Na-K-Cl COTRANSPORTER REGULATION IN THE RECTAL GLAND OF *SQUALUS ACANTHIAS*: A STUDY WITH ANTI-PHOSPHORYLATED COTRANSPORTER ANTIBODIES.

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In secretory epithelia such as the rectal gland of the spiny dogfish (*Squalus acanthias*) the Na-K-Cl cotransporter (NKCC1) functions in series with apically localized chloride channels to carry out the movement of chloride from the blood to the gland lumen. Overall secretion is dramatically increased by agents which increase cellular cAMP. During alterations in the rate of secretion, cAMP dependent protein kinase (PKA) activation of Cl channels must be coordinated with an increase in NKCC activity. Our previous studies have demonstrated that this apical-basolateral communication is achieved through alterations in intracellular Cl – a Cl decrease causes activation of NKCC through phosphorylation of the transporter by a yet-unknown kinase (Lytle and Forbush, *Am. J. Physiol.* 270: C437-48, 1996).

In the present study we have used an antibody raised against phosphorylated NKCC (anti-P-NKCC, Flemmer & Forbush, *Bull. MDIBL*, 38, 1999) to examine further the characteristics of NKCC regulation in the rectal gland through phosphorylation-dephosphorylation events, and its dependence on the intracellular chloride concentrations. This antibody detects only the phosphorylated form of NKCC1 and is so specific that it can be reliably used in dot blots. Tubules from dogfish rectal gland were prepared by collagenase treatment. Tubule suspensions were incubated in STS (in mM: 240 NaCl, 4 KCl, 2.5 $CaCl_2$, 1.25 $MgCl_2$, 1 Na_2HPO_4, 20 Hepes pH 7.8, 70 TMAO, 350 urea, 5.6 glucose) to which experimental modifications were made or substances to be tested were added. After the indicated incubation time, an aliquot of 50 µl tubule suspension was transferred to an equal volume of 1% Triton X-100 in 1M H_3PO_4, to lyse the cells and prevent further phosphorylation and dephosphorylation. 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 with a peroxidase secondary antibody and ECL (Amersham); light emission was quantitated using a CCD camera.

Under resting conditions, only one tenth of the NKCC is phosphorylated. Addition of 20 µM forskolin to the bath induced phosphorylation of the cotransporter, which reached a maximum after 5-10 min. When external potassium was increased to 80 mM to depolarize the cell and allow chloride to enter the cell, forskolin-induced phosphorylation was severely impaired. 10 µM calyculin A induced a higher phosphorylation of NKCC than forskolin. Tubules that were incubated in the presence of bumetanide 250 µM, showed twice the phosphorylation observed under resting conditions, an effect that could be attributed to either a decrease in intracellular chloride following NKCC inhibition, or to a direct conformational change caused by bumetanide, that prevents dephosphorylation of the NKCC protein. These results are in good agreement with previous results by Lytle and Forbush using ^{32}P as a tracer for NKCC phosphorylation.

To investigate chloride dependence of NKCC phosphorylation, cells were incubated in high potassium (80 mM) medium containing 0, 60, 120 or 240 mM chloride, in absence (control cells) or presence of 250 μM bumetanide. After 10 minutes, a sample was withdrawn, and 20 μM forskolin was added to the remaining tubules, followed by a second 10 min incubation period. Low external chloride dramatically increased the phosphorylation of NKCC, both in control and bumetanide treated cells (x2 at 60 mM, x3 at 0 mM), supporting the idea that reduced intracellular chloride is the signal that activates the cotransporter. Subsequent forskolin incubation induced a 3-fold increase in NKCC phosphorylation level of control cells. Surprisingly, the response to forskolin in cells incubated at 0 external chloride was significantly lower in bumetanide treated cells. This result suggests that forskolin-mediated NKCC phosphorylation is sensitive to bumetanide binding to the cotransporter.

To discriminate whether the bumetanide effect is dependent on changes in intracellular chloride, we tested the effect of bumetanide in the NKCC dephosphorylation rate in a solubilized sample. Cells were stimulated with 20 μM forskolin for ten minutes and then incubated with bumetanide for 1 minute (an aliquot was taken to check that this incubation time is not enough to increase further forskolin-stimulated phosphorylation of NKCC). Tubules were solubilized with 1% Triton-X100 (we observed this procedure stops phosphorylation but allows calyculin A-sensitive dephosphorylation to take place), and aliquots were removed at given times to study the dephosphorylation time course. As is shown in figure 1, bumetanide treated cells exhibited a lower rate of dephosphorylation than control cells, supporting the idea that bumetanide is actually reducing the availability of the phosphorylated NKCC protein to its specific phosphatase, independent of any change in cytoplasmic chloride caused by NKCC activity inhibition.

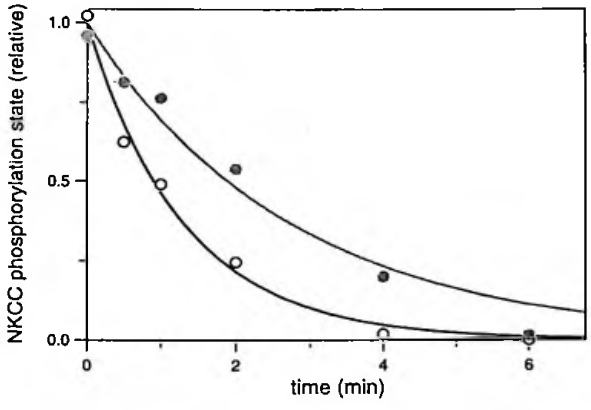

Fig 1: Bumetanide effect on NKCC dephosphorylation. Dephosphorylation rates were measured in NKCC from control cells (open circles) and bumetanide treated cells (black circles) after ten min exposure to 20 μM forskolin and solubilization with 1% Triton X-100.

In conclusion, we have applied a new tool, an anti-phosphorylated NKCC antibody, to the study of the Na-K-Cl cotransporter regulation in the dogfish rectal gland. These studies confirm observations made previously using ^{32}P incorporation. We have also found that bumetanide prevents the cotransporter from both being phosphorylated and dephosphorylated, thus raising the possibility that bumetanide binding to the cotransporter induces a conformational change in the protein that makes it less available to both the kinase and the phosphatase.

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