Metabolic and functional consequences of introducing inositol 1,4,5-trisphosphate into saponin-permeabilized human platelets

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In an earlier study we reported the effect of inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$] in releasing Ca^{2+} from highly purified human platelet intracellular membrane vesicles. [Authi & Crawford (1985) *Biochem. J.* **230**, 247–253]. We have now investigated the metabolic and functional consequences of introducing $\text{Ins}(1,4,5)\text{P}_3$ into saponin-permeabilized platelets. Washed human platelets when resuspended in a suitable medium were permeabilized with saponin (10–14 $\mu\text{g}/\text{ml}$) to allow entry of low- M_r water-soluble molecules without significant release of the cytoplasmic marker enzyme protein lactate dehydrogenase. Saponin-permeabilized platelets show identical platelet responses (shape change, aggregation and release of 5-hydroxy ^{14}C tryptamine) to both collagen (5 $\mu\text{g}/\text{ml}$) and thrombin (0.1 unit/ml) as obtained with intact cells, indicating that there is minimal disturbance to the surface membrane receptor topography for these two agonists. $\text{Ins}(1,4,5)\text{P}_3$ (1–10 μM) added to saponin-treated platelets (but not to intact platelets) induced dose-related shape change, aggregation and release of 5-hydroxy ^{14}C tryptamine which at maximal doses was comparable with responses obtained with thrombin or collagen. The cyclo-oxygenase inhibitors indomethacin and aspirin, if added prior to saponization and $\text{Ins}(1,4,5)\text{P}_3$ addition, completely inhibited both aggregation and release of 5-hydroxy ^{14}C tryptamine (EC_{50} for indomethacin, 50 nM; for aspirin, 30 μM). We believe that $\text{Ins}(1,4,5)\text{P}_3$ induces the release of Ca^{2+} from intracellular storage sites which stimulates the Ca^{2+} -dependent phospholipase A_2 releasing arachidonic acid from membrane phospholipids. Arachidonic acid is then converted to the aggregatory prostanoids (prostaglandin H_2 and thromboxane A_2) resulting in the observed responses. This concept is supported by the use of the thromboxane receptor antagonists EPO 45 and EPO 92, both of which also completely inhibit $\text{Ins}(1,4,5)\text{P}_3$ -induced responses in saponin-permeabilized platelets. Electron microscopy of the platelet preparations revealed that thrombin- and collagen-induced platelet aggregates of intact and saponized cells were identical, showing extensive pseudopod formation and dense granule release. The $\text{Ins}(1,4,5)\text{P}_3$ -induced aggregates also showed similar dense granule release but an almost total absence of pseudopod formation. These results are discussed in the light of the second messenger role of $\text{Ins}(1,4,5)\text{P}_3$ in stimulus–response coupling in platelets.

INTRODUCTION

$\text{Ins}(1,4,5)\text{P}_3$, a product of the hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate, has been shown to mobilize Ca^{2+} from intracellular storage sites in a wide variety of tissues (Streb *et al.*, 1983; Joseph *et al.*, 1984; reviewed by Berridge & Irvine, 1984). It is also known that in many activatable cells, agonist–receptor interactions at their surface membranes are associated with the breakdown of phosphoinositides. These findings have resulted in $\text{Ins}(1,4,5)\text{P}_3$ being identified as a putative second messenger molecule capable of conveying the signal generated by the agonist–receptor coupling event to intracellular Ca^{2+} sequestering sites. These Ca^{2+} storing sites are in some way stimulated by the $\text{Ins}(1,4,5)\text{P}_3$ to release the cation and thus raise the cytoplasmic $[\text{Ca}^{2+}]$ to a level which can trigger functional responses. The principal target for $\text{Ins}(1,4,5)\text{P}_3$ is believed to be the endoplasmic reticulum membrane complex which is known in many cells to sequester Ca^{2+} by an ATP-dependent mechanism.

Blood platelets have an endoplasmic reticulum-like intracellular membrane complex referred to by electron

microscopists as the dense tubular membrane system, or DTS. Isolated platelet membrane preparations, which include vesicles derived from this dense tubular membrane system, sequester Ca^{2+} in the presence of ATP (Statland *et al.*, 1969; Menashi *et al.*, 1984) and recently almost simultaneously studies from three groups led to reports of $\text{Ins}(1,4,5)\text{P}_3$ -induced release of Ca^{2+} from platelet membrane vesicles (O'Rourke *et al.*, 1985; Authi & Crawford, 1985; Adunyah & Dean, 1985). In our own studies, using highly purified human platelet intracellular membranes preloaded to steady state levels with $^{45}\text{Ca}^{2+}$, the $\text{Ins}(1,4,5)\text{P}_3$ induced the release of about 40% of the sequestered Ca^{2+} in 15–30 s with a half-maximal dose for this release of 0.25 μM - $\text{Ins}(1,4,5)\text{P}_3$ (Authi & Crawford, 1985). Following this $\text{Ins}(1,4,5)\text{P}_3$ -induced release of Ca^{2+} , there was a rapid reuptake into the vesicles with the former steady state intravesicle $[\text{Ca}^{2+}]$ restored within 30–60 s after the maximal release event. We also reported the presence in the vesicles of an active phosphomonoesterase [K_m for ^{32}P -labelled $\text{Ins}(1,4,5)\text{P}_3$ of $\sim 10 \mu\text{M}$] and suggested that the reuptake phase was probably due to phosphatase degradation of the $\text{Ins}(1,4,5)\text{P}_3$ *in situ* on the membranes.

Abbreviations used: $\text{Ins}(1,4,5)\text{P}_3$, inositol 1,4,5-trisphosphate; 5-HT, 5-hydroxytryptamine (serotonin).

The possible role of $\text{Ins}(1,4,5)P_3$ as a second messenger has also been substantiated by reports that the direct introduction of this water-soluble metabolite by micro-injection into sea urchin eggs caused exocytosis of cortical granules and the appearance of a fertilization envelope (Whitaker & Irvine, 1984) and a similar introduction into intact photoreceptor cells of *Limulus* evoked a voltage response similar to that produced by light (Fein *et al.*, 1984; Brown *et al.*, 1984).

Another procedure for introducing non-membrane-penetrating molecules into cell cytoplasmic compartments is by selective permeabilization of the plasma membrane, and indeed most studies concerned with $\text{Ins}(1,4,5)P_3$ -induced Ca^{2+} release at the whole cell level have used detergents such as saponin to permeabilize the cells without disrupting major intracellular organelles (reviewed by Berridge & Irvine, 1984). Another selective permeabilization procedure uses high-voltage discharge, and with this technique a role for Ca^{2+} in stimulating platelet secretory exocytosis (Knight & Scrutton, 1980, 1984; Haslam & Davidson, 1984) has been reported. To our knowledge the effects of $\text{Ins}(1,4,5)P_3$ on this platelet high-voltage-permeabilized model have not been described, but in a recent study by Lapetina *et al.* (1984) using saponin-permeabilized platelets it was suggested that $\text{Ins}(1,4,5)P_3$ may stimulate protein phosphorylation.

In the present paper we have complemented our earlier membrane vesicle studies with investigations of the effect of $\text{Ins}(1,4,5)P_3$ on whole human platelets permeabilized with saponin to facilitate entry of the water-soluble Ca^{2+} -releasing agent. With these saponized cells, suspended in a medium without added external Ca^{2+} , $\text{Ins}(1,4,5)P_3$ induces both aggregation and release of granule constituents in a dose-dependent manner, giving aggregation profiles identical with those obtained when saponized platelets are exposed to thrombin and collagen. Scanning electron micrographs of the aggregates, however, show interesting morphological differences. From the use of a variety of inhibitors to suppress prostanoid synthesis and to block surface membrane thromboxane receptors we propose that the release of Ca^{2+} from the intracellular membrane stores by $\text{Ins}(1,4,5)P_3$ reaches a threshold level adequate to activate phospholipase A_2 (which is resident in the same membranes) and both the aggregation and granule-release phenomena are triggered by newly synthesized thromboxane leaving the cell and binding to surface membrane receptors.

MATERIALS AND METHODS

Reagents

All experiments were carried out in solutions made up from 'Analytical' water (BDH), which in the absence of added EGTA gave $[\text{Ca}^{2+}]$ of $\sim 1 \mu\text{M}$. The following reagents were obtained from Sigma: indomethacin, aspirin, prostaglandin E_1 , saponin and human thrombin. Collagen was obtained from Hormon Chemie, München, Germany. The thromboxane receptor antagonists EPO 45 and EPO 92 were a generous gift from Dr. Robert Jones and his colleagues at the Department of Pharmacology, University of Edinburgh, and 5-hydroxy-[side chain-2- ^{14}C]tryptamine creatinine sulphate and [^3H]adenine were obtained from Amersham International. Both $\text{Ins}(1,4,5)P_3$ and inositol 1,4-bisphosphate were

prepared from red cell ghosts as described by Downes *et al.* (1982) using the modifications suggested by Irvine *et al.* (1984a).

Preparations of washed platelet suspensions

Blood was collected from healthy human donors, who had not taken any medication for at least 9 days, and was mixed with one-tenth volume of 3.8% trisodium citrate. Platelet-rich plasma was obtained by centrifugation at 200 *g* for 15 min at room temperature in polycarbonate tubes. The pH of the platelet-rich plasma was adjusted to pH 6.4 by dropwise addition of 0.3 M-citric acid (Lagarde *et al.*, 1980) and the platelets were sedimented by centrifugation at 1200 *g* for 8 min at room temperature. The platelet-poor plasma was discarded and the platelet pellet resuspended in a buffer consisting of 36 mM-citric acid, 10 mM-EDTA, 5 mM-glucose, 5 mM-KCl and 90 mM-NaCl adjusted to pH 6.5 with NaOH; prostaglandin E_1 was added to a final concentration of 100 nM. The platelets were further sedimented at 1200 *g* for 15 min and finally suspended at 2.5×10^8 cells/ml in a Ca^{2+} -free medium which resembles the cytosolic ionic environment and consists of 1 mM-glucose, 1 mM- MgCl_2 , 0.42 mM- NaH_2PO_4 , 11.9 mM- NaHCO_3 , 140 mM-KCl and 5 mM-Hepes, pH 7.35. This medium was made up in Analar water (BDH) and had a nominal $[\text{Ca}^{2+}]$ of $1 \mu\text{M}$ as checked by Ca^{2+} -electrode measurements. The platelets were then kept at 37 °C for the duration of the experiments, which were carried out within 4 h of blood withdrawal. Initially experiments involving saponin treatment were carried out in the normal Ca^{2+} -free Tyrode medium containing high Na^+ (140 mM-NaCl) and low K^+ (4 mM-KCl). However, considerable variation resulted between different platelet preparations and all the data presented in this paper have been derived from experiments using the low- Na^+ /high- K^+ buffer, which virtually eliminated the variability.

Aggregation studies on the saponin-permeabilized platelets

These experiments are carried out in siliconized aggregation cuvettes using a Payton 300B dual channel aggregometer with stirring. Typically, 400 μl of the platelet suspension was added to the aggregometer cuvette and the platelets were allowed to equilibrate for 3–5 min. Saponin was added to a final concentration of 11–13 $\mu\text{g}/\text{ml}$. The saponin concentration chosen was that subthreshold for aggregation as determined for each platelet preparation. After 2 min exposure to saponin the $\text{Ins}(1,4,5)P_3$, or other aggregation 'inducers', were then added and the aggregation profile was followed by recording continuous light transmission.

Measurement of [^3H]adenine metabolites during saponin treatment

Since platelets do not synthesize adenine nucleotides *de novo* but do so by transmembrane uptake of precursors such as adenine (Holmsen & Day, 1971), the effect of saponin treatment on the nucleotide pool was studied after labelling the cells with [^3H]adenine. The platelets in platelet-rich plasma were exposed to 1.3 μM -[^3H]adenine (10 $\mu\text{Ci}/\text{ml}$) for 1 h at 37 °C. They were then washed and resuspended as earlier described for treatment with saponin. After saponin treatment, 500 μl of ice-cold 10 mM-EDTA in 140 mM-KCl was added and the suspension was centrifuged at 12000 *g* for 2 min. The

radioactivity in the supernatants was counted by liquid-scintillation spectrometry.

Preloading with [^{14}C]5-HT for release studies

For the measurement of 5-HT release the platelets were prelabelled by using a modification of the technique of Danglemaier & Holmsen (1983). Briefly, platelet-rich plasma was equilibrated at 37 °C for 4 min, after which [^{14}C]5-HT (56 mCi/mmol) was added to a final concentration of 0.6 μM . Following a further incubation period of 5 min a second equal portion of the radioactive 5-HT was added and the incubation continued for a further 5 min. The platelet-rich plasma was then cooled to room temperature and the pH adjusted to pH 6.4 by the addition of 0.3 M-citric acid, and the platelets were washed and resuspended as earlier described. In the combined aggregation and release experiments 2 μM -imipramine was added 2 min before addition of saponin to inhibit re-uptake of released 5-HT (Danglemaier & Holmsen, 1983). This level of imipramine had no effect upon any of the agonist- or $\text{Ins}(1,4,5)\text{P}_3$ -induced responses. To determine the percentage of [^{14}C]5-HT released during the experiments the cell suspensions were fixed at the indicated times by adding 500 μl of a solution containing 3% glutaraldehyde, 6 mM-EDTA and 150 mM-KCl, pH 7.35. The fixed platelets were centrifuged for 2 min at 12000 g and the radioactivity in the supernatant measured by scintillation counting. The percentage of 5-HT released was expressed by reference to control values of the total amount of ^{14}C present in platelets not exposed to aggregation or release inducers.

Measurements of lactate dehydrogenase

In some experiments the cytosolic marker enzyme lactate dehydrogenase was measured in the suspending medium. For these studies the incubation mixtures were stopped at set times by the addition of one-tenth volume of 100 mM-EDTA and the mixtures were centrifuged at 1200 g for 2 min. The supernatants were assayed for lactate dehydrogenase by using the method of Wroblewski & La Due (1955) as described by Northover (1977). The amount of lactate dehydrogenase released was expressed as a percentage of the total activity measured in whole cells treated with Triton X-100 (0.2% final concentration).

Electron microscopy

In preparations for electron microscopy the platelet suspensions were fixed *in situ* in the aggregation tubes by the addition of 500 μl of 2.5% glutaraldehyde made up in the modified Ca^{2+} -free buffer referred to above. For scanning electron microscopy, the platelets were dehydrated and coated with gold before viewing. For transmission electron microscopy the preparations are stained with OsO_4 , dehydrated, and embedded in Araldite before viewing.

RESULTS

$\text{Ins}(1,4,5)\text{P}_3$ -induced aggregation of saponized platelets

In order to check if the saponin-permeabilized platelets behaved normally with the conventional platelet-aggregating agents thrombin and collagen, preliminary experiments were performed with saponized platelet preparations. Thrombin (0.1 unit/ml), and collagen

Fig. 1. Effect of saponin treatment of platelets prelabelled with [^3H]adenine

The platelets in platelet-rich plasma were exposed to 1.3 M- ^3H]adenine (10 $\mu\text{Ci}/\text{ml}$) for 1 h at 37 °C. The platelets were washed and treated with saponin at various concentrations (5–16 $\mu\text{g}/\text{ml}$) as described in the Materials and methods section. The adenine-related metabolites released into the supernatant at 2 min (■) and 5 min (□) after saponin treatment were expressed as a percentage of the total radioactivity present in control untreated cells.

(5 $\mu\text{g}/\text{ml}$), both added 2 min after saponin treatment, produced shape change and aggregation responses with the saponin-permeabilized platelets identical with those seen with control platelets not pretreated with saponin (results not shown). The scanning and transmission electron micrographs of the pelleted aggregates fixed by directly adding glutaraldehyde to the aggregometer cuvette were also indistinguishable. It was clear that the 'receptors' or specific surface domains for these two agonists were unaffected by saponization and excellent responses were obtained for at least 5 h after phlebotomy. Aggregation responses induced by the addition of $\text{Ins}(1,4,5)\text{P}_3$ were maintained for at least 6 h, although aggregation and release experiments were generally completed within 4 h after donation.

The amounts of saponin required to effect permeabilization as judged by the entry of $\text{Ins}(1,4,5)\text{P}_3$ varied slightly for different platelet preparations, but always fell within the range 10–14 $\mu\text{g}/\text{ml}$. Higher amounts (approx. 18–20 $\mu\text{g}/\text{ml}$) were required in the earlier experiments using high- Na^+ /low- K^+ medium; we have no satisfactory explanation for this difference, but all data recorded here were derived from the high- K^+ /low- Na^+ environment, more representative of cytosol conditions. The experiments with platelets prelabelled with [^3H]adenine to label the cytosol adenine-related nucleotide pool provided another indication of the extent of permeabilization. Fig. 1 shows the release data for [^3H]adenine metabolites with different saponin concentrations used in the permeabilization regime. Incubation with less than 10 μg of saponin/ml caused the release of less than 10% of the total cell label, suggesting limited permeabilization. Concentrations of saponin between 10 and 14 $\mu\text{g}/\text{ml}$ induced the release of 40–60% of the label, increasing to 70% with 16 μg of saponin/ml. In this context the functional responses to $\text{Ins}(1,4,5)\text{P}_3$ were only observed

Fig. 2 $\text{Ins}(1,4,5)\text{P}_3$ -induced aggregation of saponin-permeabilized platelets

Washed platelet suspensions were prepared as described in the Materials and methods section. Aliquots of the platelet suspensions (400 μl) were equilibrated in aggregometer cuvettes at 37 °C with stirring. The indicated amounts of $\text{Ins}(1,4,5)\text{P}_3$ (IP_3 ; final concentrations) were added 2 min after the addition of saponin (12 $\mu\text{g}/\text{ml}$ final concentration) and the aggregation was recorded by absorbance monitoring. Traces shown are representative of those obtained from studies with 10–15 different platelet preparations.

in platelet suspensions exposed to saponin concentrations greater than 10 $\mu\text{g}/\text{ml}$.

Fig. 2 shows the effect of adding various concentrations of $\text{Ins}(1,4,5)\text{P}_3$ 2 min after treatment with 12 μg of saponin/ml. In the control aggregation trace there is a very slow increase in light transmission, but neither shape change nor aggregation occurred for up to 7 min after the saponization. With respect to the slope of the aggregation curves it is clear from Fig. 2 that there is a dose-related induction of aggregation between 1 and 10 μM $\text{Ins}(1,4,5)\text{P}_3$. Maximally effective doses of $\text{Ins}(1,4,5)\text{P}_3$ (10 μM) produced shape change usually within 15 s, followed by rapid aggregation which was complete within 3 min. The addition of $\text{Ins}(1,4,5)\text{P}_3$ to a non-saponized platelet suspension or platelets treated with < 5 μg of saponin/ml failed to induce aggregation, suggesting that $\text{Ins}(1,4,5)\text{P}_3$ has to penetrate to an intracellular site for its effect to be observed. Preliminary studies also revealed that $\text{Ins}(1,4,5)\text{P}_3$ added between 1 and 4 min after the saponin gave consistent aggregation responses, but to standardize the procedure, and particularly for the later release experiments, the inducer was routinely added 2 min after the addition of saponin. Inositol 1,4-bisphosphate (10 μM) was totally inactive when added to either permeabilized or non-permeabilized platelet suspensions.

$\text{Ins}(1,4,5)\text{P}_3$ -induced release of [^{14}C]5-HT

Platelets were preincubated with [^{14}C]5-HT before saponin treatment according to the protocol described in the Materials and methods section. Fig. 3 clearly shows that $\text{Ins}(1,4,5)\text{P}_3$ (10 μM) induced the release of stored [^{14}C]5-HT from the saponin-permeabilized cells. The

Fig. 3. Release of [^{14}C]5-HT and lactate dehydrogenase from washed human platelets during saponin permeabilization and activation by $\text{Ins}(1,4,5)\text{P}_3$

The experimental conditions were as described in the legend to Fig. 2 and the preloading with [^{14}C]5-HT was carried out as described in the Materials and methods section. In the 5-HT release studies ($\bullet, \circ$), 2 μM -imipramine was added 2 min before the addition of saponin (12 $\mu\text{g}/\text{ml}$). In experiments with $\text{Ins}(1,4,5)\text{P}_3$ ($\circ, \square$), 10 μM - $\text{Ins}(1,4,5)\text{P}_3$ (IP_3) was added 2 min after saponin. The release of [^{14}C]5-HT is expressed as a percentage of total cell [^{14}C]5-HT (see the Materials and methods section). The release of lactate dehydrogenase (LDH) ($\blacksquare, \square$) was determined in parallel experiments in which the reaction was stopped by the addition of 10 mM-EDTA (final concentration) and after centrifugation the enzyme assay was applied to the supernatants. Release is expressed as a percentage of cell total lactate dehydrogenase activity determined after lysis with 0.2% Triton X-100. Also shown ($\blacktriangle$) is the release of [^3H]adenine (Ade) metabolites into the medium.

percentage release effected by 10 μM - and 13 μM - $\text{Ins}(1,4,5)\text{P}_3$ was $48.7\% \pm 3.5\%$ ($n = 4$) and $51.3\% \pm 1.9\%$ ($n = 3$) respectively of the total [^{14}C]5-HT present in the platelets after the preloading procedure. Control studies with saponin treatment alone resulted in $12.2\% \pm 0.6\%$ ($n = 6$) and $15.7\% \pm 2.3\%$ ($n = 6$) release of [^{14}C]5-HT after 2 and 5 min incubation respectively. The addition of inositol 1,4-bisphosphate 2 min after saponin treatment failed to show any release above the 'saponin only' control values. The corresponding values for thrombin-induced release of [^{14}C]5-HT from the saponin-permeabilized cells were: for 0.1 unit of thrombin/ml, $67.0\% \pm 0.9\%$ ($n = 6$); for 1 unit/ml, $78.5\% \pm 0.9\%$ ($n = 3$) measured 3 min after addition of the agonist. Also shown in Fig. 3 are the data from parallel measurements of the cytosol marker enzyme lactate dehydrogenase released into the supernatant of the saponin-permeabilized platelets. Measurements were made at zero time before saponization, during exposure to saponin and after the addition of $\text{Ins}(1,4,5)\text{P}_3$. It can be seen (Fig. 3) that saponin permeabilization results in release of less than 10% of the total enzyme content ($9.4\% \pm 1.2\%$, $n = 3$). The supernatant content of lactate dehydrogenase was little changed ($9.7\% \pm 2.4\%$, $n = 4$) when assayed 3 min after the addition of $\text{Ins}(1,4,5)\text{P}_3$. The 100% values for lactate dehydrogenase were

Fig. 4. Inhibition of Ins(1,4,5) P_3 -induced aggregation and [14 C]5-HT release by pretreatment of saponin-permeabilized platelets with indomethacin

The reaction conditions were similar to those described in the legend to Fig. 2. Indomethacin (various concentrations) or vehicle control (1 μ l of dimethyl sulphoxide) was added 5 min before and imipramine (2 μ M) 2 min before saponin treatment (12 μ g/ml). Ins(1,4,5) P_3 (10 μ M) (IP₃) was added 2 min after the saponin addition and the reactions were stopped by the addition of fixative after a further 3 min incubation (see the Materials and methods section). [14 C]5-HT release into the medium was measured and calculated as described in the legend to Fig. 3. Aggregation traces were representative of results obtained with about 10 different platelet separations. Arrows indicate points of addition of agents.

determined on saponized control cells which had been lysed with 0.2% Triton X-100. Fig. 3 also shows the time course for the release of [3 H]adenine-related metabolites during the period of exposure to saponin. Within 1 min of adding the saponin ~55% of the labelled nucleotides are released into the incubation medium. Little further release takes place over the following 5 min and the addition of Ins(1,4,5) P_3 does not alter the amount of nucleotides released.

Effects of cyclo-oxygenase inhibitors on the Ins(1,4,5) P_3 -induced aggregation and release in saponin-permeabilized platelets

Fig. 4 shows the effects of adding indomethacin in the range 0.01–10 μ M on the aggregation profiles produced by the addition of 10 μ M-Ins(1,4,5) P_3 to the permeabilized platelets. The indomethacin was dissolved in dimethyl sulphoxide and additions were made 3 min before treatment with saponin. Controls were performed with the carrier vehicle only. It can be seen in Fig. 4 that this drug, which is known to be a powerful but reversible inhibitor of cyclo-oxygenase, can completely inhibit both the Ins(1,4,5) P_3 -induced aggregation and the [14 C]5-HT release. The inhibition is dose-related and half-maximal inhibition of these responses occurs at ~50 nM. With a similar experimental protocol using aspirin, with the drug added before saponin permeabilization, half-maximal inhibition of the Ins(1,4,5) P_3 -induced responses (both aggregation and release) occurred at ~30 μ M aspirin with total inhibition of responses at concentrations of 50 μ M and higher (results not shown). However, aspirin, unlike indomethacin, did not inhibit the shape change even at concentrations of 100 μ M.

In experiments with three different platelet preparations the amount of thromboxane released in response to Ins(1,4,5) P_3 was measured. The platelets in the aggregometer cuvette were treated with 10 mM-EDTA and 10 μ M-indomethacin to stop the reaction 3 min after the addition of Ins(1,4,5) P_3 . They were centrifuged and the

supernatant thromboxane B₂ measured by radioimmunoassay. The three preparations gave values of 18.4, 22.9 and 34 ng of thromboxane B₂/ml. Control platelets pretreated with 10 μ M-indomethacin 3 min before saponization, which inhibited the Ins(1,4,5) P_3 -induced responses, gave supernatant thromboxane B₂ levels of 5.0, 3.4 and 3.2 ng/ml. Studies on the Ins(1,4,5) P_3 -induced release of Ca²⁺ from purified platelet intracellular membranes (Authi & Crawford, 1985) revealed no difference in the presence of 10 μ M-indomethacin (results not shown).

Effect of specific thromboxane receptor antagonists on the Ins(1,4,5) P_3 -induced responses in saponin-permeabilized platelets

The two prostanoid analogues EPO 45 and EPO 92 synthesized by Dr N. H. Wilson and his colleagues in the Department of Pharmacology, University of Edinburgh (Wilson *et al.*, 1982) have been shown completely to inhibit irreversible aggregation responses of platelet-rich plasma induced by thromboxane A₂, prostaglandin H₂ and a number of stable thromboxane mimetics. They have stated that neither EPO 45 nor EPO 92 affect the primary aggregation waves induced by ADP and platelet activating factor but do suppress the secondary phases. They also showed no effect upon cyclic AMP levels in platelet suspensions (Armstrong *et al.*, 1983, 1985). These two prostanoid analogues are regarded as specific inhibitors for the platelet surface membrane thromboxane receptor.

In the present studies the platelets were treated with the antagonist before exposure to saponin and both EPO 45 and EPO 92 at concentrations of 1.0 μ M and 0.5 μ M respectively gave identical results, completely inhibiting the aggregation (and shape change) induced by 10 μ M-Ins(1,4,5) P_3 . Similarly, both compounds lowered the secretion response down to levels closely approximating to the control secretion seen when the platelets are exposed to saponin treatment only. The data for the

Fig. 5. Inhibition of Ins(1,4,5) P_3 -induced aggregation and [14 C]5-HT release of saponin-permeabilized platelets by the thromboxane receptor antagonist EPO 92

The reaction conditions are similar to those described in the legend to Fig. 4. EPO 92 and imipramine (2 μ M) were added at 3 min and 2 min respectively before saponin treatment. Ins(1,4,5) P_3 (10 μ M) (IP_3) was added 2 min after the saponin addition. The released [14 C]5-HT was assayed and expressed as described in the legend to Fig. 3. Arrows indicate points of addition of agents.

experiments involving the addition of EPO 92 to the platelets before saponin treatment and its effect upon Ins(1,4,5) P_3 induction of aggregation and release are shown in Fig. 5. Exactly similar data were obtained for the inhibition of the Ins(1,4,5) P_3 responses when the platelets were pretreated with EPO 45 before saponinization (results not shown).

It seems clear therefore that the inhibition of surface thromboxane receptors by these specific antagonists, applied to the whole cells before permeabilization, totally blocked the aggregation and release responses induced by introducing Ins(1,4,5) P_3 directly into the cytosol compartment of permeabilized platelets.

These results so far suggest that the Ins(1,4,5) P_3 -induced release of Ca^{2+} may be associated with the production of proaggregatory prostanoids through the activation of the Ca^{2+} -dependent phospholipase A_2 and production of the precursor arachidonic acid from intracellular membrane phospholipids. However, the possibility that Ins(1,4,5) P_3 may exert a direct effect on the prostanoid-synthesizing enzymes could not be excluded. Accordingly, platelets were treated with saponin in the usual incubation medium to which had been added a Ca^{2+} -EGTA buffer to lock the free Ca^{2+} in the medium and in the platelet cytosol at a submicromolar level. A Ca^{2+} : EGTA ratio of 0.5 was used, giving a concentration of 80 nM (Fabiato & Fabiato, 1979; Lapetina *et al.*, 1984). When Ins(1,4,5) P_3 was added to the cells it was found that concentrations even as high as 40 μ M-Ins(1,4,5) P_3 failed to elicit an aggregation response. These findings suggest that the Ins(1,4,5) P_3 -induced responses seen with permeabilized platelets suspended in a nominally Ca^{2+} -free medium without EGTA present was due to a stimulated release

of Ca^{2+} from internal stores into the cytosol compartment.

In a further study, control non-permeabilized platelets were incubated with $^{45}CaCl_2$ for 4 h to label internal compartments. The cells were then permeabilized with saponin and after a short period of equilibration the ^{45}Ca present in the medium was measured. When Ins(1,4,5) P_3 was added to this platelet suspension there was a further measurable increase in output of $^{45}Ca^{2+}$ in the medium (results not shown). The fact that this was unaffected by pretreatment of the cells with indomethacin supports our view that the principal site of action of the Ins(1,4,5) P_3 introduced directly into permeabilized platelets is towards intracellular sites of sequestered Ca^{2+} (K. S. Authi, B. Evenden & N. Crawford, unpublished work).

Electron microscopy of the Ins(1,4,5) P_3 -induced aggregates of saponin-permeabilized human platelets

At various stages in the experiments the platelet suspensions (or aggregates) were fixed *in situ* in the aggregometer cuvettes for electron microscopy processing.

Scanning electron micrographs of control washed platelets (Fig. 6a) showed discrete discoid-shaped cells with no evidence of shape changes, filopodia or pseudopodia arising from their smooth surfaces. Transmission electron microscopy confirmed their discoidicity (Fig. 6b) and in many cells bundles of microtubules, believed to be involved in maintaining their discoid shape, could be seen in the apices of the cross-sectioned discs. Similarly processed saponin-permeabilized platelets were also substantially discoid, although in the occasional

platelet some small surface protruberances could be discerned and the cells appeared slightly more rounded (Fig. 6c). As with the untreated cells, the transmission electron micrographs showed transversed sectioned discocytes with an apparently full complement of intracellular granules and their microtubule integrity well maintained (Fig. 6d). Thrombin treatment of both intact and permeabilized platelets gave indistinguishable aggregates, all showing very tightly associated cells with extensive pseudopodia formation. The scanning electron micrograph of permeabilized platelets treated with thrombin (Fig. 6e) shows these changes clearly. Fig. 6(f) shows a transmission electron micrograph of a saponized platelet suspension aggregated with $\text{Ins}(1,4,5)\text{P}_3$. Most platelets in the outer region of the aggregate have secreted their granule contents whilst maintaining single cell identity. The absence of pseudopodia is quite marked. The scanning electron micrographs of $\text{Ins}(1,4,5)\text{P}_3$ -induced aggregates (Fig. 6g and 6h) also show tightly associated platelets, but almost all are spheroid without pseudopodia.

DISCUSSION

There is now considerable evidence that when platelets are activated by agonists such as thrombin the receptor-stimulus interaction induces the breakdown of membrane phosphatidylinositol 4,5-bisphosphate to produce $\text{Ins}(1,4,5)\text{P}_3$ and 1,2-diacylglycerol (Agranoff *et al.*, 1983; Graff *et al.*, 1984; Watson *et al.*, 1984; Vickers *et al.*, 1984; Siess & Binder, 1985). Diacylglycerol has been shown to be an activator of protein kinase C (reviewed by Nishizuka, 1984) and $\text{Ins}(1,4,5)\text{P}_3$ has been implicated as a second messenger molecule which can stimulate the release of Ca^{2+} from intracellular storage sites (reviewed by Berridge & Irvine, 1984). In this latter respect, studies have been made of the action of $\text{Ins}(1,4,5)\text{P}_3$ introduced into permeabilized tissues (Irvine *et al.*, 1984b; Burgess *et al.*, 1984; Prentki *et al.*, 1984a; Biden *et al.*, 1984; Hirata *et al.*, 1984) and on isolated microsomes (Prentki *et al.*, 1984b; Dawson & Irvine, 1984) and all have provided evidence for a specific action of $\text{Ins}(1,4,5)\text{P}_3$ on intracellular membranes which results in the release of sequestered Ca^{2+} .

We have now established a procedure for the permeabilization of washed human blood platelets using carefully controlled exposure to saponin. The operational range is between 10 and 14 μg of saponin/ml. The cells remain discoid and responsive to conventional agonists, and the loss of the cytosol marker enzyme lactate dehydrogenase from the permeabilized platelets does not exceed 10% of their total enzyme content. We have not been able to detect any significant difference in either the rate or extent of thrombin- and collagen-induced aggregation and 5-HT secretion responses when the permeabilized platelets are compared with untreated controls. We believe that the saponin treatment causes minimal disturbance to the surface membrane receptor topography for these two agonists and the potential fusogenic domains in the surface membrane which are required for exocytotic release of 5-HT have not been seriously compromised. The size of the pores produced by this permeabilization procedure is not at present known, but the substantial retention of LDH suggests that the majority are less than the hydrodynamic radius of this protein, but sufficient to allow entry into the

cytoplasm of the water-soluble $\text{Ins}(1,4,5)\text{P}_3$. In our investigations with these permeabilized cells we set out to examine the metabolic and functional consequences that followed the entry into the cell of $\text{Ins}(1,4,5)\text{P}_3$, having initially confirmed that $\text{Ins}(1,4,5)\text{P}_3$ had no effect on untreated whole platelets.

When $\text{Ins}(1,4,5)\text{P}_3$ is added to the suspensions of saponized platelets they aggregate in a dose-dependent manner, and at the maximal dose levels of $\text{Ins}(1,4,5)\text{P}_3$ the aggregation profiles compare well, in time of onset and magnitude of aggregation, with the responses seen when thrombin and collagen are applied to either intact or saponized platelets. Shape change is complete within 15 s after the addition of $\text{Ins}(1,4,5)\text{P}_3$ and a full irreversible aggregation takes place within 2–3 min. Moreover, the concentrations of $\text{Ins}(1,4,5)\text{P}_3$ required for full aggregation and secretion in the aggregometer compare well with the concentrations earlier found necessary to effect the release of sequestered calcium from permeabilized tissues (Streb *et al.*, 1983; Irvine *et al.*, 1984b; Biden *et al.*, 1984; Burgess *et al.*, 1984; Prentki *et al.*, 1984a). In our report of the effect of $\text{Ins}(1,4,5)\text{P}_3$ on the isolated and purified platelet intracellular membranes (Authi & Crawford, 1985) we showed half-maximal release of $^{45}\text{Ca}^{2+}$ at concentrations of $\text{Ins}(1,4,5)\text{P}_3$ of 0.25 μM . The higher concentrations of the agent required for the whole platelet responses in the present study and in tissue studies by others may indicate the existence of other binding sites within the cell for $\text{Ins}(1,4,5)\text{P}_3$ unrelated to its Ca^{2+} -releasing effect. As with the isolated intracellular membranes (Authi & Crawford, 1985), inositol 1,4-bisphosphate failed to elicit any responses with the permeabilized platelets, supporting the specificity of the action of $\text{Ins}(1,4,5)\text{P}_3$ as reported by Irvine *et al.* (1984b) in their Ca^{2+} -release experiments with permeabilized Swiss-mouse 3T3 cells. In the present investigations our findings with the use of cyclo-oxygenase inhibitors, indomethacin and aspirin and the two thromboxane receptor antagonists EPO 45 and EPO 92, all added to the platelets before permeabilization, revealed that the compounds completely inhibited the $\text{Ins}(1,4,5)\text{P}_3$ -induced responses, suggesting that they are dependent upon the synthesis of metabolites distal to the cyclo-oxygenase in the prostanoid-producing pathway.

The enzyme phospholipase A_2 is a calcium-dependent enzyme responsible for liberating arachidonic acid, the precursor for prostanoid synthesis, from phospholipids (Broekmann *et al.*, 1980; Billah *et al.*, 1980; Mahadevappa & Holub, 1984). Our earlier demonstration that in human platelets this enzyme is predominantly localized in the intracellular membranes (Lagarde *et al.*, 1981), that it shows optimum activity towards 2-arachidonyl phospholipids (Authi *et al.*, 1983), and that it may be closely associated with cyclo-oxygenase (Carey *et al.*, 1982), leads us to interpret our present findings of the $\text{Ins}(1,4,5)\text{P}_3$ -induced aggregation and secretion responses of permeabilized whole platelets as a $\text{Ins}(1,4,5)\text{P}_3$ -induced release of Ca^{2+} , which in turn activates phospholipase A_2 and thus provides the arachidonic acid for membrane-associated cyclo-oxygenase and thromboxane synthase. Newly synthesized thromboxane A_2 rapidly leaves the cell and binds to specific surface receptors on the platelets to initiate the sequence of events that result in aggregation and release of granule stored constituents. We believe that the scheme shown in Fig. 7 accounts for the rapid response that is initiated by the introduction of

Fig. 6. Electron microscopy of control and $\text{Ins}(1,4,5)\text{P}_3$ -stimulated platelets

(a,b) Scanning (a) and transmission (b) electron micrographs of control untreated washed platelets ($a, \times 3300$; $b, \times 26000$). (c,d) Scanning (c) and transmission (d) electron micrographs of washed platelet suspensions fixed 5 min after the addition of saponin ($12 \mu\text{g/ml}$) ($c, \times 3300$; $d, \times 20000$). (e) Scanning electron micrograph of an aggregate of saponin-permeabilized platelets induced by thrombin (0.1 unit/ml) ($\times 2600$). Identical results are obtained with collagen ($5 \mu\text{g/ml}$) as the agonist. (f) Transmission electron micrograph of a saponized platelet aggregate induced by $\text{Ins}(1,4,5)\text{P}_3$ ($\times 10700$). (g,h) Scanning electron micrographs of aggregates formed from saponin-treated platelets after induction by $\text{Ins}(1,4,5)\text{P}_3$ ($g, \times 1300$; $h, \times 2600$). All electron micrographs are representative of those obtained with three different platelet preparations.

Ins(1,4,5) P_3 directly into the cytosol compartment and that we have a model system that can perhaps be exploited to study the kinetic and regulatory feature of this regionally associated membrane link between Ins(1,4,5) P_3 release of Ca^{2+} and prostanoid synthesis.

Although the mobilization of Ca^{2+} from intracellular

stores is the most widely studied function of Ins(1,4,5) P_3 (reviewed by Berridge, 1984, and Berridge & Irvine, 1985) it may not be its only intracellular function. Recent studies by Whitman *et al.* (1984) showed that Ins(1,4,5) P_3 stimulated the phosphorylation of a 62 kDa polypeptide in monkey fibroblasts and brain cell lysates, and others

Fig. 7. Schematic diagram of proposed sequence of events that take place following introduction of $\text{Ins}(1,4,5)\text{P}_3$ into permeabilized platelets

The phospholipase A_2 and the enzymes which convert liberated arachidonic acid into endoperoxides and thromboxanes are predominantly located in the endoplasmic reticulum-like intracellular membrane which actively sequesters Ca^{2+} by an ATP-dependent process. The $\text{Ins}(1,4,5)\text{P}_3$ liberates Ca^{2+} from this site which activates the lipase. Thromboxane A_2 rapidly leaves the platelet and binds to surface membrane receptors to initiate release and aggregation. Abbreviations used: ER, endoplasmic reticulum; PLA_2 , phospholipase A_2 ; CO, cyclo-oxygenase; TXS, thromboxane synthase; PL, phospholipid; AA, arachidonic acid; TXA_2 , thromboxane A_2 . Receptors or surface domains for thrombin and collagen which are unaltered by saponin permeabilization are also present but not shown.

have reported the phosphorylation of two polypeptides (20 and 40 kDa) in response to $\text{Ins}(1,4,5)\text{P}_3$ added to saponin-permeabilized platelets (Lapetina *et al.*, 1984). In the latter report, since studies were not carried out using cyclo-oxygenase inhibitors, the observed phosphorylations could have resulted from secondary effects of released thromboxane A_2 binding to surface receptors rather than to a direct action of $\text{Ins}(1,4,5)\text{P}_3$ on the protein kinase(s).

There is one feature of our permeabilized platelet model that may require some caution in relating observations directly to the physiological processes which take place *in vivo*. Although in all the parameters we have

studied (i.e. shape change, aggregometry, secretion, etc.) the $\text{Ins}(1,4,5)\text{P}_3$ -induced phenomena are almost exactly analogous with those seen when thrombin and collagen are used as the stimulating agents with either intact or permeabilized platelets, the scanning electron microscopic appearance of the final $\text{Ins}(1,4,5)\text{P}_3$ -induced platelet aggregates do differ in what could be an important respect. Whereas the platelet aggregates formed by thrombin and collagen stimulation show many interacting filopodia and pseudopodia, the $\text{Ins}(1,4,5)\text{P}_3$ -induced aggregates (which are equally irreversible) show an almost complete absence of formation of pseudopodia. The platelets associate tightly as smooth-surfaced

spheres, as seen in the scanning electron micrographs; transmission electron micrographs show substantial release of granules, as do those of platelets stimulated by other agents. We cannot yet account for this quite profound difference in morphological appearance, but it has never been definitively established that the appearance of conventional stimuli-induced aggregates formed in an aggregometer cuvette necessarily reflects the true physiological situation *in vivo* that occurs when platelets fulfil their haemostatic role. These tightly associated $\text{Ins}(1,4,5)\text{-P}_3$ -induced aggregates of spheroid cells cannot be discounted and they may represent part of the early normal haemostatic picture not previously identified. One interpretation may be that in the absence of external Ca^{2+} the release of Ca^{2+} from internal stores provides a $[\text{Ca}^{2+}]$ which, though locally above the threshold level for activating phospholipase A_2 , may after equilibration with the external medium and cytosol in permeabilized cells be sub-threshold for initiating other Ca^{2+} -dependent processes involved in cytoskeletal expressions, for example, gelsolin destabilizing of actin, myosin light chain phosphorylation, reorganization of microtubules, etc. All are Ca^{2+} -dependent and involve macromolecular rearrangements. However, since thrombin and collagen stimulation of the permeabilized cells does appear to initiate a contractile-protein-dependent formation of pseudopodia without a requirement for external Ca^{2+} , the action of these agonists may lie in the co-production of other intracellular messengers (diacylglycerol or cyclic nucleotides) which are effectively bypassed when $\text{Ins}(1,4,5)\text{-P}_3$ is introduced directly into the platelet cytosolic compartment. If this proved to be so, further studies with the present permeabilized platelet model would allow a detailed examination of the $\text{Ins}(1,4,5)\text{-P}_3$ -induced events dislocated from the complexities of a bifurcated signal transduction which results when both $\text{Ins}(1,4,5)\text{-P}_3$ and diacylglycerol are released through activation and breakdown of surface membrane phosphatidylinositols by phospholipase C.

In conclusion, we present a permeabilized platelet model in which $\text{Ins}(1,4,5)\text{-P}_3$, a low- M_r water-soluble second messenger molecule, can be introduced directly into the cell for study of the metabolic and functional consequences. Further elucidation of these mechanisms may provide information about potential molecular targets for antiplatelet and antithrombotic drug design.

After submission of this typescript for review Israels & Gerrard (1985) reported in abstract form similar $\text{Ins}(1,4,5)\text{-P}_3$ -induced aggregation responses in permeabilized platelets. They suggested that the action of $\text{Ins}(1,4,5)\text{-P}_3$ may be mediated through intracellular Ca^{2+} release and thromboxane formation.

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