load derived from ingestion and gill diffusion must be handled by non-renal excretory processes (Burger, 1960). The rectal gland of the dogfish shark serves this function, controlling plasma osmolality by secreting a hypertonic fluid (Silva et al., 1996). The RGT is similar to the renal thick ascending limb of Henlé of mammalian species. A $Na^+/K^+/2Cl^-$ cotransporter, in concert with Na^+/K^+-ATPase, on the basolateral membrane and a Cl^- channel [analogous to the mammalian cystic fibrosis transmembrane conductance regulator (CFTR)] on the apical surface, control net salt secretion (Silva et al., 1996; Forrest et al., 1996). Blood flow through the artery of the rectal gland varies over a wide range *in vivo* (Kent and Olson, 1982), suggesting intermittency of gland perfusion that may reflect changes in circulatory volume and hormonal changes associated with feeding cycles and ingestion of sea water. 40 years ago, Burger suggested that vasomotor changes in the RGA might be an important factor in controlling salt secretion by the rectal gland (Burger, 1962). Subsequently, Shuttleworth demonstrated in the isolated perfused rectal gland that physiological concentrations of NE reduced perfusion flow rate in the isolated rectal gland and that agents known to stimulate secretion by the gland (VIP, adenosine + theophylline), increased efferent flow independently of their action on rectal gland secretory cells. It was concluded that blood flow through the rectal gland is normally restricted *in vivo* by physiological concentrations of catecholamines and that this effect is reversed by the vasomotor activities of substances that promote salt secretion by the rectal gland (Shuttleworth, 1983).

The RGA arises from the posterior mesenteric artery of *S. acanthias*. Studies of the anterior mesenteric artery show that acetylcholine, endothelin-1 (ET_B) and nitric oxide constrict and that cardiac naturiuretic peptide, prostaglandin E and carbaprostacyclin dilate the artery (Evans, 2001). A band of smooth muscle in the periphery of the rectal gland constricts in response to endothelin and nitric oxide and dilates following treatment with cardiac naturiuretic peptide, suggesting that the dimensions of the entire rectal gland may play a role in its secretory function (Evans and Piemarini, 2001). Because we dissected the capsule and outer layer of tubules from the gland prior to analysis and inspected the tubules microscopically before measuring $[Ca^{2+}]_i$, we are confident that smooth muscle tissue was not present in our samples.

Our initial studies explored pathways of calcium signaling in RGA and RGT (Fellner and Parker, 2001a,b). Whereas $[Ca^{2+}]_i$ of mammalian vascular smooth muscle cells, and also many epithelial cells, is relatively resistant to changes in $[Ca^{2+}]_e$ (Fellner and Arendshorst, 2000; Champigneulle et al., 1997), we were surprised to find that both RGA and RGT responded to changes in $[Ca^{2+}]_e$ with an increase in $[Ca^{2+}]_i$. Furthermore, these studies appeared to exclude voltage-gated L-type channels or Na^+/Ca^{2+} exchange operating in the reverse direction as pathways for the dependency of $[Ca^{2+}]_i$ on $[Ca^{2+}]_e$. Thus, exploration of the possibility of the presence of a CaSR on RGA and RGT was the aim of the present study.

Cloning and characterization of a CaSR from bovine parathyroid cells was achieved in 1993 (Brown et al., 1993); subsequently the CaSR was identified on cell membranes of tissues involved in the regulation of $[Ca^{2+}]_e$ such as kidney, bone and intestine. The receptor is also involved in the activation of some ion channels, control of hormonal secretion, apoptosis and cell proliferation (Brown et al., 2001). A single study in vascular smooth muscle suggests that the CaSR functions as a modulator of vasoconstriction in the spiral modiolar artery of the cochlea of the gerbil (Wonneberger et al., 2000). A number of cations (Gd^{3+}, Ni^{2+}, Pb^{2+}) are agonists for the CaSR (Wonneberger et al., 2000; Chattopadhyay, 2000; Handlogten et al., 2001; Quinn et al., 1998), as are polyamines (Quinn et al., 1997) and aromatic and L-amino acids (Conigrave et al., 2000). There is an inverse relationship between ionic strength and activity of the CaSR. Reduction in ionic strength enhances sensitivity of the receptor and an increase in ionic strength inhibits its activity (Quinn et al., 1998). This latter characteristic of the CaSR may be its major function in *Squalus acanthias*.

Our studies of both the RGA and RGT of the dogfish shark are entirely compatible with these unique characteristics of the CaSR. (1) We have shown that there is a concentration–response relationship between changes in $[Ca^{2+}]_e$ and $[Ca^{2+}]_i$. (2) Spermine activated the receptor of RGT and RGA, increasing $[Ca^{2+}]_i$. (3) Gadolinium, which is an irreversible inhibitor of SOC at concentrations of 1–3 $\mu mol\,l^{-1}$ (Broad et al., 1999), modestly increased $[Ca^{2+}]_i$ in both RGA and RGT. The response to Gd^{3+} was considerably less than that achieved by spermine, which suggests that although Gd^{3+} was able to initiate IP_3-directed mobilization of Ca^{2+} from the SR/ER, SOC that would have occurred as a consequence of depletion of SR/ER calcium stores was inhibited by the presence of Gd^{3+}. (4) Nickel, an inhibitor of Na^{2+}/Ca^{2+} exchange in millimolar concentrations and also an inhibitor of SOC (Sarosi et al., 1998), is an agonist for the CaSR (at concentrations of 0.5–5.0 $mmol\,l^{-1}$) (Adebanjo et al., 1998). We found that RGA in nominally calcium-free Ringer's solution responded to Ni^{2+} (1.0 $mmol\,l^{-1}$) with mobilization of $[Ca^{2+}]_i$ from the SR. Subsequent addition of external calcium (in the presence of 0.7 $mmol\,l^{-1}$ Ni^{2+}) further increased $[Ca^{2+}]_i$. When Ni^{2+} was added after calcium, there was inhibition of the $[Ca^{2+}]_i$ response, presumably because calcium entry *via* SOC was blocked.

The CaSR can operate *via* pertussis toxin-sensitive or -insensitive G proteins to activate phospholipase C, A or D, depending on the milieu of the cell in which it is expressed (Brown et al., 2001). Thus, both Gi and Gq proteins may be involved in the signaling pathways of the CaSR. Agonist activation of the CaSR stimulates mobilization of Ca^{2+} from the SR/ER followed by Ca^{2+} entry through non-voltage-gated

calcium channels (Nemeth and Scarpa, 1987; Wonneberger et al., 2000). We did not find evidence for CaSR-mediated L-channel calcium entry in the present study.

The majority of CaSRs identified currently are linked to G-protein coupled activation of phospholipase C (Brown et al., 1993; McNeil et al., 1998) with the generation of IP_3 and diacylglycerol. IP_3 activation of the IP_3 receptor on the SR/ER causes release of Ca^{2+} into the cytosol. To assess the participation of IP_3 generation on the response of RGA and RGT to stimulation with $[Ca^{2+}]_e$, we utilized the compound TMB-8, which inhibits the IP_3 receptor (Palade et al., 1989, Salomosson and Arendshorst, 1999), or 2-APB. The cell-permeant compound 2-APB blocks the IP_3 receptor in a variety of cell types (Missiaen et al., 2001; Ma et al., 2001) and also inhibits the SOC channel, independent of IP_3 (Gregory et al., 2001). Both TMB-8 (RGT) and 2-APB (RGA) diminished the $[Ca^{2+}]_i$ response to external calcium by approximately 50 %.

In addition, depletion of SR/ER calcium *via* non-IP_3 mechanisms should diminish the global response of RGT or RGA to external calcium because of inhibition of SOC. We employed ryanodine to stimulate the SR ryanodine-sensitive receptor and thapsigargin to inhibit reuptake of calcium into the SR by blocking the Ca^{2+}-ATPase (Fellner and Arendshorst, 1999, 2000). Both ryanodine and thapsigargin diminished the cytosolic response of RGA to external calcium by approximately 50 %. Thus, independently of the mechanism utilized to deplete the SR/ER of Ca^{2+}, the response to activation of the CaSR is diminished because of reduction in Ca^{2+} entry *via* SOC.

Further confirmation of the presence of a CaSR in the dogfish shark has been obtained in preliminary studies (Dr Marlies Betka, Marical, Portland, ME, USA, personal communication) where positive immunocytochemical staining for the CaSR was seen in both RGA and RGT of *Squalus acanthias* captured in Frenchman's Bay, ME, USA.

The mechanism by which a CaSR may influence the function of RGT is related to its effect on cAMP generation and degradation. The co-expression of a CaSR and a Ca^{2+}-inhibitable adenyl cyclase has been studied in cells of the thick ascending limb of the rat kidney (Ferreira et al., 1998). Changes in extracellular Ca^{2+}, coupled to a pertussis toxin-insensitive G-protein-activated phospholipase C, caused a dose-dependent inhibition of cAMP content both from inhibition of cAMP synthesis and from stimulation of cAMP hydrolysis (Ferreira et al., 1998). As salt secretion in RGT is dependent upon the generation of cAMP (Warth et al., 1998), agonist stimulation of the CaSR should inhibit NaCl secretion. Stimulation of salt secretion by RGT involves a complex sequence of events in which cardiac naturiuretic peptide, released from the shark heart in response to volume expansion, stimulates the release of vasoactive intestinal peptide from the neural network of the rectal gland. Vasoactive intestinal peptide then stimulates the accumulation of cAMP and promotes salt secretion (Silva et al., 1996). Cardiac naturiuretic peptide has also been shown to have a direct effect on RGT cells maintained in tissue culture (Karnaky et al., 1993).

Because the extracellular domain of the CaSR interacts with polyvalent cations and polycationic molecules such as polyamines, it was hypothesized that activation of the receptor might occur through the screening of charged side chains of acidic or basic amino acids. Furthermore, if ionic strength were increased by the addition of salts to the extracellular environment, the ability of polycations to trigger the CaSR should be decreased (Quinn et al., 1998). Such a property of the CaSR suggests that, rather than sensing changes in calcium levels exclusively, the receptor might serve as a 'salt sensor' in some cells or species. Non-mammalian vertebrates have developed specialized salt-excretory organs to maintain osmolar and volume homeostasis, such as the salt gland of birds (Shuttleworth and Hildebrandt, 1999) and the rectal gland of the shark (Burger, 1960). Only one study has examined the effect of changes in ionic strength on secretory function of the shark RG. In the isolated perfused RGT, cAMP causes a biphasic response, the second of which arises from stimulation of the $Na^+/K^+/2Cl^-$ co-transporter. Hypotonic solutions (reduction in NaCl concentration of 150 mmol l^{-1}) caused RGT cell swelling and abolished this second phase, whereas hypertonic solutions (addition of NaCl, 150 mmol l^{-1}) resulted in cell shrinkage and an increase in relative cell chloride concentration (Greger et al., 1999). These data support a role for the effect of changes in ionic strength on secretion of salt by the RGT. Our future studies will be directed at testing the effect of changes in ionic strength on salt secretion in the isolated, perfused shark rectal gland and on calcium signaling in RGA and RGT cells.

We propose that the function of a CaSR in RGA and RGT of *Squalus acanthias* is to inhibit tonically blood flow to the gland and to inhibit salt secretion (*via* a reduction in cAMP levels) by the RGT during non-feeding periods. When the shark ingests fish and sea water during feeding, the resultant increase in salt concentration (ionic strength) of the blood and interstitial spaces would then inhibit the CaSR (Quinn et al., 1998), resulting in disinhibition of RGA vascular contraction and reversal of inhibition of salt secretion. Such a control mechanism would permit the animal to efficiently recruit the function of the rectal gland only during periods of feeding.

This study was supported by a Thomas H. Maren Fellowship awarded to S.K.F. L.P. was a recipient of a Hancock County, Maine, USA, student fellowship. We thank Dr Marlies Betka (Marical, Portland, ME, USA) for kindly performing immunocytochemical studies on RGA and RGT.

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