REGULATION OF THE $Na^+K^+2Cl^-$ COTRANSPORTER IN ISOLATED *IN VITRO* PERFUSED RECTAL GLAND TUBULES OF *SQUALUS ACANTHIAS*

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Rectal gland tubules of *Squalus acanthias* were perfused *in vitro* and loaded with the pH-sensitive dye BCECF (Greger, R. and Schlatter E., *Pfluegers Arch. Eur. J. Physiol.* 402:63-75, 1984; Benning, N. et al., *Pfluegers Arch. Eur. J. Physiol.* 432:126-133, 1996). The rate of the $Na^+K^+2Cl^-$ cotransporter was measured by the ammonium pulse technique (Paulais, M. and Turner, R.J., *J. Clin. Invest.* 89:1142-1147, 1992). Ammonium is able to ride on the K^+ binding site of this cotransporter which leads to cytosolic acidification (Kikeri, D. et al., Nature 339:478-480, 1989; Bleich, M. et al., *Pfluegers Arch. Eur. J. Physiol.* 429:345-354, 1995). The rate of acidification (monitored as Δ fluorescence 488/436 nm per time) is proportional to the rate of the cotransporter. Data are expressed as mean ± 1 SEM. Paired students t-test was used to examine for statistical differences. A P-value of < 0.05 was chosen to indicate statistical significance.

In addition, transepithelial voltage (V_{te}), transepithelial resistance (R_{te}), equivalent short circuit current (I_{SC}) and basolateral membrane voltage (V_{bl}) where measured by established methods (Greger, R. and Schlatter E., *Pfluegers Arch. Eur. J. Physiol.* 396:315-324, 1983; Greger, R. and Schlatter E., *Pfluegers Arch. Eur. J. Physiol.* 402:63-75, 1984).

First, the loop diuretic selectivity of this cotransporter was examined using electrophysiological methods. It was shown that unlike the kidney type (Greger, R.: Loop diuretics, in: Handbook Exptl. Pharmacol., Springer, New York 1995), but like the colonic type (Ecke, D. et al., *Pfluegers Arch. Eur. J. Physiol.* 431:427-434, 1996), this rectal gland tubule cotransporter has a higher affinity for azosemide than for bumetanide or furosemide. The IC_{50} values were as follows: 1.2 µmol/l in case of azosemide (n=25) , 7.4 µmol/l in case of bumetanide (n=12), and 14.8 µmol/l in case of furosemide (n=50).

Next, we examined whether this cotransporter, in the absence of secretagogues, is activated by a reduction in cytosolic Cl^- activity (Greger, R. et al., *Pfluegers Arch. Eur. J. Physiol.* 402:376-384, 1984; Lytle, C. and Forbush, B., *Am. J. Physiol.* 270:C437-C448, 1996). To this end, the rectal gland tubules were preincubated for 10 minutes in a solution containing 6 mmol/l Cl^-, then 20 mmol/l ammonium (in the presence of at total of 26 mmol/l Cl^-) was added to the bath. The rate of acidification was monitored and compared in paired fashion to the rate when the tubule was preincubated in normal shark Ringer's. This solution contained in mmol/l: NaCl 278; urea 280; trimethylamineoxide (TMAO) 70; KH_2PO_4 0.4; K_2HPO_4 2.0; Ca-gluconate 2.5; $MgCl_2$ 3; D-glucose 5, Hepes 5 and was gassed with O_2. The pH was titrated to 7.4. The rate of acidification was increased significantly by a factor of two to three: 7.22 ± 1.82 versus 16.15 ± 3.27 (n=7) when the tubules where preincubated in low Cl^-.

In the next series, again in the absence of secretagogues, the effect of cell volume on the rate of the $Na^+K^+2Cl^-$cotransporter was monitored by examining the tubules in the absence and presence of additional osmolytes (mannitol 300 mmol/l, urea 200 mmol/l). The data indicate, that

cell shrinkage by manitol enhances the rate of the cotransporter significantly by a factor of 2: 9.16 ± 1.59 versus 14.7 ± 4.92 (n=6). The addition of urea had no effect: 4.58 ± 0.38 versus 4.95 ± 0.33 (n=7).

These data indicate that the $Na^+K^+2Cl^-$cotransporter can be activated in the absence of cAMP by a reduction of cytosolic Cl^- activity and/or by cell shrinkage. To examine the potential additional effect of cAMP producing agonists another series of experiments was performed. In this series 1 mmol/l Ba^{2+} was added to the bath solution. This led to a significant fall of I_{SC}: from -724 ± 119 to -225 ± 43 $\mu A/cm^2$ (n=11), an increase in transepithelial resistance from 13.8 ± 1.15 to 17.95 ± 1.73 Ωcm^2 (n=11) and a depolarization of V_{bl} from -65.6 ± 4.7 to -33.3 ± 2.8 mV (n=10). Therefore, Ba^{2+} increased cytosolic Cl^- activity by inhibiting its extrusion and enhanced cell volume by inhibiting KCl losses. Under these conditions, the addition of 0.25 mmol/l adenosine, 25 µmol/l by 8-(4-chlorophenylthio)-adenosine 3':5'-cyclic monophosphate (8CPT-cAMP), and 5 µmol/l forskolin increased the rate of acidification by a factor of two to three: 6.47 ± 1.49 versus 15.1 ± 1.51 (n=18). This increased rate in acidification like that in the other two series could be inhibited completely and reversibly by furosemide (0.5 mmol/l, n=20).

The present data indicate that the $Na^+K^+2Cl^-$ cotransporter of the rectal gland of *Squalus acanthias* can be activated by three mechanisms: fall in cytosolic Cl^- activity, a reduction in cellular volume, and cAMP dependent regulation. This study has been supported by DFG Gr 480/12.