BioMetals 1997, **10**, 157–162

Specific binding of inorganic mercury to Na^+– K^+-ATPase in rat liver plasma membrane and signal transduction

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Received 8 November 1996; accepted for publication 18 November 1996

Specific binding of Hg^{2+} to ouabain-sensitive Na^+–K^+-ATPase of rat liver plasma membrane was demonstrated with a K_a of 2.64×10^9 and B_{max} of 1.6 nmole mg^{-1} protein. The binding of mercury to the enzyme also causes significant inhibition of the enzyme, which is greater than its ouabain sensitivity. In the cytosol Hg^{2+} binding to reduced glutathione (GSH) is stimulated by GSH–S-transferase (GST), the activity of which was found to be significantly enhanced by 15 mM Na^+ and 10 mM Hg^{2+}. It is proposed that the transport of Hg^{2+} inside the cell takes place by increased dissociation of Hg^{2+} from the membrane due to greater avidity of Hg^{2+} towards cytosolar GSH binding. The GSH–Hg complex enters the nucleus where it dissociates to bind the metal response element (MRE) of the metallothionein (MT) gene to induce MT transcription.

Keywords: mercury, Na^+–K^+-ATPase, rat liver, signal transduction

Introduction

The Group II B heavy metal salts, $CdCl_2$ and $HgCl_2$, are known to stimulate metallothionein (MT) synthesis in mammals (Nordberg *et al.* 1972, Nordberg & Kojima 1979, Durnam & Palmiter 1981, Bose *et al.* 1994a). It has been reported that Hg^{2+} binds to the metal response element (MRE) of the MT gene to induce transcription (Yagle & Palmiter 1985). It was shown by Rothstein (1959) that $HgCl_2$ causes loss of K^+ ions from yeast cells and inhibits various enzymes on the cell surface by its avid binding to sulfhydryl (SH) groups on the cell surface (Rothstein 1970). Anner *et al.* (1990) reported the inhibition of renal Na^+–K^+-ATPase by mercury to have been effected through thiol groups. Anner & Moosmayer (1992) further demonstrated in liposome preparations that the intracellular part of the Na^+–K^+-ATPase molecule is the primary target for mercury action. Imesch *et al.* (1992) suggested the weakening of the membrane anchoring of the a-subunit of Na^+–K^+-ATPase by mercury, which leads to its release from the membrane preferentially in the E2 conformation and also in the E1 conformation. Thus, it is clear that mercury has a inhibitory role on the plasma membrane enzyme, which results in the elevated Na^+ concentration in the cytosol with a concomitant increase in induced synthesis of MT but no change in cyclic AMP, calmodulin and phosphodiesterase in the liver of rats treated with $CdCl_2$ (Mukhopadhyay *et al.* 1994). This observation led the authors to suggest that Na^+ has a positive role in the schematic events of MT synthesis. The present study is an attempt to elucidate the mechanism of signal transduction by inorganic mercury, which has not been proposed earlier.

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Materials and methods

Male Sprague-Dawley rats (150–200 g body weight) were housed in a well-ventilated animal facility and sacrificed using mild anasthesia under painless conditions. Liver plasma membrane was prepared according to the method of Neville (1960). Briefly, livers were perfused with Tyrode solution before homogenization in 1 mM $NaHCO_3$ at 4ºC. The homogenate was filtered through two layers of cheese cloth (mesh size 120 μm) and centrifuged. The pellet obtained was solubilized in 0.25 M sucrose and subjected to discontinuous density gradient centrifugation in a Beckman L7 55 ultracentrifuge (Beckman, Fullerton, USA) using an SW 28 Rotor. The plasma membrane fractions were collected and tested for their purity by assaying Na^+–K^+-ATPase, 5′-nucleotidase and glucose-6-phosphatase (Plummer 1988). Inorganic phospate was measured according to Fiske & Subbarow (1925) as modified by Lanzetta *et al.* (1979). Protein was assayed (Lowry *et al.* 1951) using serum albumin as standard.

The plasma membrane preparation was observed under a phase contrast microscope and found to be comprised of 5–10 μm fragments. Marker enzyme activity measurements of the membrane fragments revealed only a 5% level of contamination by the endoplasmic reticulum membranes used for the ^{203}Hg binding experiments. ^{203}Hg was procured from Bhabha Atomic Research Centre, Trombay, Bombay, India, having a specific activity of 150 mCi g^{-1}. The binding of ^{203}Hg to the plasma membrane was followed as per the protocol reported earlier (Bose *et al.* 1994b). Briefly, plasma membrane preparations containing 200 μg protein were incubated in a medium containing 5 mM $MgCl_2$; 0.1 M sucrose; 0.1% BSA in 0.01 M Na-phosphate buffer, pH 7.4, with varying concentrations of hot mercury in the absence (total binding) or presence (non-specific binding) of 1000-fold excess of cold mercury at predetermined optimum conditions of time (2 h) and temperature (30ºC) in a shaking water bath. The incubation was terminated by adding 1 ml ice cold washing medium (0.1% bovine gamma globulin and 0.1 M NaCl dissolved in 0.01 M phosphate buffer, pH 8.0) and the bound fraction was obtained by adding 20% polyethylene glycol (w/v) followed by centrifugation. The pellet was solubilized by Beckman tissue solubilizer (BTS 450) and an aliquot was taken for radioactive counting in a Beckman Automatic LS Counter (LS 6000 SC) having 95% efficiency for ^{203}Hg using a Beckman Ready Safe Liquid Scintillant.

In another set of experiments, ^{203}Hg-bound membrane fragments were separated from the unbound fractions by two column chromatography steps without using any chaotropic agent in order to ensure *in situ* Na^+–K^+-ATPase activity. Sephadex G75 and Sephacryl S 300 columns were preequilibrated with 0.01 M Na-phosphate buffer, pH 7.4. The S G75 column had a bed volume of 44 ml and void volume of 17 ml and the S300 column had a volume of 24 ml and a void volume of 10 ml. Fractions (1 ml) were eluted by the equilibration buffer at a flow rate of 10 ml h^{-1}. The eluates were analyzed for protein at 280 nm in a Beckman DU 640 Spectrophotometer and for radio-activity in the Beckman LS Counter as described earlier.

An *in vitro* assay of Hg binding to glutathione (GSH) was set up in presence or absence of Na^+ and rat liver cytosolar fraction. The reaction was followed spectrophotometrically by Ellman's reagent (Ellman *et al.* 1961). Glutathione-S-transferase (GST) activity of the cytosolar fraction was assayed according to Habig *et al.* (1974).

All chemicals were procured from Sigma, St. Louis, MO, USA. Column matrices were purchased from Pharmacia, Uppsala, Sweden. Data presented in Tables 1 to 3 are shown by the average ± SE of three replicate samples from three experiments ($n = 9$). Levels of significance were determined using Student's '*t*' test (Snedecor & Cochran 1967).

Results and discussion

The binding of ^{203}Hg to the rat liver membrane preparation was found to be highly specific, having a K_a of 2.64×10^9 and B_{max} of 1.6 nmole mg^{-1} protein (Figure 1). ^{203}Hg-bound membrane fragments were separated from the free ^{203}Hg by passing through an S G75 column; the bound fraction appeared in the void volume. This fraction was then subjected to Sephacryl S 300 gel filtration, where again the ^{203}Hg-bound membrane fragments appeared in the void volume (data not shown). Considering the filtration of the Hg-bound membrane fragments in the void volumes, the eluted fractions were analyzed for Na^+–K^+-ATPase activity and found to possess a very high activity of the enzyme. In a control set of experiments, plasma membrane prepared from the liver of untreated rat was also gel filtered through Sephadex G75 and Sephacryl S 300 columns. The Na^+–K^+-ATPase activity assayed was found to be ouabain sensitive. However, the inhibition effected by $HgCl_2$ was found to be significantly ($P < 0.001$) higher than that caused by ouabain (Table 1). Thus, our observation not only substantiates earlier reports that Hg^{2+} inhibits Na^+–K^+-ATPase (Anner *et al.* 1990, Anner & Moosmayer 1992, Imesch *et al.* 1992), but also demonstrates a highly specific binding of the metal to the enzyme. It is known that Na^+–K^+-ATPase belongs to the E_1–E_2-type of cation transporter (Pedersen & Carafoli 1987) and remains aggregated in at least dimers *in vivo* (Gennis 1989); this explains the appearance of the Hg-bound fractions in the void volumes during filtration through S G75 and S 300 columns. Further, in order to have intact membrane fragments which need not be reconstituted to demonstrate Na^+–K^+-ATPase activity *in vitro*, we did not use any

chaotropic agents to isolate the enzyme. We have already shown (Mukhopadhyay *et al.* 1994) that in Cd-treated rat liver, cytosolar Na^+ concentration increases, correlating positively with Cd-induced synthesis of MT. The increase in Na^+ now appears to be caused by the inhibition of Na^+–K^+-ATPase by specific binding of the group II B metal.

By a simple spectrophotometric *in vitro* assay it became abundantly clear that GSH binding to the Hg^{2+} is significantly ($P < 0.001$) enhanced in the presence of Na^+ and an aliquot of rat hepatic cytosol (Table 2). Since GST is a cytosolar enzyme involved in the conjugation of xenobiotics to GSH it was considered logical to investigate whether GST activity is influenced by the presence of Na^+ and Hg^{2+} in the assay protocol. Table 3 demonstrates that GST activity is significantly ($P < 0.001$) enhanced by Na^+ at an optimum concentration of 15 mM and by Hg^{2+} at 10 mM as compared with the control. The stimulation of GST activity by Hg^{2+} is further enhanced in presence of Na^+, albeit not significantly. We further investigated the binding of ^{203}Hg to GSH in the nuclear sap (data not shown) which substantiates our earlier observation on the entry of Hg^{2+} into the nucleus to bind to the DNA within 15 min post-injection (Bose *et al.* 1993). However, as is known to occur in a buffer, cytosol being a buffered biological system, GSH is stabilized in the enolic form where –C=N– enhances the coordinating power of –N– and binds avidly to Hg^{2+}. Interestingly, as reported in the present investigation, the Hg–GSH binding is further stimulated in the cytosol by Na^+ and GST.

Mercury is known to exert its biological effect by the formation of stable mercaptide bonds with the SH groups of the proteins (Hughes 1950, Schwarzenbach & Schellenberg 1965, Rabenstein 1978). Hg-sensitive thiol groups effecting membrane transport are found to be located at the inner surface of various cell membranes (Ullrich *et al.* 1973, Patzelt-Wenczler *et al.* 1975, Tse *et al.* 1985, Halbach 1990). Thus, membrane fluidity constitutes a major determinant of transmembrane flux, and the interaction of Hg with the plasma membrane also comprises a non-specific electrostatic binding of the metal to the anionic sites on the membrane (Foulkes & McMullen 1987). Since there will be enough cysteine in a protein as big as plasma membrane Na^+–K^+-ATPase the phenomenon of binding Hg^{2+} will be enhanced but at the same time the dissociation of Hg^{2+} is accelerated by the higher affinity of the metal towards cytosolar GSH (Webb 1966). The Hg–GSH complex, because of its small molecular size, can enter the nucleus through nuclear pores having a 9 nm aqueous channel which allows rapid and passive diffusion of small molecules with a molecular size of 5000 daltons or less (Alberts *et al.* 1994). Toxic metals such as Cd^{2+}, Hg^+ and Pb are known to enter the mitochondria (Southard *et al.* 1974, Mehra & Choi 1981, Trifillis *et al.* 1981, Goeri g & Klaassen 1983, Goyer 1983, Bracken *et al.* 1984, Angle *et al.*

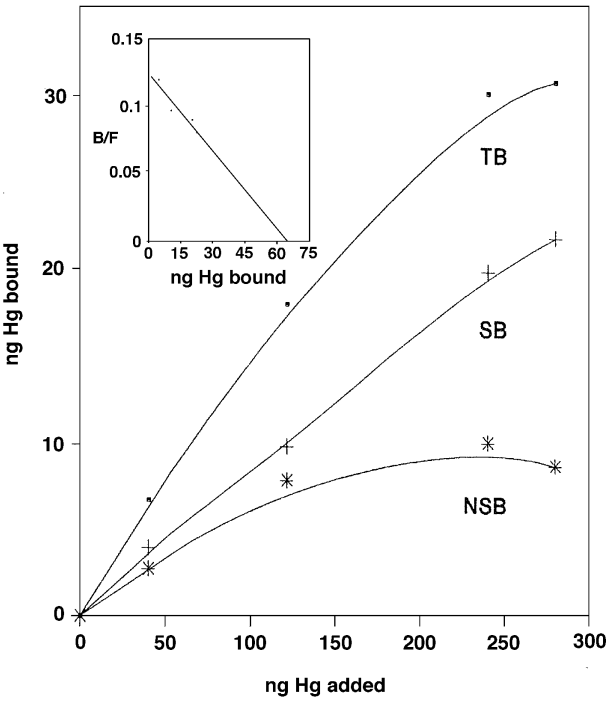

Figure 1. ^{203}Hg binding to 200 μg rat liver plasma membrane took place in an incubation medium containing 5 mM $MgCl_2$; 0.1 M sucrose and 0.1% BSA in 0.01 Na-phosphate buffer, pH 7.4, at 30°C. TB = total binding; SB = specific binding; NSB = non-specific binding. The inset shows Scatchard analysis of the data as bound/free (B/F) Hg versus bound Hg.

Table 1. Na^+–K^+-ATPase activity in rat liver plasma membrane is inhibited by ouabain and $HgCl_2$.

System	Specific activity (nmole Pi per min per mg protein)
Untreated	113 ± 1.50
1 mM Ouabain-treated	57 ± 2.28
150 nmole $HgCl_2$-treated	23 ± 1.84

Na^+–K^+-ATPase activity was assayed in a control rat liver plasma membrane preparation following the method of Plummer (1988); inorganic phosphate was measured by the method of Fiske and Subbarow (1925). The data are expressed here as $x \pm SE$ where $n = 9$. Significance of the results was compared between the untreated and the treated sets and also between the two treatments. In all cases the P value calculated from Student's t-test was < 0.001.

Table 2. Binding of $HgCl_2$ to GSH was stimulated by NaCl and liver cytosolar fraction

System	Absorbance at 405 nm
GSH	1.1843 ± 0.05
GSH + Hg^{2+a}	0.2312 ± 0.016
GSH + Na^+	0.9288 ± 0.03
GSH + Hg^{2+}+ Na^{+a}	0.2230 ± 0.01
GSH + CF	1.4877 ± 0.07
GSH + CF + Hg^{2+b}	0.2063 ± 0.01
GSH + CF +Na^+	1.3593 ± 0.16
GSH + CF + Hg^{2+} + Na^{+b}	0.1085 ± 0.01

Additions: GSH, 50 μg; CF (cytosolar fraction), 10 μg protein; Hg^{2+}, 25 μg; Na^+, 20 μg.

Ligand binding to GSH is assessed by the reduction in absorbance at 405 nm in presence of the thiol indicator, DTNB. Data are expressed as $x \pm$ SE where $n = 9$. Significance of the results was determined by Student's t-test between GSH + Hg^{2+} and GSH + Hg^{2+} + Na^+ (a = non significant) and between GSH + CF + Hg^{2+} and GSH + CF + Hg^{2+} + Na^+ ($b = P$ value < 0.001).

Table 3. Glutathione-S-transferase (GST) activity in rat liver is stimulated by Hg^{2+} and Na^+

System	μmole DNPG* per min per mg protein
Control (C)	3.6 ± 0.18
C + 15 mM Na^+	6.9 ± 0.21
C + 10 mM Hg^{2+}	8.1 ± 0.30
C + 15 mM Na^+ + 10 mM Hg^{2+}	8.3 ± 0.33

* Dinitrophenylglutathione.

GST activity was measured according to Habig *et al.* (1974) and results are expressed as $x \pm$ SE where $n = 9$. Statistical significance of the data was determined by Student's t-test. Data compared between control and treated sets were highly significant ($P < 0.001$) while those between the two treatments (C + Hg^{2+} and C + Na^+ + Hg^{2+}) were not significant although there was a slight stimulation in the GST activity.

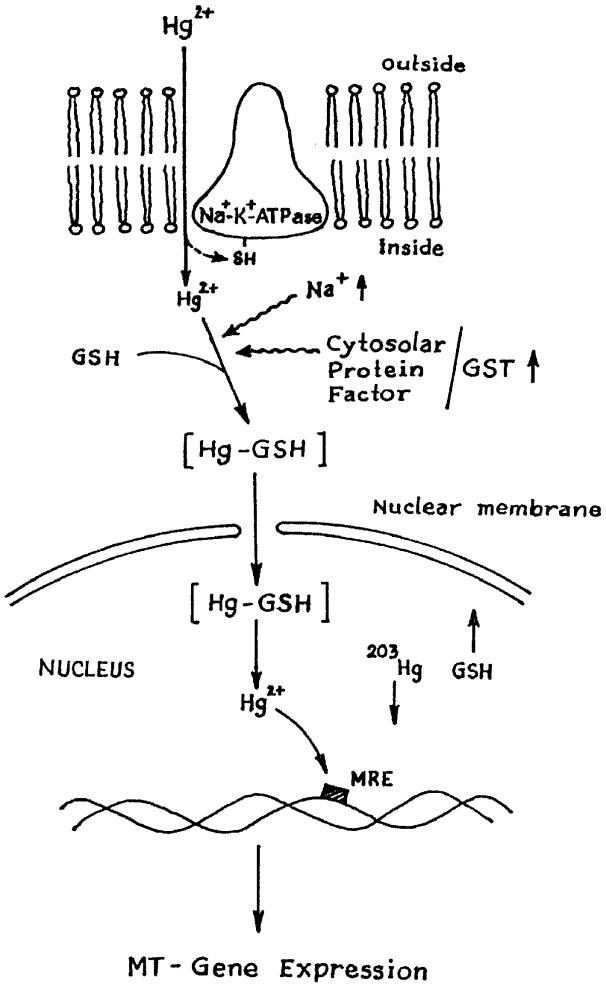

Figure 2. The model proposes a mechanism of signal transduction by inorganic mercury in the rat hepatocyte, inducing metallothionein synthesis. Hg first binds to the Na^+–K^+-ATPase at the inner surface of the membrane and then dissociates to bind to GSH which is preferred due to higher affinity of the metal for GSH. The Hg–GSH complex enters the nucleus through the 9 nm aqueous channel of the nuclear membrane where Hg induces the expression of the metallothionein gene by binding to the metal responsive element.

1993, Bose *et al.* 1993). The entry may be possible through the large number of porin molecules present in the outer membrane; these molecules are relatively non-selective channels that allow the passage of small molecules with a mass less than 10 000 daltons (Ferguson 1995). It has been demonstrated by Bose *et al.* (1993) that Hg^{2+} does bind to mitochondrial DNA; this may also be able to induce MT synthesis, as has been found in rats treated with $CdCl_2$ (Nakazawa *et al.* 1981) and Cu (Sakurai *et al.* 1993). The binding of Hg^{2+} to mitochondrial DNA may be through electron rich sites such as bases, as Hg^{2+} is considered to be a soft ion (Niyogi & Feldman 1981). From our data we propose a model (Figure 2) to visualize the events which take place when Hg^{2+} comes in contact with a hepatocyte surface, binds specifically to Na^+–K^+-ATPase and enters the cytosol to transduce its signal.

Acknowledgements

We thank the Department of Biotechnology, the Department of Science & Technology, Indian Council of Agricultural Research for funds, and the University Grants Commission for the DSA support to the department which enabled the present study.

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