Na-K-Cl COTRANSPORT IS MODULATED BY A CHLORIDE-SENSITIVE KINASE AND A CALYCULIN-SENSITIVE PHOSPHATASE IN THE RECTAL GLAND OF THE DOGFISH SHARK (SQUALUS ACANTHIAS)

Christian Lytle, Neils Ringstad, and Biff Forbush
Department of Cellular and Molecular Physiology
Yale University School of Medicine, New Haven, CT 06510

Epithelia move solutes between the compartments they separate in a selective, vectorial, and dynamic fashion. How the polarized cell coordinates solute transport at its opposing surfaces is poorly understood. We have studied "apical/basolateral cross-talk" in the chloride-secreting cells of the dogfish shark rectal gland, where chloride entry through basolateral Na-K-Cl cotransporters keeps pace with chloride exit through apical Cl channels in the face of 30-fold changes in transcellular chloride flow. We postulate that the biochemical reactions governing Na-K-Cl cotransport coordinate chloride entry and exit by sensing and correcting slight deviations in cell chloride concentration.

We have previously established that the activation state of the Na-K-Cl cotransport protein closely parallels the prevailing rate of chloride secretion (Lytle & Forbush, 1992a, Am. J. Physiol. 262:C1009). Thus, hormonally-induced changes in ion transport are accompanied by proportional changes in (a) the rate at which the rectal gland tubule cell binds [^{3}H]benzmetanide (an analog of bumetanide that binds avidly to active Na-K-Cl cotransporters) and (b) the phosphorylation state of the 195 kDa Na-K-Cl cotransport protein itself, measured by immunoprecipitation of the solubilized membrane protein from ^{32}P-labeled tubules using a monoclonal antibody (Lytle, Xu, Biemesderfer, Haas, & Forbush, 1992, J. Biol. Chem. 267:25428). The tight correlation between [^{3}H]benzmetanide binding and cotransport protein phosphorylation suggests that phosphorylation causes activation (Lytle & Forbush, 1992b, J. Biol. Chem. 267:25438).

Cotransport protein activation and phosphorylation are highly responsive to stimuli that raise intracellular cAMP (VIP and forskolin) as well as those that do not (osmotic perturbations). Dual sensitivity to cAMP and cell volume raises the possibility that hormonal activation of chloride entry represents a corrective response to cell shrinkage following primary activation of Cl channels by cAMP. However, gravimetric measurements of tubule cell water have shown that the rectal gland cell does not experience a sustained reduction in cell volume in response to secretagogues that is large enough to account for the observed increase in cotransport activity (Lytle & Forbush, 1992a).

Another factor that could regulate cotransport is cytoplasmic chloride. In support of this possibility, we have previously found that the activation state of the cotransport protein increase dramatically in response to maneuvers known to reduce cell [Cl], such as incubation of rectal gland tubules in a medium containing secretagogues or loop diuretics, or one lacking Na or Cl, or one that is hypotonic (Lytle & Forbush, 1992, Biophys. J. 61: A384). An inhibitory effect of cell chloride on the biochemical reactions which turn on the cotransport protein would also

account for the inhibitory effect of external potassium on cotransport protein activation. An example of this effect is illustrated in Fig. 1, which shows that forskolin (a potent secretagogue and adenylate cyclase agonist) increases the activation state of the cotransport protein 17-fold, but only when external [K] is low. High $[K]_o$ also blocks phosphorylation of the cotransport protein in response to forskolin (not shown). Potassium presumably inhibits by depolarizing the membrane potential, eliminating the driving force for conductive chloride exit, and preventing the decrease in cell [Cl] that normally accompanies secretion (Greger, Schlatter, Wong, and Forrest, 1984, Pflügers Arch. 402:376-384). In support of this interpretation, we find that potassium is without effect in media containing low [Cl], and that intracellular [Cl] increases 30% when forskolin-treated tubules are exposed briefly (10 min) to 80 mM K.

We now report that calyculin A, a relatively specific inhibitor of protein phosphatase 1 and 2a, is a potent activator of the Na-K-Cl cotransport protein (Fig. 1). The effect of calyculin was rapid, sustained, and partially additive with that of forskolin after 9 min. The calyculin effect was accompanied by an increase in the phosphorylation state of the cotransport protein itself (not shown). These results suggest that a robust calyculin-sensitive phosphatase operates continuously to counteract a stimulatory kinase.

Okadaic acid, another phosphatase inhibitor that is structurally different from calyculin, had no detectable effect on cotransport, even at a concentration (5 μM) ten times the maximally effective dose of calyculin. The sensitivity of the cotransport process to calyculin but not okadaic acid suggests that it is regulated by type 1 protein phosphatase. However, it remains possible that okadaic acid reaches its intracellular site of action more slowly than does calyculin.

Interestingly, the activation of the Na-K-Cl cotransport protein by calyculin was not associated with an increase in tubule oxygen consumption (data not shown). Cells treated with calyculin for 15 minutes exhibited the same response to secretagogues as did control cells. Thus, after a brief delay (~3 minutes), both calyculin-treated and control cells consumed oxygen ~10 faster in response to forskolin. The respiratory response was almost entirely blocked by bumetanide or ouabain. The ability of calyculin to activate Na-K-Cl cotransport but not transcellular chloride flow suggests that the chloride entry and exit pathways are inactivated by different protein phosphatases.

Another important feature of the calyculin effect was its dependence on the cotransport protein's prevailing state of activation. While this state is low in resting tubules, it can be reduced even further (to a level indistinguishable from zero) by incubating the tubules in a medium containing 80 mM potassium for 10 min. In tubules rendered quiescent this way, the phosphatase inhibitor had almost no effect (Fig. 1). We surmise that the kinase that activates the cotransporter is suppressed by the increase in cell [Cl] brought on by high external [K]. In support of this interpretation, we found that potassium blocked the combined effect of forskolin and calyculin A if present before and during their application (Fig. 1, bar on right). Since external potassium inhibits cotransport protein activation even when the relevant phosphatase is disabled, it must act on the pertinent kinase. Thus, we assume that external potassium acts by suppressing a chloride-inhibitable kinase

which modulates Na-K-Cl cotransport. The characteristics of this kinase and its role in apical/basolateral cross-talk are currently under investigation.

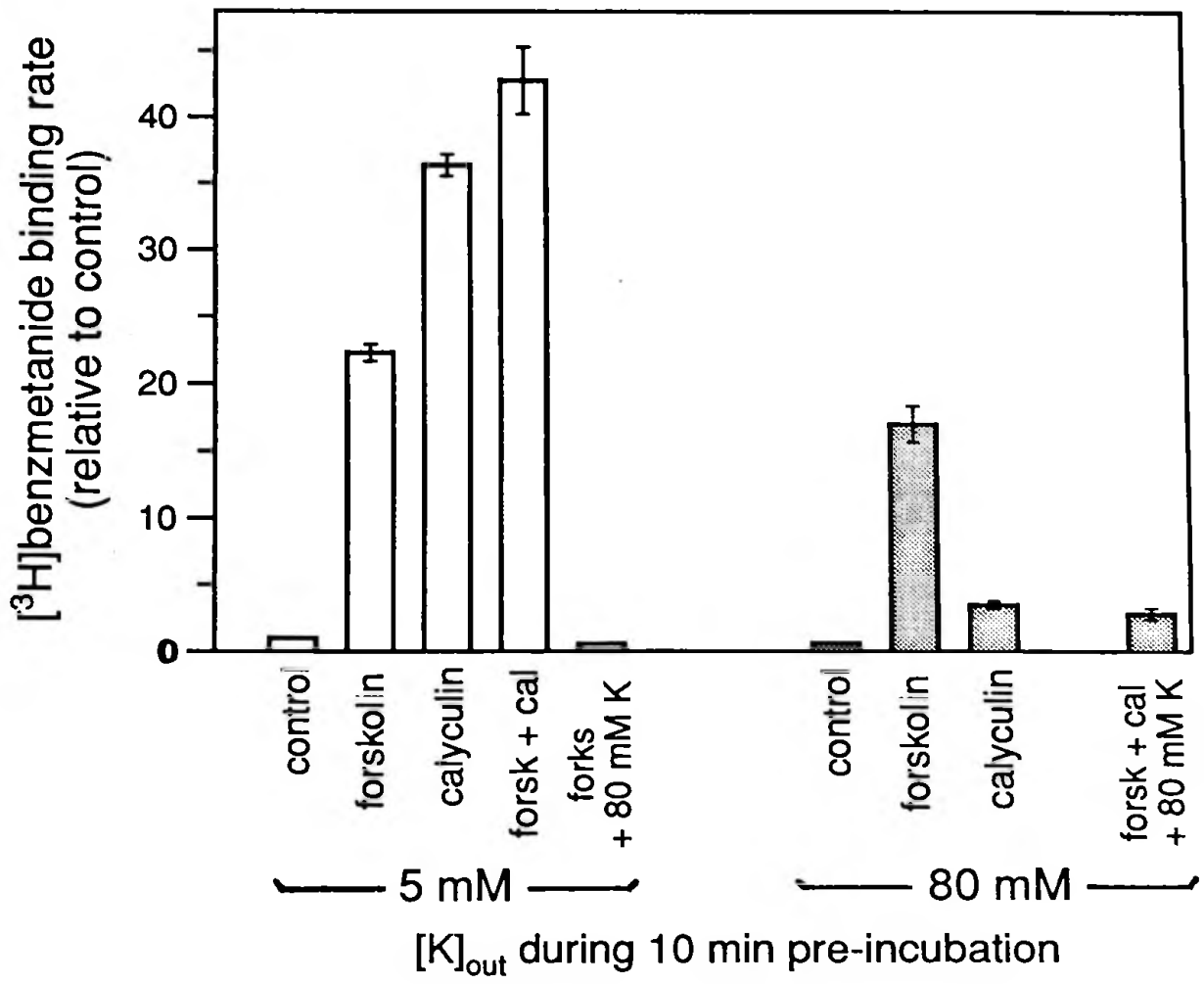

Figure 1. Activation of the Na-K-Cl cotransport protein by cAMP (forskolin) and phosphatase inhibition (calyculin A). Secretory tubules, enzymatically isolated from thin rectal gland slices, were pre-incubated for 10 min in either physiological (5 mM) or high (80 mM) potassium, then exposed to a medium containing [^{3}H]benzmetanide and forskolin (10 μM) or calyculin A (0.5 μM). The initial rate of specific [^{3}H]benzmetanide binding, a parameter proportional to cotransporter activation, was measured 6 min later over a 3 minute interval (for detailed methods, see Lytle & Forbush, 1992, Am. J. Physiol. 262:C1009). Data represent mean ± SD of triplicate measurements. Similar results were obtained in two other experiments.

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