Increased expression of procoagulant activity on the surface of human platelets exposed to heavy-metal compounds

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One of the essential roles for platelets in haemostasis is in the potentiation of blood clotting due to the contribution of anionic phospholipid from the surface of the cells, as an essential cofactor to the proteolytic reactions of coagulation (platelet procoagulant activity). Only a limited number of agonists are known to initiate platelet procoagulant activity. In this study the rate of thrombin formation on the platelet surface was observed to increase in a dose-dependent manner upon treatment of washed platelets with heavy-metal compounds. Unlike the immediate increase observed upon treatment of platelets with calcium ionophore, A23187, the change due to these agents was progressive, approaching a maximum after 10 min. The maximum-fold acceleration of the rate of thrombin formation compared with control platelets was calculated for HgCl_2 (56-fold), AgNO_3 (42-fold) phenylmercuri-acetate (24-fold) and thimerosal (14-fold), compared with 70-fold observed for calcium ionophore. The increase in procoagulant activity due to HgCl_2 coincided with a large increase

in intracellular calcium and phosphorylation of 22 and 45 kDa proteins. It is considered that the mechanism responsible for the increase in procoagulant activity is exposure of anionic phospholipids. This was detected by a 2-fold increase in the binding of ^{125}I -annexin V upon addition of HgCl_2 , compared with resting platelets (3-fold on treatment of platelets with calcium ionophore). In contrast to the generation of activity by A23187 and other known agonists of this reaction, heavy-metal compounds appeared to cause little or no release of microparticles from the platelet surface. Since HgCl_2 did not cause aggregation of platelets or significant release of serotonin, these findings may give further support to the need for exposure and ligation of glycoprotein IIb:IIIa for vesiculation to occur. Treatment of platelets with heavy metals may constitute a new approach to investigating the early changes in the cell membrane which lead to increased expression of anionic phospholipid.

INTRODUCTION

Platelets play two important roles in normal haemostasis. First, by aggregating, they constitute the initial haemostatic plug which immediately curtails bleeding from broken blood vessels. Secondly, the platelet surface can become activated and potentiate blood clotting, a property referred to as platelet procoagulant activity. This is usually observed as an increase in the rate of activation of prothrombin by factor Xa in the presence of factor Va and Ca^{2+} , referred to as the prothrombinase reaction. The change to the surface of the platelet responsible for procoagulant activity is due, principally, to a reversal of the polarity of the phospholipid membrane: the anionic phospholipids, which are normally maintained by a translocase system at a higher concentration on the inner leaflet, become exposed on the outer membrane surface (reviewed in [1,2,3,4]). The physiological importance of this property of platelets is demonstrated by the moderately severe bleeding disorder, Scott syndrome, in which stimulated platelets reveal abnormally low levels of anionic phospholipid exposure and a correspondingly lower procoagulant activity [5,6].

The generation of platelet procoagulant activity does not occur with all agonists. 'Weak' agonists such as ADP, adrenalin and platelet-activating factor hardly affect the procoagulant properties of the platelet surface even though irreversible thromboxane-dependent aggregation can proceed to near completion. In contrast, thrombin, collagen, complement attack

complex C5b-9 and calcium ionophore have been demonstrated to enhance the generation of platelet procoagulant activity in the order of potency: ionophore > collagen/thrombin > C5b-9 > collagen > thrombin [4]. Treatment of platelets with local anaesthetics (dibucaine and tetracaine) or with sulphydryl oxidizing agents (diamide or pyridyldithioethanolamine) also cause an increase in procoagulant activity which is dependent upon extracellular calcium [4].

We recently observed an increase in the procoagulant activity of U937 monocyte-like cells upon treatment with mercuric and other heavy-metal compounds [7]. Both the tissue factor activity of the cell and the ability of the surface to support the prothrombinase reaction were rapidly increased, concomitant with a large increase in intracellular calcium concentration ($[\text{Ca}^{2+}]_i$). In the present study we have identified these heavy metals as potent agonists of platelet procoagulant activity. The characteristics of induction of the procoagulant surface are distinct from that promoted by other platelet agonists in that the degree of microvesiculation is low.

EXPERIMENTAL

Materials

Human α -thrombin was obtained as a gift from Dr. J.-M. Freysinnet (Strasbourg, France), human factor X from Enzyme Research Laboratories (Swansea, U.K.) and bovine factor V

Abbreviations used: PRP, platelet-rich plasma; PKC, protein kinase C; $[\text{Ca}^{2+}]_i$, intracellular calcium concentration; $[\text{Ca}^{2+}]_i$ -HT, $[\text{Ca}^{2+}]_i$ -hydroxytryptamine; MLC, myosin light chain; $[\text{Ca}^{2+}]_{\text{cyt}}$, cytosolic calcium; Tg, thapsigargin.

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from Dr. T. Lindhout (University of Limburg, The Netherlands). Factor V was activated to factor Va as described previously [8]. Bovine annexin V was obtained from Dr. R. Donato (University of Perugia, Italy). The calcium ionophores, A23187 and ionomycin, were obtained from Sigma Chemical Co., and stock solutions in dimethyl sulphoxide were prepared and stored at -20°C until use. HgCl_2 , AgNO_3 , phenylmercuriacetate and thimerosal (sodium ethylmercurisalicylate) were obtained from Sigma Chemical Co. Soluble collagen was obtained as a solution in 0.1 M acetic acid (from Nycomed U.K. Ltd.) and was diluted in physiological buffer just before use.

Preparation of washed human platelets

Human blood was obtained by forearm venepuncture of healthy volunteers who had been denied medication for at least 10 days. Blood (5 vol.) was drawn into acid-citrate-dextrose (0.052 M citric acid/0.08 M trisodium citrate/0.183 M glucose; 1 vol.) and platelet-rich plasma (PRP) was obtained by centrifugation at 200 *g* for 15 min as previously described [9]. Prostaglandin I_2 was added to a final concentration of 20 ng/ml and the PRP was centrifuged at 1000 *g* for 10 min. The platelet pellet was resuspended in a citrate washing buffer (pH 6.5) and centrifuged at 1000 *g* for 10 min [9]. In some experiments, platelets in washing buffer were preincubated with [^{14}C]5-hydroxytryptamine ([^{14}C]5-HT) (0.1 $\mu\text{Ci}/\text{ml}$; Amersham International, Amersham, Bucks., U.K.) for 30 min (37°C) before centrifugation. Washed platelets were finally resuspended at the appropriate cell count in a Hepes-Tyrode buffer (pH 7.4) composed of 10 mM Hepes/145 mM NaCl/5 mM KCl/1 mM MgSO_4 /5 mM D-glucose (buffer A). This buffer was supplemented with 1 mM CaCl_2 in those experiments which required measurement of aggregation, dense-granule secretion or protein phosphorylation.

Platelet procoagulant activity

The ability of the platelet surface to enhance the activation of prothrombin by factor Xa in the presence of factor Va and calcium (platelet procoagulant activity) was measured by preincubating platelets (1×10^7 cells/ml) alone or in the presence of activator for 5 min at 37°C . An aliquot of platelet suspension (15 μl) was subsequently diluted 10-fold with buffer A containing Ca^{2+} , factor Va and factor Xa at final concentrations of 5 mM, 1 nM and 0.3 nM respectively. A minimal volume (10 μl) of a mixture of prothrombin (final concentration 0.42 μM) and chromogenic substrate S-2238 (final concentration 0.26 mM) was added and the increase in absorption at 450 nm observed. Experiments were performed in 96-well microtitre plates using a Molecular Devices Corporation Thermomax plate reader (37°C). The $A_{405/650}$ was recorded every 18 s for 10 min while shaking.

Separation of platelet microvesicles and time-course experiments

In order to measure the time-course of activation and the formation of microvesicles during the generation of platelet procoagulant activity, platelet suspensions ($1 \times 10^7/\text{ml}$) were treated with agonist as described above and samples were removed at timed intervals [10]. Samples were analysed for procoagulant activity as described above, directly, or as the supernatant prepared by centrifugation at 11500 *g* for 2 min. The fold activation due to the microvesicles present in the supernatant was calculated and expressed with respect to the total activity of platelet mixture before centrifugation.

Binding of ^{125}I -annexin V

Radioiodination

Annexin V was labelled by the Iodo-Gen method [11]. Iodo-Gen (Pierce Chemical Co.) was prepared according to the manufacturer's instructions and coated onto a polypropylene tube. Iodination was carried out for 10 min on ice by adding 100 μg of annexin V and 1 mCi of Na^{125}I (Amersham International, Amersham, Bucks., U.K.) dissolved in 1 ml of buffer (0.05 M Tris-HCl/0.15 M NaCl, pH 7.5). On completion, the solution was removed from the tube and ^{125}I -annexin V was isolated by chromatography on a PD-10 (G-25) column (Pharmacia) equilibrated with Hepes buffer (10 mM Hepes/0.15 M NaCl, pH 7.5) containing 0.25 % w/v of gelatin. The specific radioactivity of ^{125}I -annexin V was in the range 2700–3600 c.p.m./ng protein. Analysis of the isolated ^{125}I -annexin V by SDS/PAGE [12] and autoradiography showed two bands of 33 and 37 kDa under reducing conditions [13]. The radiolabelling efficiency was confirmed by precipitation with 20 % trichloroacetic acid. At least 90 % of the radioactivity was due to the radiolabelled protein. ^{125}I -annexin V was stored in aliquots at -70°C until use.

Binding assay

Binding of ^{125}I -annexin V to platelets was measured as described previously [14]. Platelets (10^8 cells/ml suspended in buffer A) were incubated for 15 min with ^{125}I -annexin V (100 mM, final concentration) in 0.25 ml of buffer A supplemented with 5 mM CaCl_2 and 1 mg/ml BSA. At the end of all incubations, the platelets were layered onto buffer A supplemented with sucrose (20 % w/v). The samples were centrifuged at 10000 *g* for 10 min and the tip of the microfuge tube was cut off and counted using an LKB γ -counter. Non-specific binding was measured in the presence of a 100-fold molar excess of unlabelled ligand.

Measurement of $[\text{Ca}^{2+}]_i$

$[\text{Ca}^{2+}]_i$ was determined in fura-2/AM-loaded platelets as previously described [15]. Briefly, platelets in calcium-free buffer A were loaded with fura-2/AM (2 μM ; 45 min, 37°C), washed twice by centrifugation in citrate washing buffer (pH 6.5) and resuspended to a count of $1\text{--}2 \times 10^8/\text{ml}$ in buffer A with or without added calcium (1 mM CaCl_2). Fluorescence was monitored in a Perkin-Elmer LS5 luminescence spectrometer. Free Ca^{2+} was calculated by the method of Grynkiewicz et al. [16].

Measurement of platelet aggregation and [^{14}C]5-HT secretion

Simultaneous measurement of aggregation and dense-granule secretion was carried out essentially as described previously using 300 μl aliquots of [^{14}C]5-HT-labelled platelets. Aggregation was measured by recording the extent of light transmission using an Aggreccorder II four-channel aggregometer [9].

Protein phosphorylation studies

For the measurement of protein phosphorylation, platelets ($1 \times 10^8/\text{ml}$) in washing buffer were incubated with 0.25 mCi of carrier-free [^{32}P]P $_i$ /ml for 90 min at 37°C . Following incubation,

platelets were washed twice by centrifugation (10 min; 1500 g) and were subsequently resuspended in buffer A to a final count of 4×10^8 /ml. Incubations were carried out using 100 μ l aliquots of cell suspension and were terminated with 50 μ l of Laemmli sample buffer [12]. Phosphoproteins were separated on gradient polyacrylamide gels (7–20 %) and 32 P incorporation was assessed by autoradiography (-70°C ; Fuji RX X-ray film).

RESULTS

Effect of mercury compounds on platelet procoagulant activity

After preincubation of platelets with HgCl_2 for 5 min at 37°C , the rate of prothrombin activation was enhanced (Figure 1). The observed rate of formation of thrombin was fitted, using the Enzfitter programme, to the equation:

$$A_{405} = V_0 t + \frac{1}{2} a t^2$$

where V_0 is the velocity in the absence of cells, t is the time and a is the acceleration in arbitrary units (setting the rate as one for the influence of cells in the absence of activator). Figure 2 shows the generation of procoagulant activity observed in the presence of increasing concentrations of HgCl_2 . The data are expressed as a ratio of the acceleration obtained after activation compared with control platelets. Figure 2 also illustrates the results obtained under similar conditions with the calcium ionophore, A23187, which has been previously demonstrated to promote an increase in platelet procoagulant activity [17]. The greatest acceleration was observed following treatment with the calcium ionophores, A23187 or ionomycin (results not shown). The observed increase in rate due to platelets treated with HgCl_2 is similar to that of platelets exposed to a thrombin/collagen mixture [18]. Maximal activation was observed at $4 \mu\text{M}$ HgCl_2 with a decrease observed at higher concentrations (Figure 2). Maximal acceleration due to HgCl_2 (56-fold) was greater than that observed with either thimerosal (sodiummethylmercuri-thiosalicylate) (14-fold) or phenylmercuriacetate (24-fold). AgNO_3 also stimulated procoagulant activity (42-fold). Similar results were observed whether stimulation was carried out in the presence or absence of CaCl_2 (5 mM; results not shown). In control incubations in

Figure 1 Enhancement of the rate of prothrombin activation in the presence of platelets pretreated with HgCl_2

Platelets (1×10^7 cells/ml) suspended in HEPES buffer were preincubated at 37°C in the presence (○) or absence (★) of HgCl_2 (final concentration, $4 \mu\text{M}$) or of calcium ionophore A23187 (□) ($5 \mu\text{M}$) for 5 min. The mixture was then diluted in HEPES-Tyrode buffer (pH 7.4) containing factor Xa (0.3 nM), factor Va (1 nM) and calcium ion (5 mM) in micro-well plates at 37°C . At zero time, prothrombin (final concentration $0.42 \mu\text{M}$) and chromogenic substrate S2238 (0.26 mM) were added and the increase in A_{405} was observed. Data shown here are from a single experiment with platelets from a single donor.

Figure 2 Dose-response relationship for fold acceleration of prothrombin activation by platelets pretreated with a variety of agonists

Platelets were treated with various agonists according to the procedures described in the legend to Figure 1. The fold acceleration of prothrombin activation by factor Xa was calculated as described in the Results section. ○, HgCl_2 ; ▽, thimerosal; ◇, AgNO_3 ; △, phenylmercuriacetate; □, calcium ionophore, A23187. Data are mean $\pm$ S.E.M. from six experiments performed on platelets from different donors.

Figure 3 Time-course of generation of platelet procoagulant activity by various heavy-metal agonists

Platelets were treated with various agonists according to the procedure described in the legend to Figure 1. At timed intervals samples were removed and platelet procoagulant activity measured. The mean results are shown here ($n = 4$) expressing the fold acceleration of thrombin formation as a percentage of the maximum observed during the time period of the experiment. The maximum-fold acceleration differed for each agonist as follows: ○, HgCl_2 , 54-fold ($4 \mu\text{M}$); ▽, thimerosal, 12-fold ($230 \mu\text{M}$); ◇, AgNO_3 , 41-fold ($420 \mu\text{M}$); △, phenylmercuriacetate, 25-fold ($230 \mu\text{M}$); □, calcium ionophore A23187, 70-fold ($5 \mu\text{M}$).

which each agonist was incubated with coagulation factors, in the absence of platelets, no change in rate from blank incubations was observed. Platelets treated with A23187 showed an immediate increase in procoagulant activity (Figure 3). In contrast, the increase in platelet procoagulant activity due to the heavy metals was progressive, approaching a maximum after 10 min (Figure 3). The response to HgCl_2 was unaffected by pre-treatment of platelets with indomethacin ($10 \mu\text{M}$), prostacyclin (100 ng/ml) or the cell-permeant, calpain inhibitor E-64d ($50 \mu\text{g/ml}$), but was partially reduced by the non-specific calmodulin inhibitors W7 ($40 \mu\text{M}$) and trifluoperazine ($40 \mu\text{M}$) (results not shown).

Influence of HgCl_2 upon platelet activity

Exposure of labelled platelet suspensions to HgCl_2 ($10 \mu\text{M}$) alone caused shape change and the release of approx. 5 % of the

Table 1 Influence of treatment of platelets with HgCl_2 alone or with other agonists upon shape change, aggregation and release of [^{14}C]5HT

Aliquots of [^{14}C]5-HT-labelled platelets were incubated with the indicated final concentrations of HgCl_2 alone, agonist alone or HgCl_2 and agonist. Data are mean $\pm$ S.E.M. from four to five experiments. Aggregation and percentage [^{14}C]5-HT release were quantified as described in the Experimental section. NT = not tested.

	Aggregation (%)	5-HT release (%)
Basal controls	0	0
Thrombin (0.2 U/ml)	84	35.7 ± 2.1
Collagen (20 $\mu\text{g}/\text{ml}$)	30 ± 1.5	2.4 ± 0.4
Thrombin (0.2 U/ml) and collagen (20 $\mu\text{g}/\text{ml}$)	87	51.2 ± 0.9
A23187 (1 μM)	65 ± 2.8	59.3 ± 1.3
HgCl_2 (10 μM)	Shape change (2 ± 2.3)	5.2 ± 0.4
HgCl_2 (12.5 μM)	Shape change (2 ± 1.8)	6.7 ± 3.4
HgCl_2 (10 μM , 1 min incubation) before thrombin (0.2 U/ml)	Shape change	14.4 ± 1.5
HgCl_2 (10 μM , 3 min incubation) before thrombin (0.2 U/ml)	Shape change (3.5 ± 1)	22 ± 1.5
HgCl_2 (10 μM , 6 min incubation) before thrombin (0.2 U/ml)	Shape change	26.4 ± 1.9
HgCl_2 (10 μM , 1 min incubation) before A23187 (1 μM)	20.8 ± 6.8	24.7 ± 6.5
HgCl_2 (10 μM , 3 min incubation) before A23187 (1 μM)	24 ± 6.5	28.7 ± 4.5
HgCl_2 (10 μM), thrombin (0.2 U/ml) and collagen (20 $\mu\text{g}/\text{ml}$)	Shape change (7.3 ± 0.8)	NT
HgCl_2 (10 μM , 1 min incubation) before thrombin (0.2 U/ml) and collagen (20 $\mu\text{g}/\text{ml}$)	3.5	15.9 ± 1.7

Figure 4 Protein phosphorylation in HgCl_2 -stimulated platelets

Aliquots of ^{32}P -labelled platelets were incubated with increasing concentrations of HgCl_2 for either 1 or 3 min. Reactions were terminated as described in the Experimental section and phosphoproteins were analysed by SDS/PAGE and autoradiography. The Figure shows an autoradiograph from a representative experiment. Lanes a, b, control; c, d, 10 nM HgCl_2 ; e, f, 100 nM HgCl_2 ; g, h, 1 μM HgCl_2 ; j, k, 10 μM HgCl_2 ; l, m, 100 μM HgCl_2 . Lanes a, c, e, g, i, j, l, 1 min incubation; lanes b, d, f, h, k, l, 3 min incubation. Bands at 45 and 20 kDa are indicated.

platelet [^{14}C]5-HT content (Table 1). Following treatment with HgCl_2 the cells maintained the swirling properties seen with unstimulated platelets. HgCl_2 -stimulated platelets showed modified responses following subsequent addition of other agonists (Table 1); in particular, aggregation and dense-granule secretion induced by thrombin and thrombin/collagen mixtures were inhibited by pretreatment with HgCl_2 . Although Ca^{2+} -ionophore-induced aggregation was not abolished by HgCl_2 treatment, a significant reduction in the extent of aggregation and release was observed in the presence of HgCl_2 (Table 1).

Figure 4 shows the effects of various concentrations of HgCl_2 on the extent of ^{32}P incorporation into proteins from total platelet homogenates. HgCl_2 concentrations of 100 nM and above clearly promoted the phosphorylation of the major protein kinase C (PKC) substrate, pleckstrin (approx. 45 kDa) and of a

Figure 5 Increase in $[\text{Ca}^{2+}]_i$ in platelets treated with HgCl_2

Platelets were preloaded with fura-AM and changes in $[\text{Ca}^{2+}]_i$ levels were monitored as described in the Experimental section. Data show the changes in $[\text{Ca}^{2+}]_i$ with time upon treatment of the platelet suspension with (a) HgCl_2 (5 μM) or (b) HgCl_2 (100 μM). CaCl_2 and HgCl_2 were added at the times indicated. The increase in signal at 5 min in (a) is observed upon lysis of the cells with detergent.

20 kDa protein (myosin light chain). Incorporation of ^{32}P into these proteins in response to HgCl_2 was not affected by pretreatment with W7 (100 μM) (results not shown).

Increase in $[\text{Ca}^{2+}]_i$

The increased exposure of phosphatidylserine to the outer surface of the cell upon treatment with various agonists is considered to be due, at least in part, to an increase in $[\text{Ca}^{2+}]_i$ [19,20]. The

Figure 6 Increase in $[Ca^{2+}]_i$ in platelets treated with $HgCl_2$ according to concentration

The peak concentration of $[Ca^{2+}]_i$ was measured as described in the Experimental section and the legend to Figure 5 and is shown as a function of the concentration of $HgCl_2$ (○), ionomycin (□) and thrombin (◇).

effects of $HgCl_2$ on $[Ca^{2+}]_i$ were examined using fura-2/AM-loaded platelets. (The mercury ion has been reported not to influence fura-2 AM fluorescence [21]). As shown previously in other cell types [22,23,24], $HgCl_2$ is potent in increasing $[Ca^{2+}]_i$. At $5 \mu M$ $HgCl_2$ a rapid elevation in peak $[Ca^{2+}]_i$ was observed which was maintained (Figure 5a). At higher concentrations ($> 50 \mu M$), the peak in $[Ca^{2+}]_i$ was followed by a progressive depletion of $[Ca^{2+}]_i$ reaching a minimum from which a slow increase occurred (Figure 5b, lower panel). The initial peak $[Ca^{2+}]_i$ varied according to the concentration of $HgCl_2$ as shown in comparison to varying concentrations of thrombin and ionomycin (Figure 6). We have previously observed a similar pattern of calcium elevation in $HgCl_2$ -treated U937 monocyte-like cells [7] and this effect has also been reported following treatment of cultured rabbit renal tubular cells with $HgCl_2$ [23]. The initial peak of $[Ca^{2+}]_i$ seen on exposure to $100 \mu M$ $HgCl_2$ (Figure 5b) was reduced approx. 4-fold when activation was carried out in the presence of $1 mM$ EGTA. When tested at $12 \mu M$ $HgCl_2$, the $[Ca^{2+}]_i$ peak in the absence of extracellular Ca^{2+} was only partially reduced ($870 nM$ in the presence of Ca^{2+} , $695 nM$ in the absence of Ca^{2+} , $n = 2$) indicating that the major pool responsible for $[Ca^{2+}]_i$ elevation upon treatment with $HgCl_2$ is within intracellular stores. When $[Ca^{2+}]_i$ was raised by treatment of platelets with other agonists ($2 nM$ α -thrombin, $1 \mu g/ml$ collagen, $20 \mu M$ thrombin receptor activating peptide) before treatment with $HgCl_2$, a further increase in $[Ca^{2+}]_i$ was observed. Addition of prostacyclin ($100 ng/ml$), trifluoperazine ($40 \mu M$) or W7 ($40 \mu M$) to platelets, 1 min before challenge with varying concentrations of $HgCl_2$, had no significant influence on the increase in $[Ca^{2+}]_i$.

Binding of ^{125}I -annexin and microvesicle formation

The increase of platelet procoagulant activity observed upon treatment with known agonists is due to increased expression of anionic phospholipids upon the surface of the cell [25]. In order to test this, as regards the action of mercury ion, cells were preincubated with $10 \mu M$ $HgCl_2$ and the specific binding of ^{125}I -annexin measured [14]. The results (mean $\pm$ S.E.M.; $n = 3$) showed an increase in specific binding from $13.1 \pm 3.8\%$ for untreated cells to $25.1 \pm 6.4\%$ after treatment with $HgCl_2$ and to $50.3 \pm 4.3\%$ upon treatment of platelets with A23187 ($1 \mu M$).

The exposure of phosphatidylserine is thought to arise during blebbing of the surface of the cell. This blebbing has been shown

Figure 7 The release of microvesicles from platelets treated with various agonists

Platelets were treated with various agonists according to the procedure described in the legend to Figure 1. At timed intervals, samples were centrifuged ($11500 g$ for 2 min at room temperature) and the total activity of the supernatant measured. The results are shown ($\pm$ S.E.M., $n = 4$) as a percentage of the total activity of the mixture before centrifugation. The fold acceleration for each agonist differed at each time-point (Figure 3). ○, $HgCl_2$ ($4 \mu M$); ▽, thimerosal ($230 \mu M$); ◇, $AgNO_3$ ($420 \mu M$); △, phenylmercuriacetate ($230 \mu M$); □, calcium ionophore, A23187 ($5 \mu M$).

to be associated with the formation of microvesicles which are subsequently released from plasma membrane [6,26,27]. The liberation of these vesicles, as well as the exocytosis of cell granules, involve the fusion (or fission) of opposing zones of membrane. It is thought that, at the point of fusion/fission, transient non-bilayer structures appear, allowing fast mixing of components within the bilayer. To examine this phenomenon of microvesiculation, platelets were treated with A23187 ($5 \mu M$), $HgCl_2$ ($4 \mu M$), $AgNO_3$ ($420 \mu M$), thimerosal ($230 \mu M$) or 4β -phorbol 12-myristate 13-acetate ($230 \mu M$) and samples removed at timed intervals and centrifuged ($11500 g$). The separated platelet pellets were resuspended in buffer A and the procoagulant activity of the cells and supernatant were measured. The release of microvesicles reached a maximum level within 1 min of treatment with A23187 (50% of the maximum level) and remained constant thereafter. With the heavy-metal agonists (except thimerosal) the particle release increased slowly over a 10 min period (Figure 7). With the agonists $HgCl_2$, $AgNO_3$ and phenylmercuriacetate it was possible to achieve, at certain time-points, a high degree of membrane depolarization with little or no microvesicle release (Figure 7 taken in reference to Figure 3).

DISCUSSION

The maintenance of the asymmetry of distribution of aminophospholipids in the plasma membrane is considered to be due principally to an active pump system. The putative pump protein, referred to as aminophospholipid translocase, has been demonstrated to catalyse the rapid transfer of aminophospholipids from the outer membrane to the inner leaflet [28,29]. This transport is dependent on ATP, inhibited by sulphhydryl-reactive compounds and is selective only for the L-isomer of aminophospholipids [30]. The transport is also inhibited by high levels of $[Ca^{2+}]_i$, which appears to facilitate an inside to outside movement of aminophospholipids independent of the translocase [31,32,33]. A further aspect of the generation of platelet procoagulant activity is the induction of microvesicle formation after initial blebbing of the membrane; this formation is considered to have an absolute requirement for extracellular calcium.

In the present paper we demonstrate that heavy-metal compounds are potent agonists for platelet procoagulant activity. The mechanism of this induction is probably related to the ability of these agents to oxidize sulphhydryl groups and raise $[Ca^{2+}]_i$. Inorganic mercuric ion is highly permeable in lipid bilayers [34]. For example, Gutknecht [35] estimates the permeability through a lipid bilayer at 1.3×10^2 cm/s. The ability of heavy-metal ions to cause a rapid increase in $[Ca^{2+}]_i$ has been noted previously in platelet membranes [22], kidney cells [23], skeletal [36] and cardiac muscle cells [24] and monocyte-like cells [7]. The most likely point of action is considered to be the binding of mercury to one or more free sulphhydryl groups present in Ca^{2+} -ATPases and/or Ca^{2+} release channels.

Platelets are known to contain at least two types of intracellular sarco-endoplasmic reticulum Ca^{2+} -ATPases which regulate the movement of Ca^{2+} into the stores [37]. In preliminary experiments, $HgCl_2$ (10 μ M) caused inhibition of platelet membrane Ca^{2+} -ATPases activities by 45% ($n = 2$) under conditions where 90% inhibition of the Ca^{2+} -ATPase was observed with thapsigargin (Tg, assayed as in [38]). An action of Hg^{2+} on Ca^{2+} -ATPases, alone, is unlikely to fully explain the $[Ca^{2+}]_i$ elevation seen in our studies. Indeed, the rapid elevation of $[Ca^{2+}]_i$ by Hg^{2+} (at 10 μ M) is atypical of that obtained with Tg which induces a slow increase of cytosolic calcium ($[Ca^{2+}]_{cyt}$) [38]. Thiol reagents such as thimerosal have been observed to increase Ca^{2+} fluxes by enhancing the sensitivity of the Ca^{2+} release channels to putative agonists such as inositol triphosphate and cyclic ADP ribose [e.g. 39,40] and may even affect the properties of the calcium-binding proteins releasing free Ca^{2+} in the store lumen [40]. A combination of the two above proposals may explain the rapid rise of $[Ca^{2+}]_i$ (arising mainly from intracellular stores) seen with Hg^{2+} in our experiments. At higher concentrations of mercury a reversal of the rise of $[Ca^{2+}]_i$ is seen followed later by a slower elevation (Figure 5b). We have previously observed this phenomenon upon treatment of U937 cells with $HgCl_2$ [7] and it has also been noted with proximal tubule cells [23]. A possible mechanism to explain these observations is as follows: at high concentrations of $HgCl_2$ the very rapid elevation of $[Ca^{2+}]_i$ triggers activation of (PKC) (phosphorylation of 45 kDa PKC substrate is observed, Figure 4) leading to removal of Ca^{2+} from the cytosol as observed previously, and the slower increase that follows is due to inhibition of Ca^{2+} -ATPases [38]. We are presently unable to exclude other non-specific effects as at high concentrations the procoagulant activity is also reduced (Figure 2).

Although mercury-treated platelets exhibited shape change and phosphorylation of 45 and 20 kDa proteins normally associated with platelet activation, these changes were not accompanied by an aggregatory response except at high concentrations ($> 25 \mu$ M). This is in contrast to the organomercurial, thimerosal, which as shown here for the first time, can induce platelet procoagulant activity as well as being a potent aggregator of platelets (100% platelet aggregation at 5 μ M) [41]. Platelets treated with inorganic mercury, however, maintained swirling characteristics normally observed in resting platelets, indicating that there was minimal cell damage under the experimental conditions employed. The enhanced level of phosphorylation of the major PKC substrate (45 kDa) and myosin light chain (MLC, 20 kDa) in mercury-treated platelets would suggest that this agent can either directly modulate PKC/MLC kinase activities or, more likely, that the increased phosphorylation reflects Ca^{2+} -induced enzyme activation as a direct consequence of the $[Ca^{2+}]_i$ elevation. In addition to its effects alone, mercury also inhibited the functional responses to receptor-dependent and -independent agonists (thrombin, thrombin/collagen, A23187) (Table 1). In this respect, previous

studies have shown that $HgCl_2$ significantly modifies the responses to ADP [42]. Although the mechanism of this inhibitory response is not known, it may be associated with the activation of PKC as reflected by the increased phosphorylation of pleckstrin.

A recently described feature of the generation of platelet procoagulant activity is the shedding of microvesicles from the surface due to the fusion (or fission) of opposing zones of the membrane [26]. It is considered that these changes in the membrane are related to the exocytosis of the cell granules. Although weak agonists cause such release, the increase in $[Ca^{2+}]_i$ is insufficient to inhibit translocase activity, and transport can correct the abnormal distribution of aminophospholipids [4]. According to this consideration, strong agonists induce a much higher $[Ca^{2+}]_{cyt}$, which inhibits the translocase and the scrambled partition of the phospholipids is maintained [30]. $HgCl_2$ acts as a different type of agonist in this context, since although inorganic mercury causes a large increase in procoagulant activity and $[Ca^{2+}]_i$, similar to that caused by calcium ionophore, and is capable of oxidizing sulphhydryl groups, it is unable to cause significant platelet aggregation and release. Perhaps, for this reason, substantial depolarization of the membrane can occur without microvesicle formation. This is also true of cells treated with $AgNO_3$ and phenylmercuriacetate.

Recently it has been demonstrated that microparticle formation by platelets is inhibited by monoclonal antibodies directed against glycoprotein IIb:IIIa and the peptide, RGDS [43]. The vesiculation was fully supported by the intracellular pool of fibrinogen since stimulation by collagen in the absence of plasma (no α -granule release) resulted in an inability to vesiculate, unlike treatment with the thrombin receptor peptide, SFLLRN, which can cause granule release. The shape change associated with high levels of Ca^{2+} and protein phosphorylation, but without aggregation, suggest that $HgCl_2$ induces a change to the platelet surface without the activation of the GpIIb:IIIa complex, diminishing microparticle release. Interestingly, thimerosal, which can mobilize platelet $[Ca^{2+}]_i$ and induces aggregation [41], does engender the release of microparticles. Treatment of platelets with heavy-metal ion may, therefore, constitute a useful approach with which to investigate the early changes in the cell membrane which lead to increased expression of anionic phospholipid.

These findings may also be of significance in considering the toxicological outcome of sub-acute doses of $HgCl_2$ which induce immune complex glomerulopathies in experimental animals and is a symptom of mercurial poisoning in humans [44,45]. In immunologically mediated inflammation of this type, activation of coagulation with deposition of fibrin is a common histopathological feature [46]. It is interesting to note, that in a study of the effects of single sub-toxic doses of $HgCl_2$ in rats, depletion of platelets was not observed but increased plasma levels of fibrin monomer (indicative of increased procoagulant activity) were observed for up to 3 days [47].

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