SQUALUS ACANTHIAS Na-K-Cl COTRANSPORTER (sNKCC1) DEPHOSPHORYLATION IS AFFECTED BY CONFORMATIONAL CHANGE.

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In Cl-secreting epithelial cells, including those of spiny dogfish (*Squalus acanthias*) rectal gland, activation of the Na-K-Cl cotransporter (NKCC1) is achieved by phosphorylation of threonine residues in N-terminus of the cotransporter protein. Phosphorylation by a volume- and chloride-sensitive kinase is counterbalanced by protein phosphatase 1 (PP1), which has a binding site in the N-terminus upstream of the phosphorylated residues (Behnke et al., FASEB J. 1999; 13(4): A397). We have previously developed an antibody that specifically recognizes the regulatory threonines T184 and T189 only when they are phosphorylated (anti-P-NKCC, Flemmer et al., Bull MDIBL 38:80-2, 1999). In this study we take advantage of the high specificity of anti-P-NKCC to analyze the Na-K-Cl cotransporter dephosphorylation event in the solubilized cotransporter.

Fresh tubule suspensions obtained from spiny dogfish rectal gland (2.5% cytocrit) were stimulated with 10-20 μM forskolin for 15 min at 15 °C to maximally activate the cotransporter. Tubules were solubilized in 2% Triton X-100, and incubated under several experimental conditions at 15 °C for 0-15 min. Samples from each lysate were taken at given time points into dephosphorylation stop solution (1M H_3PO_4). Aliquots of each lysate were diluted in transfer buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, 10% ethanol) and dot-blotted on Immobilon-P membrane using standard procedures. Phosphorylated NKCC was detected using anti-pNKCC a peroxidase secondary antibody and ECL (Amersham); light emission was quantitated using a CCD camera. Rate constants for dephosphorylation were determined by least-squares best fits to a single exponential process. In the course of these studies we observed a non-linear relationship between the inverse of the lysate dilution and the rate of dephosphorylation, consistent with a slowly reversible binding interaction between PP1 and NKCC1.

Bumetanide inhibition is known to increase phosphorylation of NKCC1 in rectal gland tubules (Lytle et al, Amer. J. Physiol. 1992), and we have previously observed that bumetanide causes a several-fold reduction in sNKCC1 dephosphorylation rate in solubilized rectal gland tubules

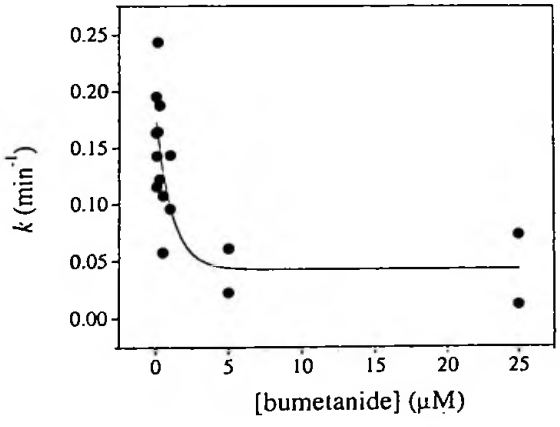

Figure 1: Rate of dephosphorylation of NKCC1 as a function of bumetanide concentration. Data from two experiments are shown together.

(Gimenez et al., Bull MDIBL 38: 83-4, 1999). In the current study we examined the concentration dependence of bumetanide in bringing about this effect. The half maximal concentration for inhibition of dephosphorylation was found to be 0.5-0.8 μM bumetanide (Figure 1), which is in good agreement with the $K_{0.5}$ for cotransporter inhibition. This is very strong support for the argument that the effect of bumetanide is to lock the transporter in a conformation in which it cannot be readily dephosphorylated by PP1.

We have also carried out experiments to examine whether the transport conformation of NKCC1 affects dephosphorylation rate. We have focused initially on anion composition because there is considerable experimental support for the hypothesis that the principal factor regulating NKCC1 phosphorylation is intracellular [Cl]. As illustrated in Figure 2, we found that when Cl was removed from the medium and replaced by nitrate, there was a 3-fold decrease in the dephosphorylation rate, similar to the effect brought about by bumetanide inhibition. It should be noted that we have not fully characterized the anion dependence and at this point it is not known whether the active anion in figure 2 is Cl or nitrate.

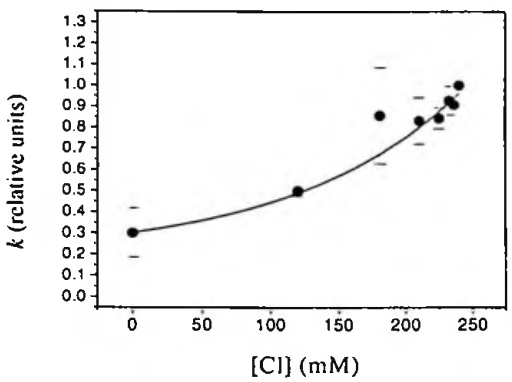

Figure 2: Rate of phosphorylation of NKCC1 as a function of Cl concentration, Cl being replaced with NO_3. Results are mean ±SEM of 3 experiments.

The anion effect in figure 2 occurs at rather high Cl concentration. If indeed Cl is the active player this would suggest a model in which only the fully loaded carrier is refractory to dephosphorylation. The effect is in the right direction to form part of the explanation of NKCC1 regulation by intracellular Cl, since higher [Cl] would promote dephosphorylation and deactivate the transporter. On the other hand, the magnitude of the effect is too small and the concentration dependence too shallow for this phenomenon to account for the majority of the regulatory behavior by intracellular [Cl].

Supported by NIH grant DK-47661.