REGULATORY RESERVE OF Na-K-Cl COTRANSPORTERS IN THE INTACT RECTAL GLAND OF THE SPINY DOGFISH, SQUALUS ACANTHIAS

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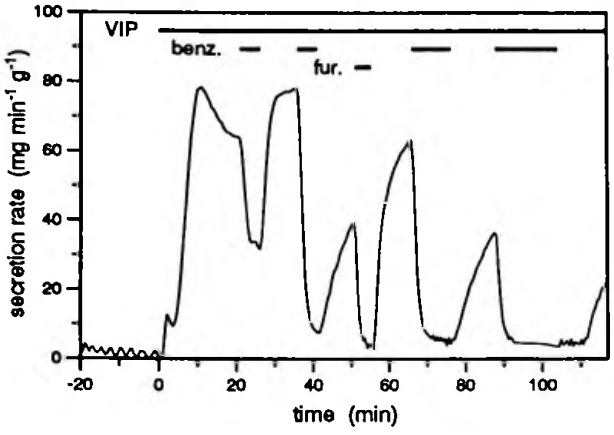
In the rectal gland of the dogfish shark as well as in other secretory epithelia, Na pumps, Na-K-Cl cotransporters, Cl channels, and K channels work together to bring about Cl secretion (Frizzell, R.A., Field, M., and Schultz, S.G. , Am. J. Physiol., 236, F1-F8, 1979; Epstein, F.H., and Silva, P. , Ann. N.Y. Acad. Sci., 456, 187-197, 1985). It is now clear that Cl channels and Na-K-Cl cotransporters must both be activated to produce a maximal secretory rate (Greger, R., Schlatter, E., Wang, F., and Forrest, J.N. Jr. , Pflugers Arch., 402, 376-384, 1984; Forbush, B. III, Haas, M., and Lytle, C. , Am. J. Physiol., 262, C1000-C1008, 1992). The data are consistent with a model in which Cl channels are activated as a primary event, and cotransporters are activated only when intracellular Cl falls (Lytle, C., and Forbush, B. III , Biophys. J., 61, 384a, 1992; Forbush, B., Behnke, R., Forbush, J., and Xu, J.-C. , Bull. Mt. Desert Is. Biol. Lab., 32, 42-44, 1993). Utilizing the intact perfused rectal gland, our recent studies have addressed two related questions regarding this process: 1) is the entire population of cotransporters activated during secretagogue-stimulated secretion? 2) Is an intracellular pool of cotransporters involved?

Are all of the Na-K-Cl cotransporters involved during secretion under our experimental conditions? To address this question we examined recovery of the secretory rate following inhibition with benzmetanide. We knew that benzmetanide binds only to activated cotransporters (Forbush, B. III, Haas, M., and Lytle, C. , ibid.; Lytle, C., and Forbush, B. III , Am. J. Physiol., 262, C1009-C1017, 1992) and that bound [^{3}H]benzmetanide dissociates slowly from the rectal gland cotransporter: $t_{1/2}$ = 40-80 min^{-1} at 15-20°C (Forbush, B. III, Haas, M., and Lytle, C. , ibid.; and Forbush, unpublished). Thus, if all cotransporters were required for secretion at the maximal rate, recovery should proceed with a slow time course following a brief inhibitory pulse with unlabeled benzmetanide. Experiments such as the one illustrated in Fig. 1 demonstrate that this expectation is not met.

We measured secretion continuously using an analytical balance sampled at 12 sec intervals (Forbush, B., Behnke, R., Forbush, J., and Xu, J.-C. , ibid.). Fig. 1 illustrates the results of a typical experiment: stimulation with VIP results in a peak in secretion within 10 min (note the low plateau at 3 min which we have previously discussed: Forbush, B., Behnke, R., Forbush, J., and Xu, J.-C. , ibid.). At 21 min, 5 μM benzmetanide was added and it rapidly produced 50% inhibition of secretion. At the end of the 5 min benzmetanide pulse, secretion rebounded rapidly, in contrast to the known low rate of benzmetanide dissociation. We attribute the discrepancy to additional cotransporters which have not been inhibited and which are rapidly activated as a conseqence of a decrease in intracellular [Cl] that occurs during benzmetanide inhibition (Lytle, C., and Forbush, B. III , Biophys. J., 61, 384a, 1992). Consistent with this, the slope of this recovery from benzmetanide inhibition is

essentially identical to the slope of the rise in secretion following VIP addition.

A second pulse of 5 μM benzmetanide at 36 min resulted in 90% inhibition of transport: this time, as well as for subsequent pulses at 66 and 88 min, recovery on removal of benzmetanide was much slower; it may be contrasted with the very fast rapid recovery after a pulse with the rapidly reversable inhibitor furosemide at 51 min. Our interpretation is that two 5 min pulses are sufficient to allow all of the cotransporters to be activated and inhibited, after which there is no longer a pool of cotransporters to be activated and produce a rapid recovery. However it is readily apparent that the rate of recovery is still ~5 fold more rapid (est. $t_{1/2}$ ~9 min) than the above-noted rate of benzmetanide dissociation. This difference is predicted if the total number of cotransporters exceeds that necessary to produce maximal flux; in that case the observed ratio between recovery and dissociation time constants is equivalent to the ratio of "steady-state activated" to total cotransporters.



Comparing the first and second benzmetanide pulses we note that 50% inhibition was reached in the first whereas 90% was attained in the second. This too is consistent with the hypothesis of an excess of cotransporters -- during the initial pulse, new cotransporters are constantly being activated as others are inhibited. Other experiments show that in one long pulse inhibition is biphasic; the ~50% inhibition level is reached rapidly, followed by a slower phase of inhibition to >90%.

Importantly, from the relative rates of recovery of secretion and of [^{3}H]benzmetanide release in these experiments we estimate that only ~20% of the total pool of cotransporters are activated in order to carry out the steady-state secretion (~2200 μeq Cl hour^{-1} gram tissue^{-1}). In addition to allowing an estimate of an upper limit on the maximal transport rate that could be attained with the available Na-K-Cl cotransporters (~11000 μeq Cl hour^{-1} gram^{-1}), this finding requires a reassessment of the translocation turnover number of the rectal gland Na-K-Cl cotransporter. The previous estimate of 66 s^{-1} (Forbush, B. III, Haas, M., and Lytle, C. , ibid.) was based on the assumption of full cotransporter activation. Since it is now clear that only 20% of the transporters mediate the steady-state flux, the estimate of turnover number must be revised upwards to ~330 s^{-1}. With a reasonable correction for the temperature difference (Forbush, B. III, Haas, M., and Lytle, C. , Am. J. Physiol., ibid.), this is in very good agreement with the value determined in duck red cells (Haas, M., and Forbush, B. III , J. Biol. Chem., 261, 8434-8441, 1986).

Is there a vesicular pool of cotransporters that is in communication with the plasma membrane? Our previous results have been consistent with the hypothesis that direct phosphorylation of the Na-K-Cl cotransporter is necessary and sufficient to bring about

its activation in the plasma membrane (Lytle, C., and Forbush, B. III , J. Biol. Chem., 267, 25438-25443, 1992), but they do not rule out a a parallel mechanism involving regulated membrane insertion of cotransporters. We have tested this possibility by examining the rate of release of [^{3}H]benzmetanide from cotransporters in the continuously perfused intact rectal gland. We reasoned that if the cotransporters were regulated by trafficking from an intramembraneous pool, the release of [^{3}H]benzmetanide to the extracellular fluid should decrease on deactivation of the cotransporter, as the transporters and [^{3}H]benzmetanide are internalized. We used control pulses of furosemide to prevent released [^{3}H]benzmetanide from rebinding to cotransporters and examined [^{3}H]benzmetanide release in continuous perfusion. In these experiments we found no significant difference (<10%) in the dissociation rate under activated and non-activated conditions. These results appear to be inconsistent with the trafficking hypothesis and thus lend support to the idea that the rectal gland Na-K-Cl cotransporter is regulated entirely by direct phosphorylation.

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