EFFECT OF MERCURIC CHLORIDE ON THE Na-K-ATPASE OF THE RECTAL GLAND OF SQUALUS ACANTHIAS

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Na-K-ATPase is a transport protein that spans the plasma membrane of all cells. The many cysteine residues in the amino acid chain of the alpha subunit of the enzyme can potentially bind mercury. The binding to cysteine residues is the way in which mercury inhibits most enzymes. There are many reports in the literature on the inhibition of Na-K-ATPase by mercury. Most of these reports have relied on indirect assessments of the activity of the enzyme or have measured its activity directly using the coupled enzymatic assay of Barnett. Previous experiments have suggested that the effect of mercury to inhibit chloride transport in the shark rectal gland may be at a site other than Na-K-ATPase (Silva P, et al. Comp. Biochemistry Physiol. 103C:579-585, 1992). To examine this question further we measured the effect of mercury on the activity of rectal gland Na-K-ATPase in vitro, using the coupled enzymatic assay of Barnett, and the effect of mercury on oxygen consumption and rubidium uptake by isolated rectal gland tubules.

Sodium potassium ATPase was measured by the method of Barnett in rectal gland plasma membranes (Barnett, R. E. Biochemistry, 9:4644-4648, 1970). For each preparation two rectal glands were removed and all connective tissue dissected out and discarded. The glands were placed in 35 ml ice-cold homogenizing solution of the following composition (in mM): Sucrose 250, Hepes 20, pH 6.8, and homogenized for one minute in a Waring blender on ice. The homogenate was filtered through four layers of gauze and brought to a final volume of 35 ml with ice-cold homogenizing solution. Plasma membranes were then prepared by differential centrifugation, resuspended in 450 µl of homogenizing solution, passed 3-4 times through a 25 ga. needle and stored frozen until used.

Isolated rectal gland tubules were prepared as previously described (Silva P, et al. Am. J. Physiol. 265:R439-46,1993). Two rectal glands were used for each tubule preparation. The rectal glands were perfused in vitro with 100 ml of shark Ringer's. The glands were then perfused with 10 ml of shark Ringer's containing 0.2% collagenase and 10% fetal calf serum. The glands were sectioned longitudinally and minced into 1 mm cubes with a razor blade. The minced tissue was then incubated in shark Ringer's containing collagenase and fetal calf serum in the same proportions given above, at room temperature while constantly stirring for 45 min. The tissue digest was then centrifuged at 50 x g for 1 min. in a refrigerated centrifuge to remove undigested tubules. The supernatant was then spun at 500 x g for three min. to harvest the cells. The cells were washed twice in shark Ringer's and kept on ice until used.

Rubidium uptake was measured in isolated rectal gland tubules preincubated for 60 min. in K-free shark Hepes of the following composition (in mM) Na, 280; Cl, 280; phosphate, 1; Ca, 2.5; Mg, 1; sulfate, 0.5; urea, 350, Hepes 20, pH 7.6, with 10 mM glucose at 25°C. Uptake was started by the addition of 0.5 mCi ^{86}Rb diluted in K-free shark Hepes. 300 µl aliquots of the incubate were removed at 0, 30, 60 seconds and diluted into stop solution consisting of choline chloride 280 mM, Hepes 20 mM, pH 7.6, ouabain 0.1 mM. The samples were layered over 1 ml of dioctyl phtallate:silicon oil 1.75:1 and spun for 20s in an Eppendorf centrifuge. Radioactivity in the supernatant was measured to determine specific activity of the bath. The tip of centrifuge tube was cut and radioactivity measured in a gamma counter. Protein content of cell suspension was measured using Bio-Rad assay.

Oxygen consumption was measured as previously described in a constant temperature (25°C) chamber using a Clark type polarographic electrode connected to a recorder (Silva P, et al., Miner. Electrolyte Metab. 12:286-92,1986). The rate of oxygen consumption was calculated from the tangent of the recorded slope of the oxygen consumption, the solubility of oxygen in shark Ringer's at 25°C, the barometric pressure, the volume of incubation solution in the measuring chamber, generally 2 ml, and the wet weight of the cells. The wet weight of the cells was determined at the end of the experiment by removing a measured aliquot of the cell suspension, spinning it down in a tared centrifuge tube, removing the supernatant, and reweighing the tube. Glucose 5 mM, pyruvate 10 mM and acetate 2.5 mM were used as exogenous substrates. Oxygen consumption was measured under basal conditions and the rate of oxygen consumption was allowed to reach a steady rate. $HgCl_2$ was then added and the rate of oxygen consumption allowed to reach a new steady state. Once this new steady state was established, ouabain 1 mM was added and the rate of oxygen consumption determined again. Ouabain-sensitive oxygen consumption was calculated as the difference between the rate of oxygen consumption under basal or stimulated conditions and the rate after ouabain.

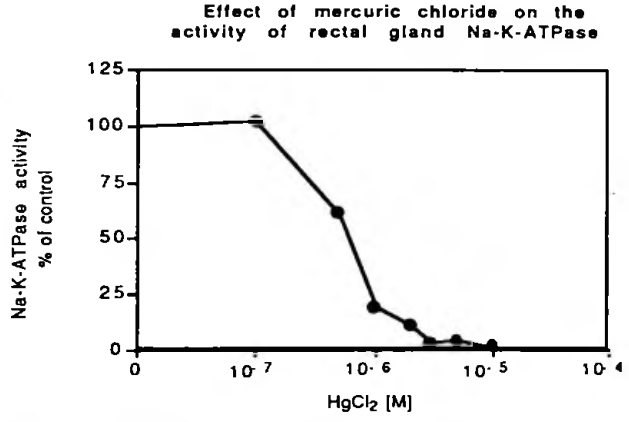

Figure 1. Effect of mercuric chloride on the activity of rectal gland Na-K-ATPase. Shown is a representative experiment showing that mercuric chloride inhibits the enzyme on a dose dependent way. The experiment was done using the Barnett assay.

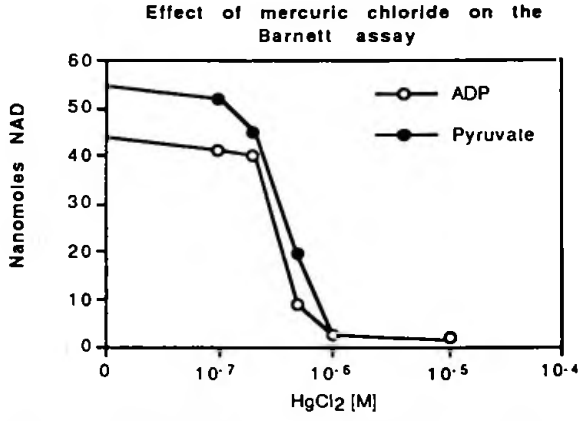

Figure 2. Effect of mercuric chloride on the Barnett assay. Mercury inhibits the oxidation of NADH, the indicator of the Barnett assay, in a dose dependent way when either ADP or pyruvate are used as substrates thus bypassing the initial Na-K-ATPase step. Shown are two representative experiments.

When the coupled assay of Barnett is used, mercury inhibits the activity of Na-K-ATPase in a dose dependent way (Figure 1). Figure 2 shows that mercury also inhibits the oxidation of NADH, the indicator of the Barnett assay, in the same dose dependent way when either ADP or pyruvate are used as substrates, bypassing the initial Na-K-ATPase step. Figure 3 shows that mercury inhibits the initial rates of the Barnett assay when ADP is the substrate. When the initial rates of the assay are measured using pyruvate as the substrate there is no inhibition until the concentration of mercury reaches 0.1 mM. These results suggest that mercury inhibits the pyruvate kinase mediated step of the assay and to a much lesser extent that of lactic dehydrogenase. When the Barnett reaction initial rates are measured with ATP as substrate, in the presence of an ATPase source, inhibition is observed at concentrations of mercury of 5×10^{-6} M, essentially similar to those seen in the regular assay. Thus, the coupled assay of Barnett should not be used for the assessment of the effect of mercury on Na-K-ATPase because its intermediary steps are inhibited by mercury, the pyruvate kinase mediated step more so than that of lactic dehydrogenase.

To further assess the effect of mercury on Na-K-ATPase we measured the uptake of rubidium into isolated rectal gland tubules, a measurement of enzyme activity in vivo. Figure 4 shows that mercuric chloride does not inhibit the uptake of rubidium into the tubular cells until the concentration of the latter reached 0.1 mM. This result suggests that the activity of Na-K-ATPase is not inhibited in an intact cell preparation until very large concentrations of the metal are present in the environment. At 10^{-4} M mercury not only completely inhibits ouabain sensitive rubidium uptake but also ouabain insensitive uptake suggesting that its effect is non-specific.

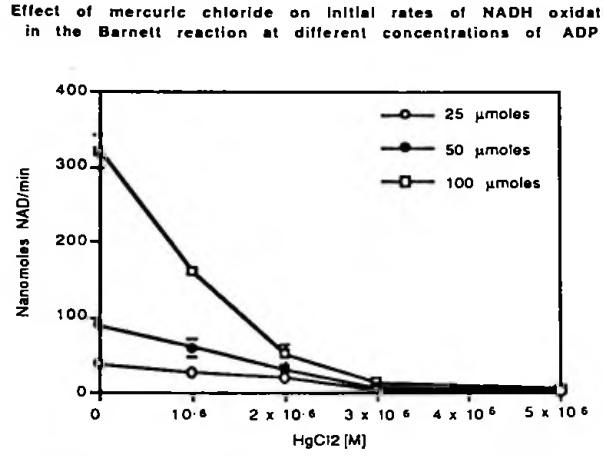

Figure 3. Effect of mercuric chloride on the initial rates of NADH oxidation in the Barnett assay at different concentrations of ADP. Mercuric chloride inhibit the initial rates of NADH oxidation in a dose dependent way. Representative experiments.

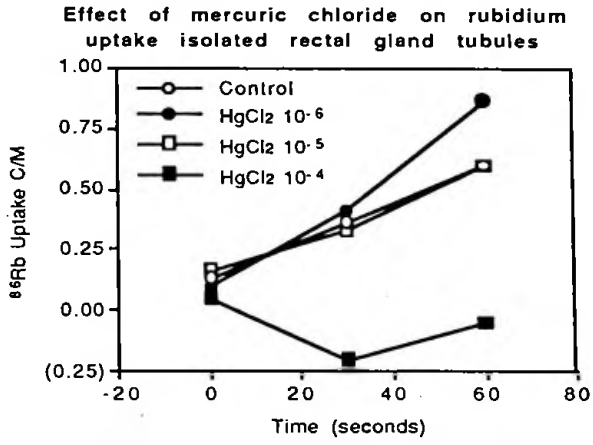

Figure 4. Effect of mercuric chloride on rubidium uptake by isolated rectal gland tubules. Mercuric chloride inhibits ouabain sensitive rubidium uptake only at 10^{-4} M. At this concentration it inhibits also ouabain insensitive uptake. Representative experiments.

To determine whether this effect of mercury was a non-specific effect on cellular metabolism or specific inhibition of rubidium uptake we measured oxygen consumption by isolated rectal gland tubules. Figure 5 shows the results. Mercury inhibits oxygen consumption at concentration of 10^{-5} M and greater. At a concentration of 10^{-4} M mercury inhibits not only ouabain sensitive, that is that moiety of oxygen consumption that relates to transport, but also ouabain insensitive oxygen consumption, that is oxygen consumption that is not related to transport. Moreover, at 10^{-4} M, mercury causes a progressive reduction in oxygen consumption after an initial stimulation indicating that the metal is altering cellular metabolism in a non-specific way.

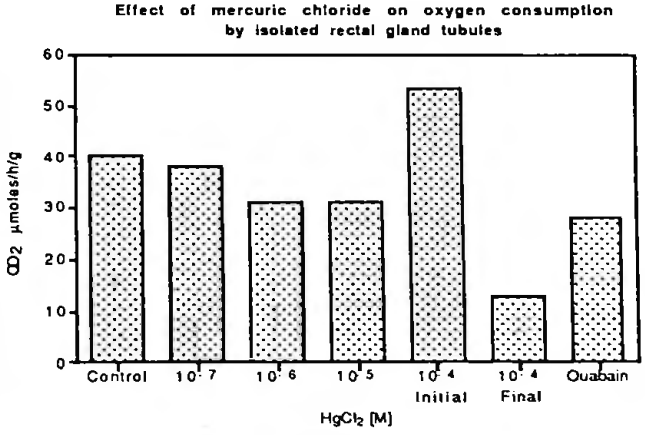

Figure 5. Effect of mercuric chloride on oxygen consumption by isolated rectal gland tubules. Mercuric chloride inhibits oxygen consumption at concentration greater than 10^{-5}M. At 10^{-4} it stimulates initially (uncouples) and then inhibits. Shown are representative experiments.

The mechanism of the inhibition of inorganic mercury on chloride transport by the rectal gland can be at the level of Na-K-ATPase with the resulting reduction in the driving force for entry of sodium, chloride and potassium via the cotransporter. The present experiment suggest that Na-K-ATPase is not the site of inhibition by mercury. The activity of the enzyme needs to be examined in vitro as well as in vivo under conditions that measure directly the activity of the enzyme using a kinetic assay and

also in a functional assay such as rubidium uptake. The partial reactions of the enzyme and its associated activities, such as paraphenyl phosphatase activity, could be used to clarify the effect of mercury on the Na-K-ATPase.

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