EVIDENCE FOR A CRITICAL FUNCTIONAL ROLE OF CYSTEINE RESIDUES IN THE SHARK (*SQUALUS ACANTHIAS*) Na-K-2Cl COTRANSPORTER EXPRESSED IN COS-7 CELLS

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Previous studies have shown that inorganic mercury inhibits reversibly the activity of the Na-K-2Cl cotransporter when analyzed in plasma membrane vesicles isolated from shark rectal gland (Kinne-Saffran, E. et al., *Bull. MDIBL* 33:93-94, 1994) or in intact human colonic T84 cells (Kinne-Saffran, E. and Kinne, R.K.H., *Bull. MDIBL* 36:19-20, 1997). These results lead to the hypothesis that cysteine residues located in an extracellular hydrophilic domain of the transporter are the target of the mercury inhibition. According to the sequence data derived from cloning experiments and the hydropathy profile proposed for the transporter (see Payne, J.A., and Forbush III, B., *Curr. Opin. Cell Biol.* 7:493-503, 1995 for review) the extracellular loop between transmembrane segments TM7 and TM8 is particularly rich in cysteine residues. We therefore established a cell system in which the Na-K-2Cl cotransporter can be transiently expressed and analyzed the effect of replacement of the cysteine residues by serine on the functional activity of the transporter.

For cloning of the *nkcc* gene into the eukaryotic vector pZeoSV (Invitrogene, Leek, The Netherlands) the pBluescript KS plasmid (Strategene, La Jolla, CA), containing 5260 bp shark rectal gland cDNA in Eco RI site (pNKCC1; gift from Dr. B. Forbush) was used. This plasmid was digested with XbaI and XhoI and the 5260 bp fragment was inserted into pZeoSV vector digested with SpeI and XhoI. Mutant 1 in which four cysteines in position 536, 541, 550, and 555 aa were mutated to serines was prepared by mutagenesis using Chameleon^{TM} Kit (Stratagene). Transfection of cells was performed in 6-well plates according to the Superfect Transfection Reagent protocol provided by Quiagen GmbH (Hilden, Germany). About 2 µg DNA were used per 3 x 10^5 cells. Expression time was 2 days. Uptake of ^{86}Rb into nonconfluent cell cultures was studied at 25°C as described previously (Kinne-Saffran, E. and Kinne, R.K.H., *Bull. MDIBL* 36:19-20, 1997). The bumetanide-sensitive uptake in the presence of 0.2 mM bumetanide was considered to represent the activity of the Na-K-2Cl cotransporter. Protein content determined in each well of the 6-well plate was used as reference point.

In a first series of experiments the activity of the Na-K-2Cl cotransporter was determined in various cell types that potentially could be transfected with the cotransporter. As depicted in Table 1, T84 cells showed the highest total ^{86}Rb uptake followed by COS-7 cells, BALB c3T3 cells, MDCK cells, and CHO cells. The portion of the bumetanide-sensitive ^{86}Rb uptake was, however, smallest in COS-7 cells and largest in T84 cells. Therefore, COS-7 cells were chosen for the subsequent transfection experiments.

Table 1. ^{86}Rb uptake into various cell lines. Effect of bumetanide (0.2 mM)

Cell line	Rb uptake		
	no bumetanide	0.2 mM bumetanide	bumetanide sensitive
T84	9954 ± 193	4734 ± 313	5220
COS-7	9645 ± 517	7232 ± 1106	2413
BALB c3T3*	8760 ± 843	4792 ± 462	3968
MDCK**	7512 ± 551	3895 ± 607	3617
CHO	5423 ± 1150	1737 ± 82	3686

Uptake was determined for 2' at 25°C in the absence or presence of 0.2 mM bumetanide. Cells were preincubated for 90 min at 25° in media containing gluconate instead of chloride (Kinne-Saffran, E. and Kinne, R.K.H., *Bull. MDIBL* 36:19-20, 1997). Uptake is given in cpm per mg protein. Mean values of 4 determinations ±S.D. are given. * Gift from Dr. Thomas O'Brien, Lankenaus Medical Research Center, Wynnewood, PA; ** gift from Dr. W. Minuth, University of Regensburg, Germany. T84 cells were obtained from ATTC, COS-7 and CHO cells from DSMZ.

As demonstrated in Table 2 COS-7 cells transfected with the wild-type cDNA of the cotransporter show a 3.9-fold higher bumetanide-sensitive ^{86}Rb uptake than cells transfected with the empty vector. Cells transfected with the vector coding for a cysteine-free extracellular loop (mutant 1) had the same transport activity as the sham-transfected cells.

Table 2. Expression of shark rectal gland Na-K-2Cl cotransporter in COS-7 cells

Transfectant	Bumetanide-sensitive ^{86}Rb uptake (cpm/2 min · mg protein)	n
empty vector	1930 ± 220	4
wild-type	7697 ± 760	4
mutant 1	1978 ± 110	4

Mean values ±S.D. from n determinations are given.

These results demonstrate that the cysteine residues in the extracellular loop between TM7 and TM8 are essential for the functional expression of the shark rectal gland Na-K-2Cl cotransporter in a heterologous cell culture system. Further experiments are required to elucidate whether they play a role in determining synthesis, sorting and/or activation of the transporter or are critical for the transport function per se, as hypothetized from the inhibitor studies.

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