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Mercuric Chloride ($HgCl_2$) and Methyl Mercury (CH_3HgCl) Block Sodium Transport in the Isolated Skin of the Toad *Pleurodema thaul*

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The influence of heavy metals on the isolated toad skin has been demonstrated in previous papers (Suwalsky et al. 1998, 1999); thus, both Cu(II) and Al(III) decreased ion transport across the skin, the effect of the former metal being irreversible. Mercury is an element of great pharmacological and toxicological importance and is widely used in industry and in medicine. It exists in three forms: elemental mercury (Hg^o), inorganic mercury [Hg(I), Hg(II)], and organic mercury (CH_3Hg^+). Environmental mercury and mercury compound contamination has increased dramatically in developed countries (Remez et al. 1999). Toxicity can result from industrial exposure or commercial application (Olivero and Solano 1998; Mion and Pierin 1998). Human exposure results in an impairment of coordination, reduced cognition, tremor and abnormal reflex activity (Albers et al. 1988).

The surface membrane is a diffusion barrier and protects the cell interior from environmental changes. Ligands on the cell surface or inside the cell can aid in transmitting information to the cell interior; therefore cell membrane functions are susceptible to the actions of heavy metals. Evidence has accumulated that heavy metal ions interact with ionic channels, receptors and their enzymes. $HgCl_2$ inhibited $Na^+/K(^+)$-ATPase in oocytes at micromolar concentrations (Wang and Horisberger 1996) and caused serious destruction of the cell membrane of human lymphocytes and monocytes (Steffensen et al. 1994). The thiazide-sensitive NaCl co-transporter is a specific site of action for submicromolar doses of mercury (Wilkinson 1993). Although the methylated form of mercury is retained at higher levels in higher trophic organisms (Kim et al. 1996) its toxicity is apparently less than that of the inorganic form; $HgCl_2$ was more effective in reducing Ca^{2+} channel currents in dorsal root ganglion neurons (Leonhardt et al. 1996).

There are no studies on the effect of inorganic and organic mercury on the ion permeability of the amphibian skin, which has been instrumental in the understanding of ion transport across membranes and the mechanism of action of numerous agents to which cellular membranes are exposed, and

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has been accepted as a prototype of a transporting epithelium. According to the two membrane hypothesis, Na^+, the main ion transported, diffuses into the cells at the outer (mucosal or apical) membrane driven by its electrochemical gradient and is actively extruded across the inner (serosal or basolateral) membrane by a $Na^+/K(^+)$-ATPase in exchange for K^+ (Rytved et al. 1995). Active Na^+ transport is measured by the amount of current (short-circuit current, Isc) necessary to keep the transmembrane potential difference (PD) at zero. It has been shown (Kiss and Osipenko 1994) that Hg(II) at micromolar concentrations decreases Na^+ permeability by interacting with sulfhydryl groups of channel proteins. The current work seeks to determine and compare the effects of $HgCl_2$ and CH_3HgCl on the bioelectric parameters of the isolated toad skin, in accordance with the hypothesis that these compounds are capable of interacting with and perturbing the structure and functions of the cellular membranes of the epithelium.

MATERIALS AND METHODS

Experiments were performed on toads of the species *Pleurodema thaul* of either sex (15-25 g) collected from fresh water ponds during the spring and summer seasons. They were kept in chlorinated tap water at room temperature (18-22°C) at least 24 hr prior to use and fed on sow bugs (*Oniscus asellus*). The toads were pithed and sections of the abdominal skin were removed, rinsed and mounted between two halves of a Ussing perspex chamber (Ussing 1994): a circular area of 1 cm^2 was exposed to 3 mL Ringer's bathing solution on each side. The composition of the solution was (mM): Na^+ 114, K^+ 2.5, Cl^- 117.5, Ca^{2+} 2.0, HCO_3^- 2.3 and glucose 11. The bathing medium was oxygenated by a stream of air. The Isc was monitored with non-polarizable Ag/AgCl electrodes placed at 15 mm distance from the epithelium and connected to a voltage clamp circuit (G. Métraux Electronique) set to keep the PD across the skin at zero mV. The PD was measured with calomel-agar electrodes at intervals of 2 min. for 4 sec. Both parameters were displayed on a two-channel Cole-Parmer recorder. Experiments commenced 30 min. after the electric parameters of the skin had reached a steady level. Aqueous solutions of either $HgCl_2$ (Riedel de Haën, Seelze, Germany, lot 21280. MW 271.5) or CH_3HgCl (Pfaltz & Bauer, Inc., Conn., USA, M 21890, MW 251.1) were applied to the solution bathing the outer and inner surfaces of the skin in the final concentrations indicated in the text. Values are expressed as means ± standard error of the mean (S.E.M.). Statistical analysis was performed by means of Student's paired *t* test.

RESULTS AND DISCUSSION

Both mercuric compounds were effective when applied in either the outer or the inner bathing solution. Figure 1-A shows that a near maximal

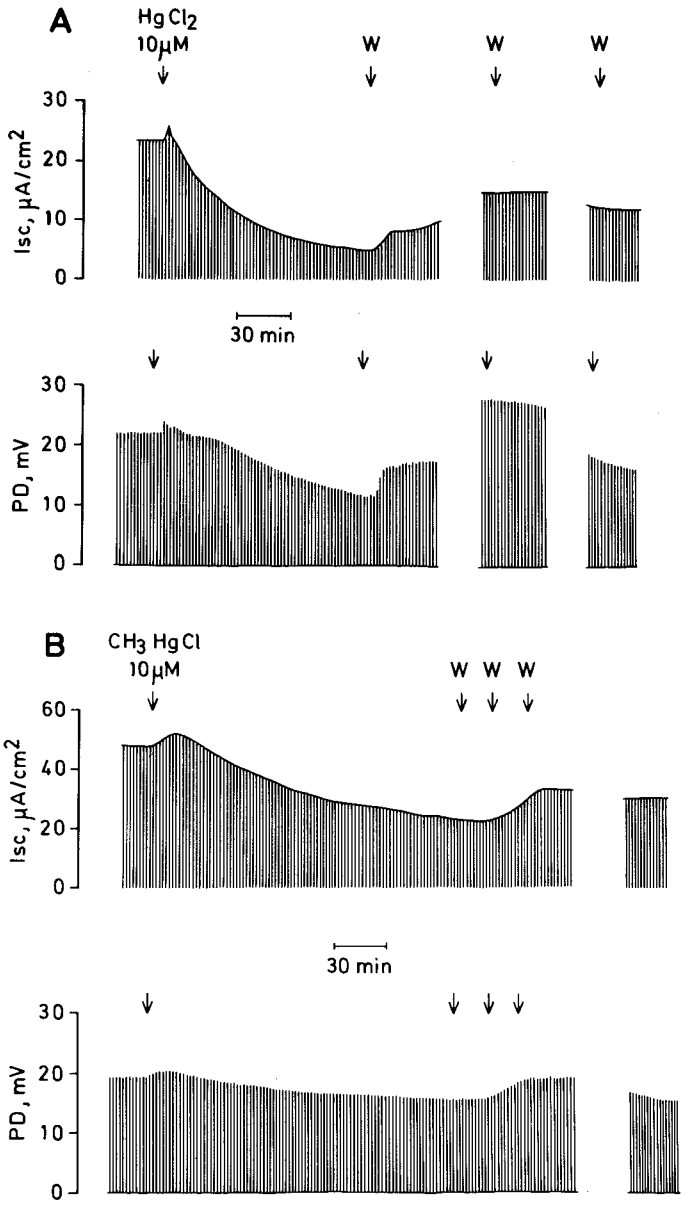

Figure 1. Single experiment illustrating the time course of the action of the two mercuric compounds applied in the outer bathing solution, on the electrical properties of the isolated toad skin. Isc = short-circuit current; μA = microamperes; PD = potential difference; mV = millivolts; W = washout. (A) effect of 10 μM $HgCl_2$; (B) effect of 10 μM CH_3HgCl.

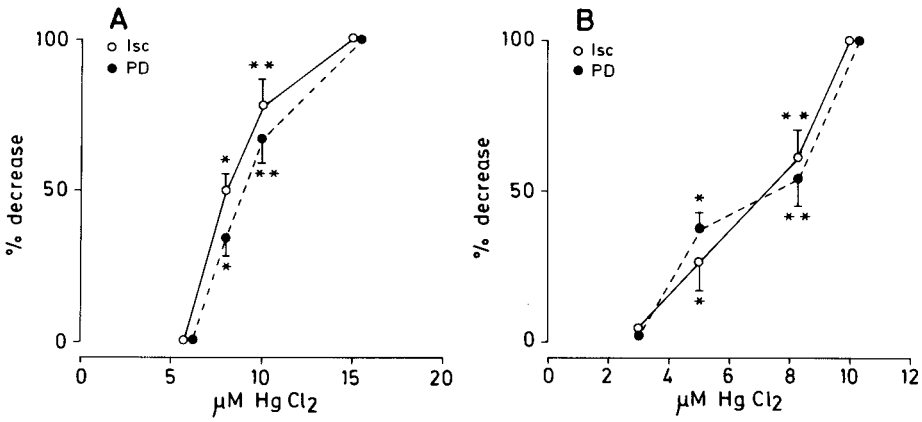

Figure 2. Inhibitory effects of increasing concentrations of $HgCl_2$ on the electrical properties of the isolated toad skin. Results are expressed as percent decrease in control values. Each point represents means ± S.E.M.; n = 6. Isc = short-circuit current; PD = potential difference. * $P < 0.01$; ** $P < 0.001$ (Student's paired *t* test). A, outer surface: 5.6, 8.0, 10.0 and 15.0 μM. B, inner surface: 3.0, 5.0, 8.3 and 10.0 μM.

concentration of $HgCl_2$ (10 μM, outer solution) gave rise to a slight, non-significant increase in the electrical parameters which lasted about 9.6 ± 2.5 min. (n = 6), followed by a gradual decline, which reached a trough in 113 ± 3 min. (n = 6). This effect was irreversible in spite of partial recovery after repeated washout of the skin. Addition of 10 μM CH_3HgCl to the outer solution also decreased the electrical parameters, although the effect was slightly less; time to trough was 192 ± 3 min. (n = 13) and partial recovery was obtained after washout (Figure 1-B).

The magnitude and duration of the responses was concentration-dependent. Figure 2-A shows that $HgCl_2$ (outer solution) elicited a decrease of the electrical parameters which was only partially reversible at concentrations ranging from 8-10 μM. Slightly lower concentrations (5-10 μM) added in the inner solution were also followed by partly reversible decrease of both parameters (Figure 2-B). At maximal concentrations of 10 (inner) or 15 (outer solution) μM, electrical activity of the skin was abolished. CH_3HgCl was more effective when applied in the outer solution (Figure 3-A); a 10 μM concentration reduced the electrical parameters by about 50% whereas a 20 μM concentration was necessary in the inner solution to decrease both parameters by 50% (Figure 3-B). These effects were only partially reversible after repeated washing. Results show that inorganic mercury was more damaging to the isolated toad skin than

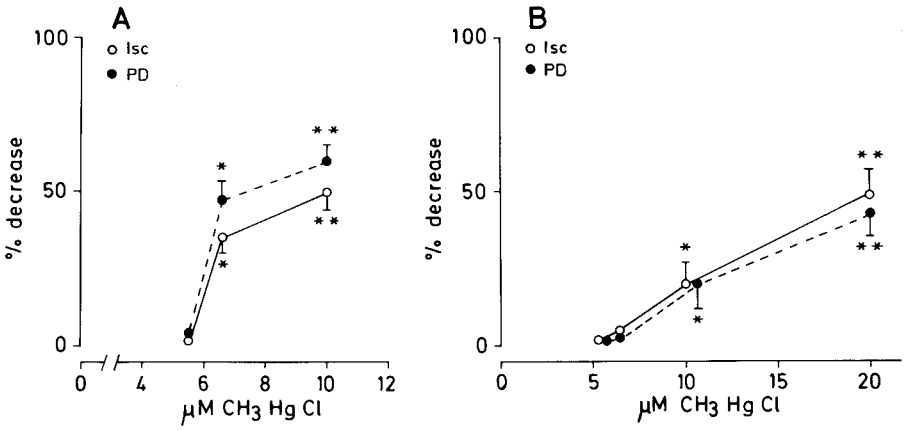

Figure 3. Inhibitory effects of increasing concentrations of CH_3HgCl on the electrical properties of the isolated toad skin. Results are expressed as percent decrease in control values. Each point represents means ± S.E.M., n = 13. Isc = short-circuit current; PD = potential difference. * $P < 0.05$; ** $P < 0.01$ (Student's paired *t* test). A, outer surface: 5.6, 6.6 and 10.0 μM; B, inner surface: 5.6, 6.6, 10.0 and 20.0 μM.

organic mercury. The greater bioaccumulation of methyl mercury over mercuric chloride, ascribed to higher lipid solubility, makes organic organic mercury. The greater bioaccumulation of methyl mercury over mercuric chloride, ascribed to higher lipid solubility, makes organic mercury the main chemical form in food chains reaching higher organisms (Girault et al. 1997). Nonetheless, inorganic mercury blocked synaptic transmission of CA_1 neurons in hippocampal slices of the rat more quickly than did organic mercury (Yuan and Atchison 1994). The acute effects reported for the concentrations used may be connected to toxic effects in human subjects after a few hours' exposure to mercury: lethal blood levels are in the range of 0.4-22 μg/mL (Von Burg 1995). The results could be due to strong inhibition of the active transport of ions and possible disruption of the cell membrane. The observation that the compounds were active at both sufaces of the skin suggests that the metals permeated the membrane (Gyori et al. 1991). Mercury, principally Hg(II), shows affinity for epithelial cells such as the skin epithelium (Von Burg 1995; Wang and Horisberger 1996).

Functions associated with the cell membrane are susceptible to the action of heavy metals which interact with ligands forming integral parts of biological molecules in aqueous solution (Kiss and Osipenko 1994). A number of investigations argue for several molecular sites of action of mercury toxicity, which are active simultaneously: a) entirely lipid with a loss of bilayer integrity; b) a lipid perturbing effect, leading to interaction with and conformational changes of proteins; c) interaction with ion

channel or receptor proteins (De Biasi et al. 1993; Büsselberg et al, 1994); d) binding to ionic channel-receptor complexes facing the cytoplasm; e) decrease in the water permeability of the protein channels which form aquaporins (Heymann and Engel 1999). The first mechanism, involving loss of bilayer integrity, implies decreased resistance across the bilayer; in the current experiments resistance increased significantly (for $HgCl_2$ 69 ± 7, n = 6, $P < 0.001$) except at the concentrations which abolished the electrical parameters of the skin. This finding could indicate that disruption of the bilayer was not present at the lower concentrations used in the work, and is not the case for CH_3HgCl since this compound did not change the resistance significantly.

Fluorescence and NMR imaging showed that $HgCl_2$ displayed strong affinity for the primary amino groups of phospholipids and that CH_3HgCl binds to the phosphate group in anionic lipids inducing decrease in fluidity and integrity loss (Girault et al. 1997). X-ray diffraction showed that $HgCl_2$ induced molecular disorder of model membrane phospholipids and fluorescence spectroscopy confirmed its interaction with phospholipid amino groups (Suwalsky et al. 2000, in press), which also affected the packing of the acyl chains at the deep hydrophobic core of these layers: the fluidity perturbation and the induction of a change in the electric field of the core should affect the activity of membrane proteins, particularly of ion channels. Therefore this evidence favours a lipid-perturbing effect which changes protein conformation. Inhibition of $Na^+/K(^+)$-ATPase by Hg(II) has been confirmed (Wang and Horisberger 1996). Recent investigations (Jacoby et al. 1999) have shown that $HgCl_2$ inhibits $Na(^+)$-$K(^+)$-$Cl(^-)$ cotransport in cultured epithelial shark and human cells by reacting with cysteinyl sulfhydryl groups of the transporter.

The third mechanism refers to interaction of mercuric compounds with ionic channel proteins; the binding of mercury to SH^- groups of proteins leads to a variety of effects due to different accessibility of the compounds to thiol groups instrumental to membrane transport, which appear to be located at the inner surface of the membrane (Delnomdedieu et al. 1989; Halbach 1990). The irreversible toxicity found in the present study is probably due to an intracellular action involving such groups: Hg(II) enters through Na^+ or Ca^{2+} channels (Arhem 1980) and is able to block Na^+ open channels by binding to these groups. Since protein receptors and ion-channel receptor-protein complexes possess SH^- groups, mercuric compounds can act at both sites.

The transport of water is coupled to net transepithelial Na^+ transport by local osmosis (Nielsen 1997) in the presence of an osmotic gradient maintained by the Na, K pump and therefore the decreased fluidity of the phospholipid bilayer exposed to mercury could point to blockage of the apical water channels in the skin (Grosso et al. 1993; Grosso and De

Sousa 1994), possibly correlated with changes in intracellular chloride (Grosso et al. 1994).

The decrease of the bioelectric activity of the isolated toad skin following exposure to mercuric compounds is in accordance with the interference of ion transport, principally Na^+, at several sites. Loss of bilayer integrity was probably responsible for the abolition of skin activity at the maximal concentration used; at lower concentrations the second mechanism may be important since $HgCl_2$ strongly perturbed the structure of phospholipids present in both outer and inner monolayers of cell membranes, leading to conformational changes in proteins.

To conclude, mercury is a major, ubiquitous and persistent contaminant causing increased morbidity and mortality in humans. A concentration of mercury in blood in excess of 0.20 μM (Klaassen 1996) suggests the need for environmental evaluation and assessment of possible adverse health effects. Biological membranes are the initial targets of this pollutant: understanding their interactions at the molecular level is of primary importance to interpret the results of bioaccumulation. Results obtained in this work may be interpreted as reflecting irreversible inhibition of the active transport of ions, principally Na^+, by $HgCl_2$ and CH_3HgCl and they confirm previous reports of the toxicity of mercuric compounds at micromolar concentrations on membrane structure and function.

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