COORDINATE MODULATION OF THE Na-K-Cl COTRANSPORTER AND OF THE SECRETORY RATE IN THE INTACT RECTAL GLAND OF THE SPINY DOGFISH, SQUALUS ACANTHIAS

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As in other salt-secreting epithelia, NaCl secretion in the rectal gland of the dogfish shark is mediated by four transport pathways: Na pumps, K channels, Cl channels (CFTR), and Na-K-Cl cotransporters (Epstein, F.H., and Silva, P., Ann. N.Y. Acad. Sci., 456, 187-197, 1985). While many previous studies have focused on Cl channels as the element which is central to regulation of secretion, we have determined that the Na-K-Cl cotransporter is modulated by a process involving direct phosphorylation of the transport protein (Lytle, C., and Forbush, B. III, J. Biol. Chem., 267, 25438-25443, 1992). Our studies have utilized the fact that the cotransporter binds loop diuretics only in its activated state -- both in the intact rectal gland (Forbush, B. III, Haas, M., and Lytle, C., Am. J. Physiol., 262, C1000-C1008, 1992), and in collagenase-isolated tubules (Lytle, C., and Forbush, B. III, Am. J. Physiol., 262, C1009-C1017, 1992) we have found a dramatic stimulation of [^{3}H]benzmetanide binding by hormonal secretagogues like VIP (from 11 to 104 pmol/mg total protein in the intact gland). Here we report the results of experiments in which we have determined the time course of cotransporter activation and the rate of secretion in the intact gland.

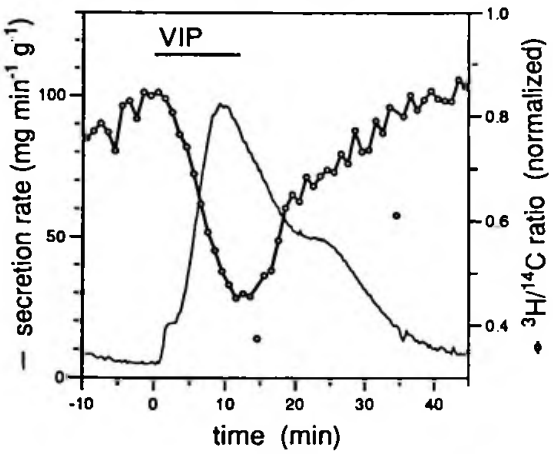
Because of the high density of Na-K-Cl cotransporters in the rectal gland, a substantial fraction of [^{3}H]benzmetanide in the perfusion solution binds to transporters and is removed during the passage of the solution through the gland. It is fortuitous that this fraction is between 10 and 60%, depending upon the level of cotransporter activation. Thus we have been able to perfuse with [^{3}H]benzmetanide and monitor the rate of [^{3}H]benzmetanide binding continuously as the arterial-venous difference, using 1 min collection intervals. We use [^{14}C]inulin as a marker for total perfusate volume, and express the amount of [^{3}H]benzmetanide in the perfusate as a normalized [^{14}C]inulin/[^{3}H]benzmetanide ratio.

We have verified that the fraction of [^{3}H]benzmetanide removed in passage through the gland exhibits the characteristics of a simple flow/binding model. Removal of [^{3}H]benzmetanide from the perfusate is completely blocked ([^{3}H]benzmetanide/[^{14}C]inulin > 0.95) on addition of furosemide or bumetanide at high concentrations to compete for binding sites. The fraction of [^{3}H]benzmetanide removed is inversly proportional to flow rate in a saturable manner predicted by a simple flow/binding model. Finally, the fractional rate of [^{3}H]benzmetanide removal is independent of [^{3}H]benzmetanide concentration over a wide range, consistent with pseudo-first order binding kinetics. Thus it is possible to measure the fractional rate of [^{3}H]benzmetanide binding by using high specific activity, and a concentration of [^{3}H]benzmetanide which is too low to appreciably inhibit secretion (1-5 nM, less than 0.03 of K_i). The method is limited by a low frequency time constant equal to the dissociation time constant of [^{3}H]benzmetanide from the transporter (> 30 min); in

practice this can be partially circumvented by periodically increasing the concentration of the [^{3}H]benzmetanide/[^{14}C]inulin mixture.

We have also developed a simple and sensitive gravimetric measurement of the rate of secretion. The duct effluent is conducted through an 8 in length of PE-90 tubing to a beaker on the pan of an analytical balance. Readings from the balance (time constant ~5 s) are continuously monitored with a PC computer, and the incremental weight is recorded as the rate of secretion at 15 s intervals.

Fig. 1. Rate of secretion and [^{3}H]benzmetanide binding in an intact rectal gland



The results of a typical experiment utilizing these techniques are shown in Figure 1; similar results were obtained in more than 15 other experiments. The gland was perfused with normal ringer solution containing the [^{3}H]benzmetanide/[^{14}C]inulin mixture and supplemented with porcine VIP (50 nM) during the interval from 0 min to 12 min. After initiation of perfusion with VIP, the rate of secretion (light line) is seen to increase rapidly after a ~1 min delay; however during the interval from 1 min to 2 min, the rate pauses at a temporary plateau which is only ~15% of the maximal rate. During this period, the rate of [^{3}H]benzmetanide binding does not increase significantly, as determined from the rate of [^{3}H]benzmetanide removal from the perfusion solution (open circles). After the 2 min time point, both secretion rate and [^{3}H]benzmetanide binding rate increase rapidly to a peak and again decline, first to a plateau (see 20-25 min), and then to baseline levels. In other experiments (not shown), it was noted that when VIP perfusion was maintained for a longer period, the rates were maintained at the level of the (20-25 min) plateau.

We are particularly interested in the events in the first few minutes. Our explanation, based on previous experiments (Forbush et al., 1992; Lytle and Forbush, 1992a, 1992b) is as follows: On stimulation with VIP, PKA-mediated phosphorylation of Cl channels results in a rapid increase in secretory rate by the 1 min time point. However the net rate is limited by the still-dormant Na-K-Cl cotransporters (1-2 min). Lowered intracellular Cl is the necessary stimulus for phosphorylation of the Na-K-Cl cotransporter (via kinases other than PKA (Lytle and Forbush, 1992a; Lytle, C., and Forbush, B. III, Biophys. J., 61, 384a, 1992)), and as the Na-K-Cl cotransporter is activated, secretion increases at the same time.

This proposal is supported by the results of several related experiments. When perfusate Cl is decreased to 20 mM to decrease intracellular Cl, the cotransporter is activated but the rate of secretion is not increased (Forbush et al. 1992; Lytle and Forbush, 1992c). In experiments similar to that in Fig. 1, when stimulation with VIP in normal ringers follows a period of perfusion with low Cl, there is no initial plateau -- near-maximal secretion is reached within 2 min (not shown). We attribute this to the

fact that the transporter had been pre-stimulated during exposure to low Cl.

Exposure of rectal gland cells to 80 mM K abolishes cotransporter activation (Lytle and Forbush, 1992c) -- presumably because it prevents cellular Cl loss. In the intact gland we find that 80 mM K blocks both cotransporter activation and secretion in the presence of VIP. On subsequent return to normal Ringers, the time course of increase in secretion and cotransporter activation is essentially the same as in Fig. 1, except that the initial 1 min lag is absent (data not shown). This indicates that high K_{ext} blocks an early step in the activation sequence, consistent with the above model and with the hypothesis that high K_{ext} blocks Cl loss.

In a preliminary experiment, exposure to calyculin A, an inhibitor of protein phosphatase, had an effect similar to that of exposure to low Cl, in that it activated the cotransporter without activating secretion and abolished the early plateau phase in secretion. This is consistent with the above model, and with an increase in phosphorylation level of the cotransporter. However in other experiments the calyculin A effect was smaller -- there is evidence that the variability was due to a bad lot of calyculin A.

In summary, our experiments are consistent with the hypothesis that both Cl channels and Na-K-Cl cotransporters must be activated to produce a maximal secretory rate. The present data provide temporal evidence strongly in favor of a model in which Cl channels are activated as a primary event, and cotransporters are activated only when intracellular Cl falls. The correspondence of the secretory rate and the level of cotransporter activation provide an indication that Cl entry through the cotransporter is a rate limiting step in the process of secretion under most conditions.

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