REGULATION OF Na-K-Cl COTRANSPORT IN THE Cl-SECRETING CELLS OF THE SHARK (SQUALUS ACANTHIAS) RECTAL GLAND

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The rectal gland secretes a salty fluid profusely when exposed to agents which enrich intracellular [cAMP] (forskolin, vasoactive intestinal peptide, adenosine). Models of epithelial secretion customarily envision chloride exit as the primary determinant of secretory output, a belief bolstered by the recent discovery of apical chloride channels activated by cAMP via protein kinase A (Greger, Schlatter, and Gogelein, 1985, Pflügers Arch. 403: 446-448). However, since transmembrane ion gradients change little during rectal gland secretion (Greger, Schlatter, Wong, and Forrest, 1984, Pflügers Arch. 402: 376-384), the cell must replenish chloride ions as soon as they are lost without an increase in the force driving inward Na-K-Cl cotransport. The compensatory chloride uptake must therefore involve activation or recruitment of otherwise dormant Na-K-Cl cotransport units.

Our test of this prediction employed secretory tubules enzymatically liberated from thin rectal gland slices, and two probes: (1) [^{3}H]benzmetanide, a potent inhibitor of Na-K-Cl cotransport which binds avidly only to active cotransporters, and (2) a panel of monoclonal antibodies which selectively recognize the 200 kDa cotransport protein. Our studies on intact perfused glands (Forbush, Haas, and Lytle, 1992, Am. J. Physiol. in press) and isolated tubules (Lytle and Forbush, 1992, Am. J. Physiol. in press) established that secretagogues evoke a dramatic (13-fold) increase in [^{3}H]benzmetanide binding site density. Thus, hormonal modulation of rectal gland secretion must involve a coordinated activation of chloride entry basolateral Na-K-Cl cotransporters) and exit (via apical Cl channels).

Binding studies also revealed that the cotransporter is activated by osmotically-induced increases or decreases in cell volume (Lytle and Forbush, 1992, ibid.). In fact, a 45% reduction in cell water content proved to be as effective as secretagogues in promoting [^{3}H]benzmetanide binding, and a 40% increase in cell water was half as effective.

Our recent studies suggested that the functional residency and/or turnover rate of the cotransport protein is controlled by direct phosphorylation (Lytle and Forbush, 1990, J. Cell Biol. 111:312a). Using monoclonal antibodies that selectively immunoprecipitate the 195 kDa cotransport protein (Lytle, Xu, Zhu, Haas, and Forbush, 1990, J. Gen. Physiol. 96:44a), we found that secretagogues or osmotic perturbations produce parallel changes in cotransporter activation state and phosphorylation state (i.e, tubule [^{3}H]benzmetanide binding site density and cotransporter ^{32}P content co-vary).

How chloride entry keeps pace with chloride exit in the face of 30-fold changes in transcellular chloride flow is poorly understood. The sensitivity of the cotransport process to both cAMP and cell volume raised the possibility that hormonal activation of chloride entry represents a corrective response to cell shrinkage following primary activation of Cl channels by cAMP. Our results, however, did not support the "volume-coupling" model. First, to produce the amount of cell shrinkage required to raise [^{3}H]benzmetanide binding to the level evoked by secretagogues (via isotonic KCl loss), the rectal gland cell would have to jettison at least 3 times as much chloride as it possesses. Second, gravimetric measurements of cell water indicated that the rectal gland cell does not experience large sustained shifts in cell volume during secretion as predicted by the volume-coupling model.

An attractive alternative is that cytoplasmic chloride both participates in and modulates the cotransport process. Studies with ion selective microelectrodes do in fact suggest that $[Cl]_i$ declines slightly upon secretion (Greger et al., 1984, ibid.). A sensitivity to $[Cl]_i$ itself would account for the apparent activation of cotransporters by maneuvers known to reduce $[Cl]_i$, such as exposure of the rectal gland cell to furosemide (Greger et al., 1984, ibid.),

to low $[Cl]_o$ (Forbush et al., 1992, ibid.), or to low osmolality (Lytle and Forbush, 1992, ibid.). It would also account for the observation that barium, which prevents conductive K exit (and therefore Cl exit) during secretion, blocks cotransporter activation by secretagogues (Forbush et al., 1992, ibid.). Our hypothesis that $[Cl]_i$ modulates cotransport is supported by four additional observations:

First, we found that pre-incubation of rectal gland tubules in media lacking Na or Cl (NMDG or gluconate substitutions, respectively), maneuvers which reverse the normal direction of Na-K-Cl cotransport and lower $[Cl]_i$, increased [^{3}H]benzmetanide binding site density. This response clearly differs from the stimulatory effect of substrate ions on loop diuretic binding since all [^{3}H]benzmetanide binding assays were conducted in normal shark Ringers over a brief interval immediately folowing pre-incubation. Moreover, this effect of ion omission developed gradually over a 10 min interval, was rapidly reversible, and elevated binding to a level about half that produced by secretagogues alone.

Second, the activation of cotransporters by secretagogues was highly sensitive to external potassium — for example, raising $[K]_o$ from 5 to 80 mM abolished the effect of forskolin on [^{3}H]benzmetanide binding to isolated tubules. The inhibition by K_o was half-maximal at 40-50 mM, reversible, and unrelated to its role as a cotransported substrate (K only stimulated binding to isolated membranes). Besides blocking cAMP-induced [^{3}H]benzmetanide binding, high $[K]_o$ blocked cAMP-induced cotransporter phosphorylation. High $[K]_o$ is also known to depolarize the membrane potential (Greger et al., 1984, ibid.), to elevate intracellular Cl, and to enlarge the rectal gland cell (Kleinzeller et al., 1985, J. Comp. Physiol. 155: 145). However, the effect of high $[K]_o$ on cotransporter activition cannot be due to cell swelling since (a) swelling itself activates the cotransporter, and (b) K_o causes the cell to swell gradually but blocks cotransporter activation immediately. An established link between $[K]_o$ and cotransporter regulation is $[Cl]_i$ — since K_o depolarizes the membrane potential, it should prevent or retard chloride exit through apical cAMP-induced Cl channels.

Third, cotransporter activation by cell shrinkage, like that by secretagogues, was blocked by raising $[K]_o$, but at 5-fold lower concentrations (IC_{50} ~10 mM). Importantly, this inhibition by K_o required both Na and Cl externally. An explanation for this phenomenon emerged from estimates of intracellular ion concentrations before and after cell shrinkage — when the medium was rendered hypertonic by the addition of 580 mM sucrose, the cells lost about half their water by osmosis, cytoplasmic ions were concentrated, and the net driving force for Na-K-Cl cotransport shifted from one overwhelmingly favoring salt entry to one favoring salt loss. We conclude that high K_o acts by fortifying the driving force for inward cotransport — this, in turn, maintains $[Cl]_i$high enough to suppress activation of auxiliary cotransporters.

Fourth, when nominally-active cotransporters in quiescent rectal gland tubules were inhibited with the loop diuretic bumetanide, a maneuver which reduces $[Cl]_i$ (Greger et al., 1984, ibid.), the phosphorylation state of the cotransport protein increased 3-fold within 10 min. Tubules exposed to hypertonicity, secretagogue, or low $[Cl]_o$ for this duration exhibited a 4.3-fold, 8.8-fold, or 5.2-fold increase in cotransporter phosphorylation (n = 3).

In summary, our observations suggest that: (1) cytoplasmic chloride depletion activates the Na-K-Cl cotransport protein by promoting its phosphorylation; (2) hormonal activation of cotransport represents a corrective response to a reduction in $[Cl]_i$ following primary activation of apical Cl channels by cAMP; (3) activation of the cotransporter by cell swelling is a response to intracellular chloride dilution; (4) activation of the cotransporter by cell shrinkage is a response to a reduction in $[Cl]_i$ via outward Na-K-Cl cotransport; (5) $[Cl]_i$ may be the signal that coordinates chloride entry and exit during secretion.

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