A LOW DOSE OF MERCURY INHIBITS PHOSPHORYLATION OF THE NA-K-CL COTRANSPORTER IN THE RECTAL GLAND OF THE DOGFISH SHARK, *SQUALUS ACANTHIAS.*

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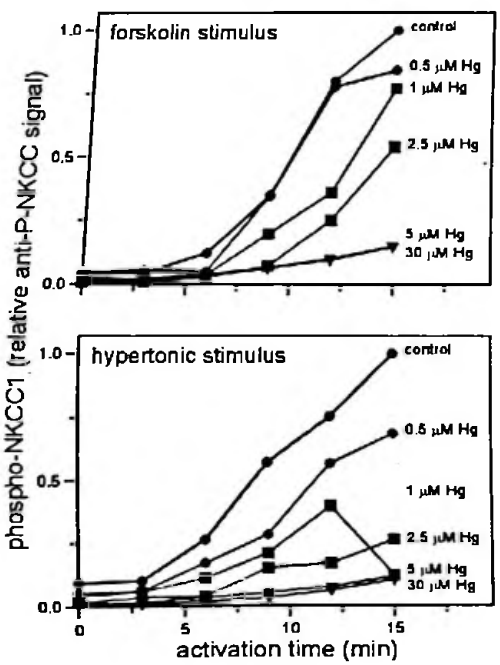
The Na-K-Cl (NKCC1) cotransporter is essential for epithelial salt and water secretion as well as for the maintenance of cell volume and [Cl] in a wide variety of animal cells. We and others have previously shown that Hg inhibits the function of the NKCC1 in shark rectal gland at concentrations near 25 μM (Jacoby et al.,Am. J. Physiol., 277, C684-92, 1999; Kinne-Saffran and Kinne, Biochim. Biophys. Acta., 151, 442-451, 2001). On the other hand, short circuit current in rectal gland cell monolayers is inhibited at Hg concentrations below 10 μM, and this effect has been attributed to an effect on shark CFTR (Ratner et al; Bull MDIBL 37:20-22, 1998). When forskolin is used as a stimulus, NKCC1 is activated by phosphorylation in response to ionic changes secondary to CFTR activation, and it would thus be expected that NKCC1 activation would be very sensitive to Hg. In contrast, NKCC1 is also activated by hypertonicity, but this does not involve CFTR, and might not be expected to be Hg sensitive. Here we report the effect of inorganic mercury on the phosphorylation state of NKCC1. The results do not fit the simple hypothesis above, but give new insights into the action of Hg on regulation of cellular processes. They also suggest that up-regulation of the Na-K-Cl cotransporter may be the critical point at which mercury affects secretion in the rectal gland.

These experiments were accomplished in isolated rectal gland tubule cells, using a polyclonal antibody (anti-P-NKCC) that detects the cotransporter when it is phosphorylated at T_{184} and T_{189} (Flemmer et al; Bull. MDIBL 38:80-2, 1999). Shark rectal gland tubules were prepared as described previously. NKCC1 was activated in the tubule cells either by addition of 50 μM forskolin or by shrinkage in 575 mM sucrose, and $HgCl_2$ was added either in a preincubation, concurrent with activating additions, or concurrent with lysis of the cells with 1% Triton. All experiments were terminated by addition of 1M H_3PO_4/1% Triton and the samples were analyzed by dot blotting and development with anti-P-NKCC.



We found that preincubation with inorganic Hg completely prevents phosphorylation of NKCC1 in rectal gland tubule cells. With a 4% cytocrit, the $K_{0.5}$ for this effect of mercury was about 35 μM. We found however that by reducing the cytocrit we proportionately reduced the $K_{0.5}$ for mercury, demonstrating that Hg absorption by the tubule cells is a

Figure 1. The effect of inorganic Hg on phosphorylation of NKCC1. These are individual determinations of NKCC1 phosphorylation activated either by forskolin or hypertonic medium as marked in tubules at 0.06% cytocrit. Representative of results of three experiments.

limiting phenomenon. At very low cytocrits (0.06%), $K_{0.5}$ was found to be 1-2 μM and it appears from the available dataset that this is the limiting value for Hg sensitivity of cotransporter activation.

We examined cotransporter phosphorylation with a hypertonic stimulus as well as with forskolin. To our surprise, both modes of activation were inhibited by Hg at 1-4 μM and the $K_{0.5}$s were indistinguishable from one another in internally paired experiments (Fig. 1). This finding strongly supports a model in which a single inhibited process occurs after the point of convergence of the two regulatory stimuli; a likely candidate is the cotransporter-kinase, which is yet unidentified.

Since hypertonic stimulation of NKCC1 does not involve CFTR, these findings demonstrate that Hg inhibition of NKCC1 phosphorylation is not due to inhibition of CFTR. On the contrary, we note that NKCC1 activation is necessary for transepithelial ion transport to occur, and for generation of short-circuit current in an Ussing chamber system. Thus we propose that the Hg inhibition of short-circuit current measured by Forrest and coworkers (Ratner et al; *op cit*) in rectal gland tissue culture is most likely due to inhibition of NKCC1 activation, as well as to direct inhibition of CFTR as proposed.

In the course of these experiments it was noted that while mercury inhibits activation of NKCC1 by hormonal and hypertonic stimuli, by itself it actually increased activation of NKCC1 in the resting state. The $K_{0.5}$ of this effect was >40 μM, but was difficult to determine, since maximal activation was not reached even at 100 μM Hg. A similar observation has been made for the activation of JNK by Hg in LLC-PK1 cells, where the phosphorylation of JNK is increased by exposure to 10 μM Hg (Matsuoka, M. et al. Toxicol. Sci. 53: 361-368, 2000). As to the mechanism of the activation of NKCC1, it is possible that it is achieved (a) by inhibition of the phosphatase which dephosphorylates NKCC1, (b) activation of the kinase, or (c) an effect upstream of NKCC1 regulatory phosphorylation/ dephosphorylation.

To investigate the possibility that the relevant phosphatase is inhibited by Hg (a, above), the rate of dephosphorylation of NKCC1 was examined following solubilization of the rectal gland cell with 1% Triton. This method was used previously to demonstrate that PP1 is the phosphatase that is involved in NKCC1 dephosphorylation (Darman et al, J. Biol. Chem., 276, 34359-34362, 2001). In the current experiments it was found that Hg strongly inhibited dephosphorylation of NKCC1 with a $K_{0.5}$=28±2 (n=3) without preincubation and $K_{0.5}$ = 15 μM (two of three experiments) following a 10 min preincubation. Presuming that the somewhat different dose-dependence is due to the broken vs. intact cell difference, this result provides a clear explanation for activation of NKCC1 by Hg in the resting state. This work was supported by NIH DK 47661 and NIEHS P30-ES 3828.