cAMP Activates the Sodium Pump in Cultured Cells of the Elasmobranch Rectal Gland

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ABSTRACT

The inorganic ion content of rectal gland cells cultured from Squalus acanthias was studied by electron probe analysis in order to determine the effect of stimulation by cAMP. Cell sodium was reduced by 30% ($P < 0.01$) at 8 min after exposure to dibutyryl cAMP and theophylline and remained low at 25 and 33 min. Chloride content also fell significantly with stimulation. Although cAMP may activate several transport sites, the results are consistent with a direct effect of stimulation to increase the activity of the sodium pump in shark rectal gland.

Key Words: Rectal gland, chloride secretion, sodium, potassium-ATPase, cAMP, electron probe analysis

The function of the elasmobranch rectal gland is to maintain salt homeostasis by the active secretion of chloride, which is under neurohumoral control. The process of secretion has been extensively studied (Figure 1) (1). Chloride entry into the cell occurs via a basolateral Na⁺-K⁺-2Cl⁻ cotransporter and is indirectly energized by the creation of a favorable gradient for Na⁺ entry by the Na⁺-K⁺ pump, which is located in the basolateral membrane at exceptionally high density. Na enters the lumen across a paracellular pathway. In addition, there are luminal Cl⁻ and basolateral K⁺ channels that permit ion exit.

Salt secretion by the rectal gland can be stimulated as much as 40-fold by cAMP (2) and by substances that act via adenylate cyclase, particularly vasoactive intestinal peptide (VIP) (3). There is strong indirect evidence that this is accompanied by enhanced activity of (Na,K)-ATPase. For example, stimulation by cAMP is associated with a sixfold increase in ouabain-sensitive oxygen consumption by the whole gland (4) and an abrupt rise in ouabain-sensitive $^{86}$Rb⁺ uptake by dispersed rectal gland cells (5). The mode of activation of (Na,K)-ATPase, however, is not clear. An increase in (Na,K)-ATPase turnover might be driven either by a rise in cellular Na⁺ activity or by an increase in pump activity or in the number of enzyme units, or both. A key question, therefore, is whether cell sodium increases, decreases, or remains the same when rectal gland secretion is increased.

By using electron probe analysis, we made serial measurements of the electrolyte content of cultured rectal gland cells, before and during stimulation, in an effort to determine the mechanism of stimulation. We found a highly significant fall in internal Na⁺ content in stimulated rectal gland cells. These results support a direct effect of stimulation on the sodium pump.

METHODS

Rectal gland cells were placed in culture at the Mount Desert Island Biological Laboratory (MDIBL) during the summer season. Experiments and electron probe analysis of the cultured cells were subsequently performed at the National Electron Probe Resource for the Analysis of Cells (NEPRAC) at Harvard Medical School in Boston.

Rectal Gland Cell Culture

The culture of rectal gland cells is done by the protocol developed by Dr. John Valentich at MDIBL (6). The rectal gland of the spiny dogfish is removed with sterile instruments and by a clean technique. As much connective and vascular tissue as possible is trimmed from the gland at that time. The gland is immediately placed in chilled, sterile shark Ringer’s for transport from the dock work area to the tissue culture facility. The remainder of the preparation of
sterile rectal gland tubules is performed at the tissue culture facility at the MDIBL, by a sterile technique. The gland is placed in a Petri dish, which is dried, chilled, and embedded in ice, and is sliced horizontally into sections which are 2 to 3 mm thick. The terminal 1 to 2 mm of each gland is discarded, because of a high ratio of connective tissue to tubules. The slices are then minced by using a scissoring motion with surgical blades. The minced tissue is then transferred into a conical tube and washed three times with 20 mL of sterile shark Ringer's by gentle manual shaking. The composition of shark Ringer's solution is as follows (millimolar): Na, 288; Cl, 289; K, 5; Ca, 5; Mg, 3.3; H2CO3, 24; urea, 350; glucose, 5; and phenol red, 1.25 µg/mL, gassed with O2 (95%) and CO2 (5%). The minced gland is then suspended in a collagenase solution (0.2% in 20 mL of sterile shark Ringer's) and aspirated several times through a wide-bore pipette to break up clumps and inadequately minced tissue. The mince is incubated in collagenase at room temperature (25°C) for 1 h on a mechanically inverting rack, is stopped midway to agitate vigorously and aspirate the tissue, and is stopped again to prevent clumping and aid digestion. At the end of the incubation, the suspension is washed with sterile shark Ringer's by centrifuging at 200 x g at 4°C for 45 s. The supernatant is discarded, and the pellet is resuspended in approximately 20 mL of sterile shark Ringer's. The digested mince is then agitated with a large-bore pipette so that tubules are released and float in the supernatant. After the larger fragments of minced tissue are allowed to settle, the tubules suspended in the supernatant are harvested and kept on ice. The addition of fresh sterile shark Ringer's, the agitation of the mince, and the harvest of tubules in suspension are repeated several times until the tubule yield appears to decline. The accumulated tubules are then washed and finally suspended in 20 mL of sterile shark Ringer's from which two 1-mL aliquots are removed to determine the total yield by wet weight. The average yield is 250 to 500 mg wet wt per gland.

The tubules are ultimately suspended in medium containing equal parts of Dulbecco's modified Eagle's medium and Ham's F12, supplemented with 5% Nuserm (Collaborative Research, Bedford, MA), 1% ITS (insulin, transferrin, selenium) (Collaborative Research), and penicillin/streptomycin (100 U/mL/100 µg/mL). The tubules, at a density of approximately 10 mg/mL, are finally seeded onto fibronectin-coated silicon chips, which have been prepared several hours earlier in a 35-mm Petri dish. Cells were kept in a 25°C incubator.

After the tubules are firmly attached (generally after at least 48 h), the chips are packaged in styrofoam containers and flown to NEPRAC at Harvard Medical School and Brigham and Women's Hospital in Boston, in refrigerated packing (wet ice). Experiments were performed within 10 to 14 days of initial plating.

Cells grow as an outgrowth of randomly distributed tubule fragments. Chips had one or more confluent patches of cells but never formed a confluent monolayer covering the entire chip. Visually, transporting cells were the predominant cell type, with occasional central duct epithelium cells also present, as described by Valentich and Forrest (6).

Experiments

Experiments were performed at room temperature (25°C). At time 0, medium supplemented with the stimulatory agents [the cAMP analog, dibutyryl cAMP (db-cAMP) (1 mM), theophylline (0.5 mM), and VIP (5 x 10^-8 M)] was added to some of the dishes. Chips with adherent cells were removed before the addition of the stimulatory agents and at 8, 25, and 33 min after the addition of stimulatory agents or vehicle. Immediately after removal, the cells were washed by dipping each chip into four successive beakers of swirling distilled water for 2 s each and were then immediately frozen in liquid nitrogen, as previously described (7, 8). Cells were then freeze-dried and stored under vacuum until electron probe analysis was performed.

Wavelength-dispersive x-ray spectrometry was performed with a Cameca MS 46 electron probe. Counts for phosphorus, sodium, potassium, and chloride were obtained with a beam diameter of 15 µm.
Beam location was guided by direct microscopic visualization. The intensity counts for each element were normalized for spectrometer efficiencies, and cell sodium, potassium, and chloride were expressed per unit of phosphorus counts (as molar ratios) to normalize for the cell mass encompassed by the electron beam. The value at a given time point represents the mean value of a population of cells analyzed individually. No significant difference was found among the ion contents of unstimulated cells at 0, 8, and 25 min by the Newman-Keuls test. Therefore, these groups were pooled and used as the control group for comparison with stimulated cells.

Reagents

Except for Nu-serum and ITS (Collaborative Research), the fibronectin, media, salts, and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Statistics

The normalized Na, K, and Cl contents and the P content at each time point after stimulation were compared with the values of the pooled controls by Dunnett’s test. Because there were several time points involved, we used multiple sample comparison testing.

RESULTS

In preliminary experiments, ouabain-inhibitable oxygen consumption of cultured rectal gland cells increased from 3.3 ± 5.0 to 41 ± 4 µmol of O₂/mg wet wt/h (N = 7; mean ± SE) within 2 to 3 min when 1 mM db-cAMP and 0.5 mM theophylline were added. That these cells respond promptly to stimulation is consistent with studies by Valentich and Forrest that show that voltage and short-circuit current across sheets of cultured cells increase within 2 min of the application of VIP and forskolin (6).

Intracellular Na and Cl contents of stimulated cells and the unstimulated time controls are listed in Table 1. There was a highly significant fall of about 30% in cell Na content with stimulation. The Cl content also decreased significantly at all time points with stimulation as compared with that in unstimulated controls (Table 1; Figure 2).

Potassium content (Table 1; Figure 2) did not change significantly with stimulation at the earliest time point (8 min) but did fall later by 6%. It is important to note that there were no significant differences in phosphorus measurement among groups (Table 1). The large dispersion in phosphorus is due to the normal variation in the thickness of the cell fraction encompassed by the electron beam, which randomly scans the cells.

**Table 1. Ion contents of cultured rectal gland cells**

<table>
<thead>
<tr>
<th></th>
<th>mean ± SE</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium (mol/mol of P)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.31 ± 0.01</td>
<td>465</td>
</tr>
<tr>
<td>Stim, 8 min</td>
<td>0.20 ± 0.01b</td>
<td>149</td>
</tr>
<tr>
<td>Stim, 25 min</td>
<td>0.21 ± 0.02b</td>
<td>166</td>
</tr>
<tr>
<td>Stim, 33 min</td>
<td>0.18 ± 0.01b</td>
<td>307</td>
</tr>
<tr>
<td>Chloride (mol/mol of P)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.93 ± 0.01</td>
<td>465</td>
</tr>
<tr>
<td>Stim, 8 min</td>
<td>0.83 ± 0.02b</td>
<td>149</td>
</tr>
<tr>
<td>Stim, 25 min</td>
<td>0.86 ± 0.02c</td>
<td>166</td>
</tr>
<tr>
<td>Stim, 33 min</td>
<td>0.81 ± 0.02b</td>
<td>307</td>
</tr>
<tr>
<td>Potassium (mol/mol of P)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.33 ± 0.01</td>
<td>465</td>
</tr>
<tr>
<td>Stim, 8 min</td>
<td>1.30 ± 0.02</td>
<td>149</td>
</tr>
<tr>
<td>Stim, 25 min</td>
<td>1.27 ± 0.02c</td>
<td>166</td>
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<tr>
<td>Stim, 33 min</td>
<td>1.25 ± 0.02b</td>
<td>307</td>
</tr>
<tr>
<td>Phosphorus (fmol/100 µg)</td>
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<td></td>
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<tr>
<td>Control</td>
<td>28.47 ± 0.93</td>
<td>465</td>
</tr>
<tr>
<td>Stim, 8 min</td>
<td>32.56 ± 1.91</td>
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</tr>
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<td>Stim, 25 min</td>
<td>28.34 ± 1.60</td>
<td>166</td>
</tr>
<tr>
<td>Stim, 33 min</td>
<td>31.68 ± 1.27</td>
<td>306</td>
</tr>
</tbody>
</table>

* Stim, stimulated.

p < 0.01 as compared with control.

p < 0.05 as compared with control.

**Figure 2.** The intracellular contents of Na⁺, Cl⁻, K⁺, and P of cultured rectal gland epithelial cells, as measured by electron probe analysis. The stimulated values were obtained 8 min after the addition of db-cAMP, theophylline, and VIP. For the controls, N = 465; for the stimulated cells, N = 149.

DISCUSSION

By electron probe analysis, a technique that permits the measurement of ion content in individual cells, we studied rectal gland epithelium in primary...
culture. Of major importance is the finding of a fall in sodium content with stimulation. A significant decrease in intracellular chloride accompanied the drop in intracellular sodium. The marked fall in intracellular sodium indicates that by 8 min after the addition of stimulatory agents, the increase in pump activity is not driven by high levels of internal sodium.

The results reflect the shifts in the intracellular ion content of transporting cells after stimulation. Although a second cell type (central duct epithelium) is present in very small numbers (6), histograms of sodium content in both the control and stimulated states reveal that the cells behave functionally as one population (data not shown). No pockets of trapped extracellular fluid were noted visually nor were there regions of high sodium content and low potassium content. Such pockets would not be expected because cells did not form a confluent monolayer across the entire chip. The basolateral surface has adequate access to the bathing medium, as is evidenced by a rapid influx of sodium and efflux of potassium after ouabain application (B.J. Cohen, C. Lechene, unpublished observations).

The electron probe measures sodium content per mole of cellular phosphorus rather than sodium activity in cell water of cytoplasm. In theory, therefore, it is conceivable that if cellular volume contracted by more than 30%, the concentration of sodium in cell water would remain undiminished. This seems unlikely, however, because measurements of intracellular water in rectal gland cells have shown either no change (9,10) or a minimal (5%) decrease (10,11) in intracellular water after stimulation by cAMP. In unpublished experiments on freshly isolated rectal gland tubules, Lytle found no significant difference in cell volume between quiescent cells and cells stimulated with 20 μM forskolin when measured 10, 15, and 20 min after stimulation (C. Lytle, personal communication). In addition, acute volume loss is generally effected through loss of potassium. Potassium content in our experiments was unchanged in the early time points (at a time when sodium content had already fallen) and fell only 6% over the course of the rest of the experiment. Thus, it is almost certain that the early fall in sodium content that we measured could not be accompanied by a rise in sodium activity because of a simultaneous volume decrease.

We cannot entirely exclude the possibility that stimulation of the pump occurs because of an increase in sodium activity due to the release of compartmentalized sodium in response to stimulation. Because sodium content fell by about one third in our experiments, this mechanism could explain our results only if at least one third of total sodium content were released from compartmentalization. Although there has been considerable speculation that sodium may be compartmentalized to some degree, there is no precedent that we know of for such a release of sodium from an intracellular compartment. Furthermore, a survey of the literature in this area reveals a large range of reported activity coefficients for sodium. This large variation suggests a methodologic pitfall. Because the calculated activity coefficients all depend on comparisons of measured activity with chemically measured intracellular sodium concentrations, we suggest that all may suffer from varying degrees of contamination by extracellular sodium.

cAMP stimulation might theoretically act on any of four different sites in the rectal gland cell: the cotransport-mediated entry of Na and Cl, the Cl and K exit channels, and the Na pump [(Na,K)-ATPase]. Because the pump is exquisitely sensitive to small changes in intracellular Na (12), the rate of pump activity could increase as a result of an increase in the passive entry of Na. That intracellular sodium falls in response to stimulation is clearly of critical importance in demonstrating that the sodium pump is stimulated directly by cAMP.

Previous evidence bearing on this point is conflicting. By using classical chemical methods to calculate the intracellular electrolyte content of whole tissue, Silva et al. reported a fall in intracellular sodium and chloride in slices of isolated perfused rectal glands harvested 30 min after stimulation with cAMP and theophylline (10). On the other hand, Greger and Schlatter using microelectrode measurements of single isolated perfused rectal gland tubules, found an increase in cell sodium activity from 2 to 4 min after stimulation (13). They concluded that the primary event after stimulation was an increase in apical chloride conductance, resulting in a fall in intracellular chloride activity, followed by an increase in sodium entry through the Na-K-2Cl cotransporter and a consequent rise in intracellular sodium concentration, which stimulated the sodium pump. The results presented here indicate that, by 8 min after exposure to cAMP and theophylline, intracellular sodium content had fallen below the basal level present in resting cells. This change cannot be accounted for by a concomitant increase in apical chloride conductance or membrane depolarization because of the absence of sodium channels in this tissue (14) and, therefore, points strongly to direct stimulation of the sodium pump. Several possibilities could account for the difference between our findings and those of Greger and Schlatter. First, our measurements of content began at 8 min after stimulation whereas those of Greger and Schlatter were made within the first few minutes. Direct pump stimulation may have a slower onset than the chloride conductance effect. It is also possible that the difference in the preparation used is responsible (dissected tubules versus cultured cells). If both sodium entry and (Na,K)-
ATPase were stimulated independently, the net effect on intracellular sodium content could be an increase, a decrease, or no change, depending on the relative degree of stimulation of the two processes. A difference in the relative sensitivity of these two transport systems to stimulation in the two preparations could explain the divergent results. Both sets of experimental data agree that there is little or no change in internal content/activity of potassium with stimulation, even though the active uptake of potassium must be increased. Given the likelihood that chloride exit is enhanced by stimulation, the resulting cell depolarization may facilitate potassium exit by an increase in its driving force.

Further evidence for a direct effect of hormonal stimulation on (Na,K)-ATPase comes from ouabain-binding studies. Ouabain binding to a high-affinity site is increased in rectal gland slices (5) and dispersed cells (14) after treatment with db-cAMP and theophylline. The increase in ouabain binding is demonstrable even in the presence of furosemide and after the removal of Na+ or Cl− from ambient solutions—maneuvers that block active transport (5). Such experiments suggest a direct action of cAMP on the binding properties of the transport enzyme.

Precedents for a direct hormonal effect on (Na,K)-ATPase can be found in the actions of thyroxine (15), aldosterone (16,17), and insulin (18,19). In rat adipocytes, for example, stimulation of (Na,K)-ATPase by insulin does not affect sodium entry but, rather, alters the sodium affinity of the pump (18). Stimulation of the β-adrenergic receptors of skeletal muscle with epinephrine also increases (Na,K)-ATPase activity in association with a fall in cell sodium (20) in a way that is independent of the number of pumps (21).

cAMP may simultaneously activate a number of separate membrane events in rectal gland cells that operate together to produce active secretion of chloride. An initial opening of chloride channels and an increase in apical chloride conductance, resulting in membrane depolarization, have been well documented (22). The late fall in cell potassium is likely the result of increased potassium exit via the basolateral channel, which may be enhanced directly by stimulation per se or facilitated by an increase in the driving force for potassium exit secondary to an increase in the apical chloride conductance. An increase in the passive entry of sodium and chloride via the Na+−K+−2Cl− cotransporter also occurs (23) and is apparently associated with an increase in basolateral binding sites for bumetanide analogs (B. Forbush and S. Lear, unpublished observations). This probably explains the initial increase in Na+ activity noted by Greger and Schlatter in micro-punctured perfused rectal gland tubules immediately after stimulation (13). In addition, however, activity of the sodium pump itself appears to be enhanced, so that after some minutes, internal sodium can be lowered below baseline levels at the same time that active sodium transport, driven by basolateral (Na,K)-ATPase, is greatly increased. It is not yet clear whether this is accomplished by the insertion of additional enzyme units into the plasma membrane or by the activation of enzyme(s) already in place.

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