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In vitro binding of inorganic mercury to the plasma membrane of rat platelet affects Na^+-K^+-ATPase activity and platelet aggregation

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Abstract

Hg^{2+} binding to ouabain-sensitive Na^+-K^+-ATPase of rat platelet membrane was specific with a K_a of 1.3×10^9 moles and B_{max} of 3.8 nmoles/mg protein. The binding of mercury to Na^+-K^+-ATPase also inhibits the enzyme significantly ($P < 0.001$), which is greater than its ouabain sensitivity. Further in the cytosol of washed platelets conjugation of reduced glutathione (GSH) to Hg^{2+} is correlated dose dependently (25, 50 and 100 pmoles) to enhanced GSH-S-transferase (GST) activity. It may be concluded from the present *in vitro* experiments that mercury binds specifically to thiol groups present in the platelet membrane Na^+-K^+-ATPase, inhibits the enzyme and induces changes in platelet function, namely, platelet aggregation by interfering with the sodium pump.

Introduction

Mercury is a serious environmental pollutant which can induce a wide spectrum of toxic effects in mammals both physiologically and biochemically (Imura *et al.* 1980; Ghosh & Bhattacharya 1992; Bose *et al.* 1993). It is known that this heavy metal can alter the enzymatic activities by binding to functional groups (sulfhydryl, carboxyl, imidazole, etc.) or displacing the metal associated with the enzyme (Rothstein 1970; Viarengo 1985). Several studies are available on the effect of mercury on Na^+-K^+-ATPase activity (Rothstein 1959; Klonne & Johnson 1988; Anner *et al.* 1990; Anner & Moosmayer 1992) but only a few have addressed the effect of heavy metals on platelet aggregation (Jamaluddin & Thomas 1992; Chaudhury *et al.* 1996; Vinaya Kumar & Bhattacharya 2000; Vinaya Kumar *et al.* 2001). Recently, Vinaya Kumar & Bhattacharya (2000) reported that under *in vitro* treatments (5, 10 & 20 pmoles) with Hg^{2+} and As^{3+} the rate of ADP-induced aggregation accelerated dose dependently but was inhibited by Cd^{2+} treatment in the presence of Ca^{2+}, suggesting the involvement of heavy metals in platelet aggregation. Further studies revealed that Hg^{2+} inhibits adenylate cyclase and stimulates phosphodiesterase activity with a concomitant increase in the rate of platelet aggregation, suggestive of a specific role of cellular cyclic AMP in the event of platelet aggregation. Moreover, the prostaglandin/thromboxane receptors in mammalian platelets have essential sulfhydryl groups (Dorn 1990) therefore Hg^{2+} binding to platelet membrane is an expected phenomenon which may affect platelet aggregation by interacting with the membrane Na^+-K^+-ATPase. This prompted us to address the *in vitro* binding pattern of mercury to rat platelet plasma membrane and its influence on the activity of Na^+-K^+-ATPase.

Materials and methods

Animals

Adult healthy male Sprague-Dawley rats (175–200 g body weight) were used for the present study. Animals were segregated at the rate of four animals per cage in a well ventilated animal room as per standard protocol

(Inglis 1980) and water and food were made available *ad libitum*. Permission was obtained from the Animal Ethics Committee of the University before initiating the experiments. Animals were maintained in humane conditions and sacrificed without causing cruelty.

Chemicals

Sucrose, bovine serum albumin, bovine γ-globulin, sodium chloride, magnesium chloride and polyethylene glycol (PEG) were purchased from E. Merck (India) and were of analytical grade. [^{203}Hg] mercuric nitrate (specific activity 8.32 mCi/mg) was supplied by the Board of Radiation and Isotope Technology (BRIT), Department of Atomic Energy, Government of India, Mumbai, India.

Preparation of platelet-rich plasma (PRP)

Blood was obtained by cardiac puncture from ether anesthetized male rats using a 5 ml disposable plastic syringe containing 0.1 ml of 3.8% sodium citrate. PRP fraction was obtained by centrifugation at 1,000 rpm for 10 min at room temperature using polypropylene conical tubes (Vinaya Kumar & Bhattacharaya 2000). The PRP (3×10^8 cells ml) was diluted with 25 mM Tris-HCl buffer (pH 7.5) to have an optimum preparation of 3×10^6 cells ml of PRP.

Preparation of washed platelet suspension

Washed platelets were prepared according to the method of Ashida & Abiko (1979) and Hayashi *et al.* (1998) with slight modification (Vinaya Kumar & Bhattacharya 2000). Briefly, the citrated PRP was centrifuged at 2,000 rpm for 10 min at 4 °C in a Remi cooling centrifuge (Model C-24). The pellet was resuspended in 25 mM Tris-HCl (pH 7.5) containing 130 mM NaCl, 0.1% glucose, 3.5 g/l BSA and 1.5 mM EDTA, and the suspension centrifuged at 2,000 rpm for 10 min. The process was repeated thrice to have a final suspension of 3×10^6 cells/ml referred as washed platelet suspension.

Preparation of platelet membrane fraction

Platelet membrane was prepared by following the method of alternate abrupt cold and heat shock treatments according to Haslam & Lynham (1972) with slight modification. Briefly, the samples (washed platelet suspension) were completely frozen in a freezing mixture and subjected to thermal shock for 30 min at 37 °C in a shaking water bath (Techne Cambridge, SB-16). The lysed platelets were then centrifuged at 15,000 rpm and 4 °C for 20 min in a Remi cooling centrifuge. The pellet obtained is the platelet membrane fraction which was resuspended in 25 mM Tris-HCl (pH 7.5) containing 130 mM NaCl. The membrane fraction was stored at −80 °C to study the binding kinetics of Hg^{2+} to the specific binding protein or receptor on the platelet membrane surface. Further, the platelet membrane preparation was tested for purity by assaying marker enzymes (Bhattacharya *et al.* 1997). The protein content of the membrane was measured according to the method of Lowry *et al.* (1951) using serum albumin as standard.

Radioassay of mercury binding to platelet membrane

The binding of radiolabelled mercury (^{203}Hg as mercuric nitrate in nitric acid solution having a specific activity of 8.32 mCi/mg, procured from the Board of Radiation and Isotope Technology, Mumbai, India) to the isolated platelet membrane was followed according to the method of Bhattacharya *et al.* (1997). Briefly, 150 μg plasma membrane protein from untreated rat platelet was incubated with varying concentrations of ^{203}Hg in the absence (total binding) or presence of 1,000 fold excess of cold Hg to measure the non specific binding. The tubes with a final volume of 700 μl made up with the incubation medium (0.01 M Na-phosphate buffer, 5 mM $MgCl_2$, 0.1 M sucrose and 0.1% (w/v) BSA, pH 7.4) were incubated at 37 °C for 2 h in a shaking water bath and the reaction terminated by adding 1 ml ice cold washing medium (0.1% bovine gamma globulin and 0.1 M NaCl dissolved in 0.01 M Na-phosphate buffer, pH 7.4). The membrane bound ^{203}Hg was precipitated by adding 1 ml of 20% ice cold PEG followed by centrifugation at 10,000 rpm for 10 min. The supernatant was carefully discarded by aspiration and the steps were repeated as described above. The final pellet was suspended in 1 ml ice cold washing medium, solubilized with 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.

Preparation of Na^+-K^+-ATPase enriched membrane fragments

^{203}Hg-bound fractions of Na^+-K^+-ATPase enriched membrane fragments were obtained by following two

chromatography steps using Sephadex G 75 (SG-75) and Sephacryl S-300 (S-300) columns (Pharmacia, Sweden) in a Biologic LP protein purification system (Biorad Pacific Ltd., Hong Kong). SG-75 (1 × 58 cm) and S-300 columns (1 × 30 cm) were previously equilibrated with 0.01 M Na-phosphate buffer, pH 7.4. The SG-75 column had a bed volume of 44 ml and void volume of 17 ml and the S-300 column had a bed volume of 24 ml and a void volume of 10 ml. Fractions of 2.5 ml each were eluted by the equilibration buffer at a flow rate of 10 ml/h. No chaotropic agent was used in order to have the intact membrane fragments, which will need no reconstitution to demonstrate Na^+-K^+-ATPase activity *in vitro* (Bhattacharya *et al.* 1997). The eluates were analyzed for protein at 280 nm in a Beckman DU 640 Spectrophotometer and for radioactivity in a Beckman Automatic LS Counter as described earlier.

Estimation of Na^+-K^+- ATPase

Na^+-K^+-ATPase activity was assayed in the enzyme enriched fractions of rat platelet membrane following the method of Plummer (1988). Briefly, 2 ml of incubation buffer containing 10 mM NaCl, 120 mM KCl and 30 mM imidazole (pH 7.0) was mixed with 20 μg enzyme protein and 2 mg ATP and incubated at 35 °C for 5 min. The incubation was terminated by chilling the reaction mixture in ice and then by adding 8% cold TCA. An aliquot of 2 ml was taken for inorganic phosphate determination following the method of Fiske & Subbarow (1925) as modified by Lanzetta *et al.* (1979). Briefly, 2.3 ml of distilled water was added to 2 ml of the aliquot collected to which 0.5 ml ammonium molybdate was mixed. Subsequently 0.2 ml reducing reagent (1.5 M $NaHSO_3$, 0.01 M Na_2SO_3 and 10 mM aminonaphthol sulphonic acid) was added and the mixture was incubated for 10 min at room temperature. Finally, the liberated inorganic phosphate was measured at 660 nm in a Beckman DU 640 Spectrophotometer.

Determination of reduced glutathione (GSH) and glutathione-S-transferase (GST)

An *in vitro* assay of Hg (25, 50 and 100 pmoles) binding to GSH was done in cytosolar fraction of washed platelets. The reaction was followed spectrophotometrically by Ellman's reagent (Ellman *et al.* 1961). Briefly, 0.5 ml of GSH sample was added to 2.5 ml DTNB (5,5′-dithiobis-2-nitrobenzoic acid) solution (5 mg/50 ml 0.05 M Na-phosphate buffer, pH 7.6). After 5 min the intensity of the yellow coloured GSH-DTNB adduct (GSTNB) was measured at 405 nm in a Beckman DU 640 Spectrophotometer. GSH content of the sample was calculated from a freshly prepared standard curve and expressed in terms of nmole/mg protein. GST activity of the cytosolar fraction was assayed according to Habig *et al.* (1974). The reaction mixture (3 ml, at 25 °C) contained 0.1 M potassium phosphate buffer, pH 6.9, 1 mM GSH and 1 mM CDNB (1-chloro-2,4-dinitrobenzene) with 0.4 mg protein. The reaction was initiated by adding CDNB and the linear increase in absorbance was recorded for at least 3 min. A concurrent enzyme blank was also recorded. Enzyme activity was calculated from the extinction coefficient of 9.6 mM^{-1} cm^{-1} for DNPG (GSH-CDNB adduct) at 344 nm in a Beckman DU 640 Spectrophotometer and expressed in terms of DNPG produced/min/mg protein.

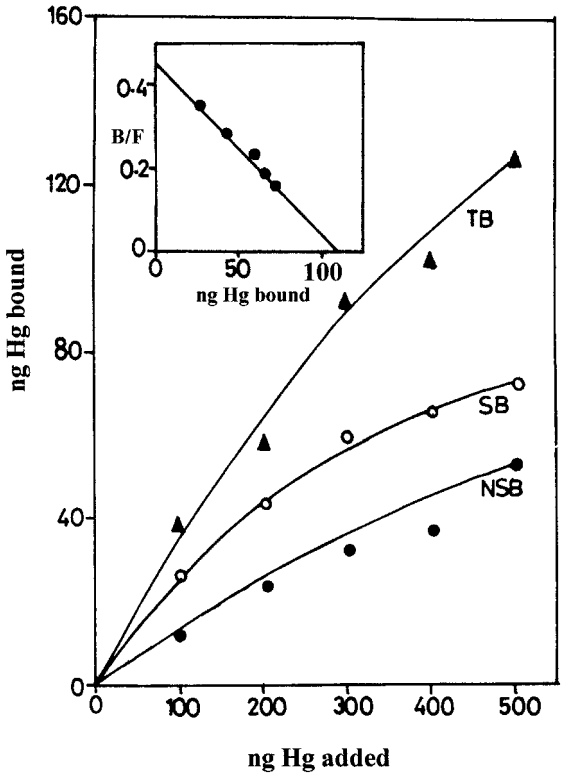

Fig. 1. ^{203}Hg-binding to rat platelet plasma membrane took place in an incubation medium containing 5 mM $MgCl_2$; 0.1 M sucrose and 0.1% BSA in 0.01 M Na-phosphate buffer pH 7.4 at 37 °C. TB = Total binding; SB = Specific binding; NSB = Nonspecific binding. The inset shows Scatchard analysis of the data as bound/free (B/F) Hg versus bound Hg.

Table 1. In vitro Na^+-K^+-ATPase activity in Sephadex G-75 and Sephacryl S-300 fractions of platelet plasma membrane fragments

System	SG-75 fraction 5 (nmole Pi per min per mg protein)	S-300 fraction 3 (μmole Pi per min per mg protein)
Untreated	104 ± 1.38	35 ± 2.34
Ouabain-treated (1 mM)	48 ± 1.92*	16 ± 1.42*
$HgCl_2$-treated		
25 ng	40 ± 3.12*	14 ± 1.57*
50 ng	27 ± 2.16*	9.5 ± 2.26*
100 ng	15 ± 1.27*	6.2 ± 2.34*

Values are expressed as $x \pm$ SE ($n = 15$) and asterisks indicate significant differences ($P < 0.001$) from untreated.

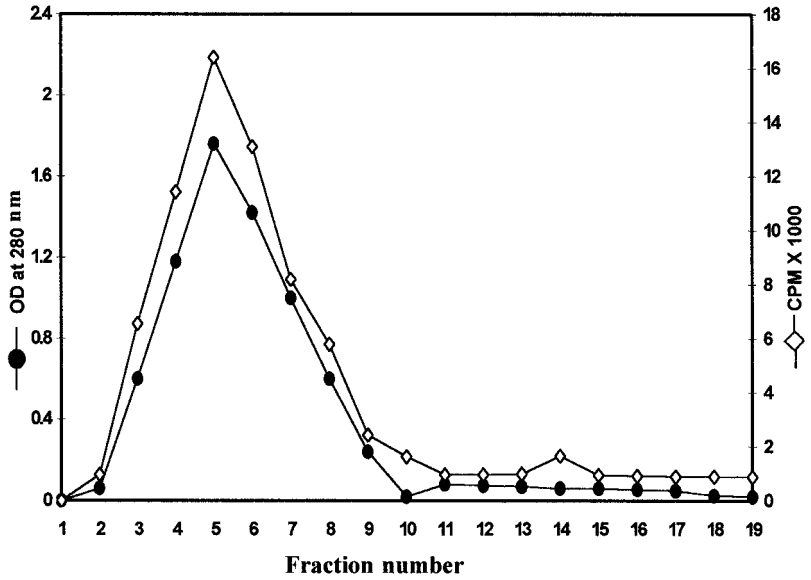

Fig. 2. Separation of ^{203}Hg bound rat platelet membrane fragments in Sephadex G-75 column. [1000 CPM = 5.8 ng ^{203}Hg]. Assay conditions are as described in the text.

Statistical analysis

All data (Tables 1 to 3) are expressed as mean ± SE which were further analysed for statistical significance by Student's '*t*'-test (Snedecor & Cochran 1967).

Results and discussion

Scatchard analysis of specific binding of ^{203}Hg to the rat platelet membrane demonstrated an Association Constant (K_a) of 1.3×10^9 moles and Maximum Binding Capacity (B_{max}) of 3.8 nmoles/mg protein (Figure 1). Our data clearly show ^{203}Hg binding to rat platelet membrane to be highly specific as reported earlier in two different cellular systems, rat liver (Bhattacharya *et al.* 1997) and fish oocyte (Mondal *et al.* 1997). Mercury is known to bind avidly with (-SH) containing biomolecules and high sulphur binding strength of mercury has been observed in a metal cytotoxicity study (Denizeau *et al.* 1990). The involvement of thiol groups in platelet membrane may also explain the high binding capacity of mercury to the membrane (Dorn 1990) and remarkable enhancement of the rate of platelet aggregation with ADP as agonist (Vinaya Kumar & Bhattacharya 2000). More recently, Vinaya Kumar *et al.* (2001) demonstrated by scanning electron microscopy profuse pseudopodia formation on the surface of washed platelets, enhancing the aggregation rate on treatment with 10 pmoles of Hg^{2+}.

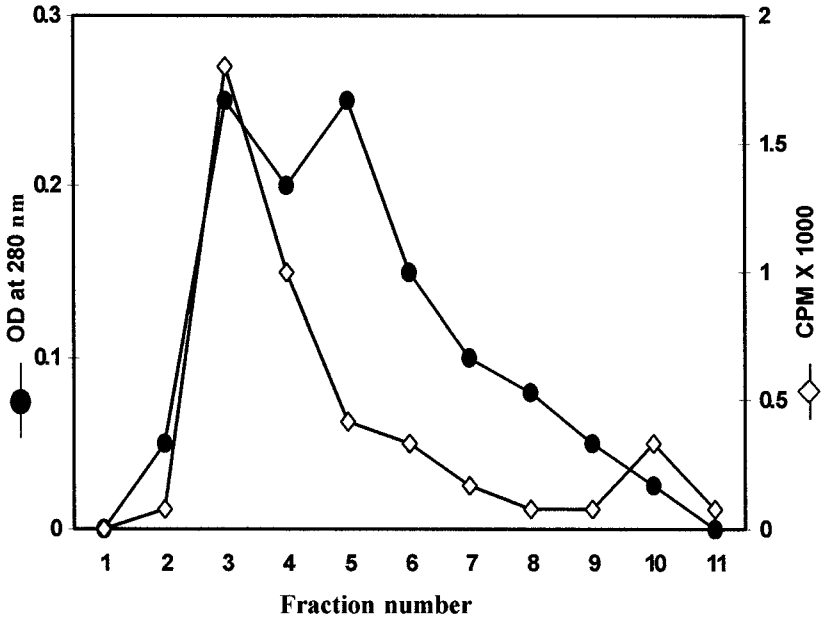

Fig. 3. Separation of ^{203}Hg bound rat platelet membrane fragments in Sephacryl S-300 column. [1000 CPM = 5.8 ng ^{203}Hg]. Assay conditions are as described in the text.

These findings suggest that inorganic mercury also have specific binding sites in the rat platelet membrane to induce changes in platelet function.

Column chromatography revealed that the fractionated ^{203}Hg-bound Na^+-K^+-ATPase enriched membrane fragments possess a very high molecular weight (Figure 2) in the range of 300,000 dalton (Figure 3), and Hg^{2+} dose dependently inhibits this ouabain sensitive Na^+-K^+-ATPase (Table 1). These observations not only substantiate that Hg^{2+} inhibits Na^+-K^+-ATPase as reported earlier (Anner *et al.* 1990; Anner & Moosmayer 1992; Imesch *et al.* 1992; Bhattacharya *et al.* 1997; Mondal *et al.* 1997) but also demonstrates a high specific binding of the Group II B metal to the enzyme as reported in rat liver (Bhattacharya *et al.* 1997) and fish oocyte (Mondal *et al.* 1997). Since this enzyme 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), the Hg-bound platelet membrane fractions appeared in the void volumes during gel filtration through both SG-75 and S-300 columns. The inhibition of Na^+-K^+-ATPase by heavy metals such as mercury and cadmium is explained by their high binding capacity to the platelet membrane due to involvement of thiol (-SH) groups as substantiated by earlier reports (Bhattacharya *et al.* 1997; Mondal *et al.* 1997; Lionetto *et al.* 1998). It has been reported that platelet aggregation is altered by inhibition of Na^+-K^+-ATPase through formation of stable mercaptide bonds with the -SH groups present in the platelet membrane (Hughes 1950; Rabenstein 1978; Dorn 1990).

Table 2. Reduced glutathione (GSH) profile in platelets (3×10^6 cells ml) treated *in vitro* with 25, 50 and 100 pmoles of Hg.

System	pmoles	GSH (nmole/mg protein)
Control		15.41 ± 0.25
Hg-treated	25	10.37 ± 0.15*
	50	6.48 ± 0.33*
	100	4.70 ± 0.25*

Values are expressed as $x \pm$ SE ($n = 3$) and asterisks indicate significant differences ($P < 0.001$) from control.

Further from a simple spectrophotometric *in vitro* assay it is clear that Hg^{2+} binding to platelet decreased GSH level (Table 2) significantly ($P < 0.001$) in a dose dependent manner below the control (32%, 57% and 69%) and concomitantly enhanced GST activity by 22%, 57% and 92% above the control (Table 3). The cytosol of washed platelets being a buffered biological system, Hg^{2+} binding to GSH is catalyzed by GST as substantiated by earlier reports with cadmium and selenium (Se) compounds (sodium selenite and sodium selenate) on glutathione-

Table 3. Glutathione-S-transferase (GST) activity in platelets treated *in vitro* with 25, 50 and 100 pmoles of Hg.

System	pmoles	GST (μmoles DNPG*/min/mg protein)
Control		0.04 ± 0.016
Hg-treated	25	0.049 ± 0.004*
	50	0.063 ± 0.007*
	100	0.077 ± 0.008*

*Dinitrophenylglutathione
Values are expressed as $x \pm$ SE ($n = 3$) and asterisks indicate significant differences ($P < 0.001$) from control.

related enzymes (glutathione peroxidase, glutathione-S-transferase [GST] and glutathione reductase [GR]) in human and pig blood platelets (Mukhopadhyay *et al.* 1988; Zbikowska *et al.* 1997). Moreover, GSH is stabilized in the enolic form where -C=N-enhances the coordinating power of -N- and binds to Hg^{2+}. Hg-GSH conjugation is preferred over metal binding to the membrane – SH groups due to the higher affinity of Hg to GSH (Webb 1966). Interestingly, Hg-GSH conjugation by GST is stimulated by the increased Na^+ concentration owing to inhibited Na^+-K^+-ATPase activity as reported earlier in Hg-treated rat liver (Bhattacharya *et al.* 1997).

It is concluded that such *in vitro* studies could contribute to the understanding of the cellular events which may take place when Hg^{2+} comes in contact with a platelet surface, binds specifically to the membrane Na^+-K^+-ATPase and enters the cytosol to transduce its signal in the form of platelet aggregation (Feinberg *et al.* 1977) by cytosolar increase in Na^+ concentration. However, it is difficult to postulate a clear signal in platelets on the basis of the few reports available on the effect of xenobiotics on platelet aggregation and warrants further studies to address the specific regulatory mechanism of xenobiotic-platelet interactions.

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