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Inositol 1,4,5-trisphosphate (IP_3) induced rapid formation of thromboxane B_2 in saponin-permeabilised human platelets: mechanism of IP_3 action

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The mechanism of IP_3-induced activation of saponin-permeabilised platelets has been examined. Saponin permeabilisation resulted in the leakage of low-M_r substances into and from the cells without loss of cytoplasmic proteins. Addition of IP_3 rapidly induced a dose-related formation of thromboxane B_2 and release into the medium, leading to the responses of shape change, aggregation and [^{14}C]5HT release. These responses were inhibited by the thromboxane A_2 receptor antagonist AH23848. The IP_3-induced release of ^{45}Ca from intracellular stores was not affected by indomethacin. Synthesis of thromboxane was inhibited if Ca^{2+} elevation was prevented by using Ca-EGTA buffers during permeabilisation. These studies indicate that IP_3-induced activation was due to Ca^{2+} mobilisation leading to phospholipase activation and thromboxane synthesis.

Permeabilized platelet; Inositol 1,4,5-trisphosphate; Ca^{2+} mobilization; Thromboxane synthesis

1. INTRODUCTION

Inositol 1,4,5-trisphosphate has now been identified as an important intracellular second messenger molecule that can effect the release of Ca^{2+} from intracellular stores in a wide variety of tissues [1]. In some tissues this ability of IP_3 to raise the cytosolic [Ca^{2+}] has been correlated with functional events, e.g. exocytosis of cortical granules in sea urchin eggs [2] and voltage responses in photoreceptor cells of *Limulus* [3,4]. It has also been shown to release Ca^{2+} from platelet intracellular membranes [5–7] and more recently we have demonstrated that IP_3 can initiate certain functions such as shape change, aggregation and release of granule-stored [^{14}C]5HT when added to saponin-permeabilised platelets [8]. These properties are comparable to those initiated by conventional agonists applied to intact platelets. By using inhibitors of cyclooxygenase and thromboxane receptor antagonists we also demonstrated [8] that these IP_3-induced responses are dependent upon the formation of thromboxane and its rapid release and binding to surface receptors on the platelets. These findings led us to suggest that IP_3-induced release of Ca^{2+} from an intracellular membrane storage site (the platelet endoplasmic reticulum-like complex) is followed by activation of phospholipase A_2, release of arachidonic acid and the formation of thromboxane A_2 by cyclooxygenase and thromboxane synthase. All these enzymes have been shown to be located in the same intracellular membranes which have the capacity to sequester Ca^{2+} through the action of a ($Ca^{2+} + Mg^{2+}$)-ATPase [9–13].

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Here, our studies using saponin-permeabilised platelets have been extended to confirm that IP_3 triggers prostanoid synthesis through Ca^{2+} mobilisation rather than by any direct effect upon the synthetic 'enzymes' involved.

2. EXPERIMENTAL

2.1. *Reagents*

AH23848 was a gift from Glaxo Group Research, Ware, Herts. ^{45}Ca was obtained from NEN (4–50 Ci/g Ca). All other reagents and chemicals were from sources described earlier [8].

2.2. *Preparation of washed platelet suspensions*

Preparation of washed human platelets and labelling of the dense granule constituents with [^{14}C]5HT were carried out as in [8], except that the final resuspension buffer was of the following composition: 1 mM glucose, 140 mM KCl, 1 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 6 mM $NaHCO_3$ and 10 mM Hepes (pH 7.4) made up in Analar water (BDH) at a cell concentration of 2.5×10^8 cells/ml. This platelet suspension was kept at 37°C, gassed with O_2/CO_2 (95%/5%) every 30 min and experiments were completed within 4 h of blood donation. Only donors who were certain that they had taken no aspirin-containing medicaments during the previous 10 days were used.

2.3. *Saponin permeabilisation and aggregation experiments*

Unless otherwise indicated the following experimental protocol was carried out at 37°C using a Payton 300B dual-channel aggregometer. Typically, 400 μl platelet suspension was equilibrated in aggregation cuvettes for 3 min and saponin was added at a final concentration of between 12 and 14 μg/ml. After 2 min either IP_3 or the different agonists were added and reactions were followed for another 4 min. In the [^{14}C]5HT-release experiments 2 μM imipramine was also added 2 min before saponinisation. Reactions were terminated by the addition of 500 μl of 3% glutaraldehyde, 6 mM EDTA, 150 mM KCl (pH 7.4). The [^{14}C]5HT released into the supernatant was determined by liquid scintillation counting after centrifugation at $12000 \times g$ for 3 min.

2.4. *Measurement of thromboxane B_2 in media*

These were duplicate experiments carried out in parallel to those with platelets not prelabelled with any isotopes. For these studies reactions were terminated in the aggregation cuvettes by the addition of 10 mM EDTA and 10 μM indomethacin. After centrifugation at $12000 \times g$ for 3 min the supernatants were assayed for thromboxane B_2 (TXB_2) by radioimmunoassay [14] after separation of non-polar materials on a Sep-Pak column.

2.5. *$^{45}Ca^{2+}$ labelling and release studies*

^{45}Ca labelling of intact platelets was carried out using buffers modified to increase uptake of ^{45}Ca as used by Steiner and Tateishi [15] and Brass [16]. Platelets were isolated from PRP as described above and resuspended in buffer of composition 140 mM KCl, 1 mM $MgCl_2$, 1 mM glucose, 0.42 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, 10 mM Pipes (pH 6.5) containing 1 mg/ml BSA and 100 nM PGE_1. The cells were incubated for 4 h at room temperature in the presence of 2 μCi/ml ^{45}Ca (4–50 Ci/gCa) and 20 μM cold $CaCl_2$. At the end of this incubation period the cells were sedimented at $500 \times g$ for 20 min and washed in a medium containing 120 mM KCl, 5 mM $MgCl_2$, 10 mM Pipes, 5 mM EGTA, 100 nM PGE_1 adjusted to pH 6.5, centrifuged at $1000 \times g$ for 15 min and resuspended in the nominally Ca^{2+}-free KCl medium used for the aggregation studies at 2.5×10^8 cells/ml. The uptake of ^{45}Ca and its efflux from the cells followed very similar kinetics (not shown) to those described by Brass [16]. The ^{45}Ca distributed between the cytosolic and intracellular pools. However, after labelling, the cytosolic pool quickly reached equilibrium with the external environment ($t_{½}$ ~17 min) whilst the intracellular storage pool loses Ca^{2+} much more slowly ($t_{½}$ ~313 min). Experiments on IP_3-induced release of ^{45}Ca were carried out between 30 and 90 min after final resuspension of the cells when there had been only a slight decrease in the ^{45}Ca associated with the cells as measured by rapid filtration through 0.45 μm Millipore membrane filters. Reaction conditions were similar to those used in the aggregation experiments. The 400 μl aliquots of platelet suspensions were incubated in the presence of 14 μg/ml saponin at room temperature and after 2 min exposure to the detergent, IP_3 or other Ca^{2+}-mobilising agents were added and the reac-

tions terminated at set times by rapid filtration through 0.45 μm Millipore filters. The filters were washed twice with 10 ml of a buffer containing 120 mM KCl, 5 mM $MgCl_2$, 10 mM Pipes, 5 mM EGTA (pH 7.4), air dried, and the radioactivity remaining on the filter counted by liquid scintillation counting.

3. RESULTS

Addition of IP_3 (1–15 μM) to washed human platelets permeabilised by saponin treatment rapidly induced shape change, aggregation and dense granule release [8]. These effects with IP_3 were only observed with permeabilised cells and are not seen with intact cells. They were also not observed with inositol 1,4-bisphosphate (IP_2). Under these conditions 15 μM IP_3 produced shape change and aggregation profiles comparable to those produced by 0.1 U/ml thrombin and approx. 50% of the previously incorporated [^{14}C]5HT was released. The relationship between IP_3 concentration added and thromboxane produced has been examined and fig.1a shows the formation and release of immunoreactive TXB_2 measured in the supernatants, 4 min after the addition of varying amounts of IP_3 to platelets permeabilised with 14 μg/ml saponin. This figure shows a dose-related increase in the formation of TXB_2 from 5.4 ± 2.8 ng TXB_2/ml (basal levels, n = 6) to approx. 140 ng TXB_2/ml in the presence of 15 μM or greater amounts of IP_3. The addition of 0.1 U/ml thrombin to saponised platelets produced 225 ± 34 ng TXB_2/ml (n = 3). Fig.1b shows a time curve of thromboxane release into the medium over 4 min; 30% of the total released appears in 15 s and around 70% in 30 s. In this context IP_3-induced shape changes are generally seen within 15 s of adding IP_3 to the permeabilised platelets.

To complement our earlier studies where we showed that IP_3-induced platelet functions were totally inhibited by pretreating the platelets at the whole cell level with TXA_2 receptor antagonists EPO92 and EPO45 before saponisation, we have investigated the effect of another TXA_2 receptor-blocking agent AH23848. This compound has been shown to antagonise platelet responses induced by U46619, TXA_2 and PGH_2 as also activation by collagen and arachidonate [17]. In the present studies,

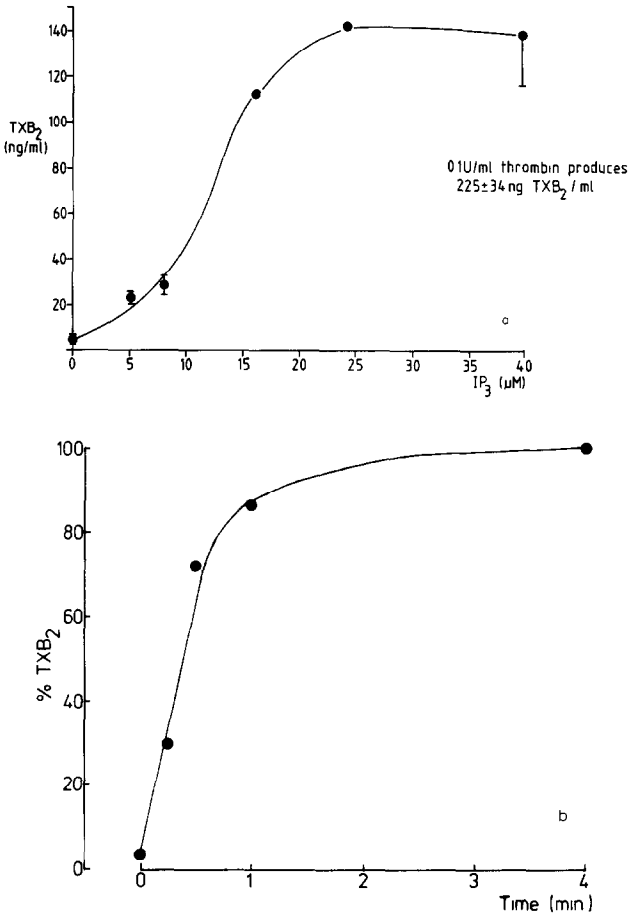

Fig.1. (a) IP_3-induced TXB_2 formation in saponin-permeabilised platelets. Washed human platelets were incubated in aggregation cuvettes at 37°C (400 μl, 2.5 × 10^8 cells/ml). Saponin was added at 14 μg/ml and followed after 2 min by various concentrations of IP_3 (0–40 μM). The reactions were stopped by 10 μM indomethacin and 10 mM EDTA after 6 min and following centrifugation the TXB_2 present in the supernatant was assayed as described in section 2. Points represent mean ± SE (n = 3) or mean of duplicates. Similar results were obtained in 3 different preparations. (b) IP_3-induced formation of TXB_2 as a function of time. Reaction conditions are similar to those in (a). The concentration of IP_3 used was 15 μM. Points represent mean of duplicate observations with similar results obtained in 2 other preparations.

AH23848 was extremely effective in inhibiting the IP_3-induced responses and fig.2 shows a typical aggregation trace where AH23848 was added to intact cells 2 min before saponin. The suppression of

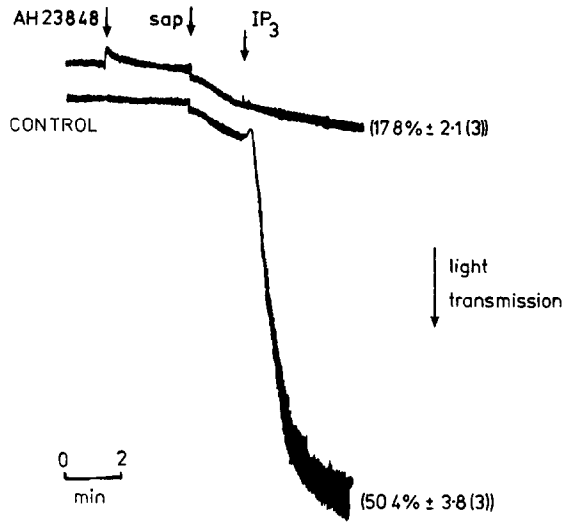

Fig.2. Inhibition of IP_3-induced platelet responses by AH23848. Reaction conditions are similar to those in fig.1a except that platelets were prelabelled with [^{14}C]5HT. The agent AH23848 (30 nM) or carrier vehicle ($NaHCO_3$, 0.1%) was added 3 min before saponin, and 15 μM IP_3 added 2 min after saponin. Figures in parentheses indicate % [^{14}C]5HT release. Traces are representative of at least 7 different platelet preparations.

IP_3-induced aggregation was dose-dependent over the range 3–30 nM AH23848 with total inhibition at concentrations of 30 nM and over. It was noticeable, however, that at concentrations of the drug in excess of 30 nM, weak shape change responses were observed, but even at 10-fold higher concentrations (300 nM) these were not accompanied by either aggregation or 5HT release. A second equal aliquot of AH23848 (300 nM) to intact cells added 3 min after the first addition of the drug did not produce shape change, probably indicating that all of the receptor sites had been occupied by the first addition and that the shape change effect was not due to a non-specific detergent action of the drug. The weak shape change effect was also inhibited by the previously used TXA_2 receptor antagonist EPO92 ($\geq$5 nM) if added 3 min prior to AH23848 (300 nM). Partial agonist activity of AH23848 has also been reported with some isolated smooth muscle preparations [17].

To explore further our concept that the IP_3 induced functional responses operate through Ca^{2+} mobilisation and thromboxane production, Ca/EGTA buffers were included in the suspension media during saponisation. Permeabilisation was carried out in the presence of 80 nM free Ca^{2+} (Ca/EGTA ratio of 0.5 with EGTA final concentration 1.0 mM, pH 7.4; see [18]). The Ca/EGTA buffer rapidly equilibrates with the cytosol when the plasma membrane is permeabilised by saponin and under these conditions concentrations of IP_3 (up to 40 μM) failed to elicit shape change, aggregation and 5HT release was reduced to basal levels (16.4% ± 2.4, n = 3). Additionally with 40 μM IP_3, thromboxane production (measured as TXB_2) was inhibited by 92% [12.0 ± 1.3 ng/ml (n = 3) compared with 140 ± 23 ng/ml (n = 3) in the absence of EGTA buffer], i.e. rose little above the basal level, suggesting that any increase in cytosolic [Ca^{2+}] was prevented by the presence of the EGTA and no significant activation of phospholipase A_2 takes place.

The addition of exogenous Ca^{2+} (20–500 μM without EGTA) also activates saponin-permeabilised platelets in a dose-dependent manner giving similar responses to that produced by IP_3. In seven different platelet preparations 500 μM Ca^{2+} added 2 min after saponin caused shape change, aggregation and the release of between 47 and 67% of previously incorporated [^{14}C]5HT (54.4% ± 8.2, n = 7). As with the IP_3-induced responses, Ca^{2+}-induced responses were similarly inhibited by indomethacin and thromboxane receptor antagonists.

To demonstrate clearly that IP_3 induces Ca^{2+} release from intracellular stores, intact platelets were first pre-incubated with ^{45}Ca for 4 h (as described in section 2). The platelets were then washed in the EGTA-containing medium to remove any surface-associated cations and resuspended in the nominally Ca^{2+}-free medium used in aggregation experiments. In such uptake experiments the ^{45}Ca distributes between the cytosol and the intracellular membrane stores with perhaps a small amount in the granule pool [16]. The cells were then permeabilised as before with 14 μg/ml saponin. The loss of ^{45}Ca through permeabilisation amounted to approx. 3% of the total cell ^{45}Ca after 6 min incubation. However, if IP_3 is added to the cell suspension 2 min after saponisation, release of ^{45}Ca is measurable at 30 s, and 20% of the cell associated ^{45}Ca is released

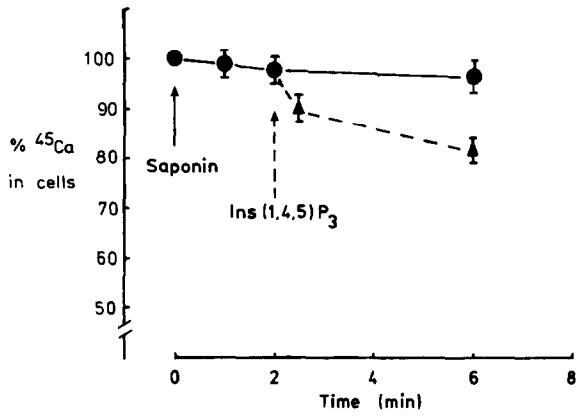

Fig.3. IP_3-induced release of ^{45}Ca from saponin-treated platelets. Intact platelets were labelled with ^{45}Ca and washed as described in section 2. Aliquots of platelet suspensions were incubated with saponin for various times up to 6 min (●—●). Reactions were terminated by rapid filtration through 0.45 μm filters and the radioactivity of the cells on the filter counted by liquid scintillation. IP_3 (15 μM) was added at 2 min (▲---▲) and reactions terminated as before. 100% ^{45}Ca represents 8305 cpm/10^8 cells. Points represent mean ± SE of triplicate determinations.

Table 1

Release of ^{45}Ca from saponin-permeabilised platelets

Experimental conditions	^{45}Ca associated with the platelets after filtration and washing	% release
Control cells	5931 ± 51 (100)	–
Cells + saponin (14 μg/ml)	5778 ± 106 (97.0)	3.0
Cells + saponin + IP_3 (15 μM)	5020 ± 311 (84.6)	15.4
Cells + saponin + IP_3 + indo (10 μM)	4736 ± 52 (79.8)	20.2
Cells + saponin + IP_3 + 80 nM Ca-free	4863 ± 77 (82.0)	18.0
Cells + saponin + A23187 (5 μM)	3182 ± 281 (53.6)	46.4
Cells + saponin + thrombin (0.2 U/ml)	3071 ± 462 (51.7)	48.3

Saponin was added at zero time. IP_3, thrombin or A23187 was added 2 min after saponin and the reactions stopped at 6 min by rapid filtration through a 0.45 μ filter. Figures in parentheses refer to % associated with cells. Data are means ± SD (n = 3). indo, indomethacin

after 4 min (fig.3). This IP_3-induced release is believed to have its origin in an internal store. This Ca^{2+} release is dose-related over the range 0.1–15 μM IP_3 and in control experiments with IP_3 added to intact cells no release of ^{45}Ca was detected. Indomethacin (10 μM), a reversible cyclooxygenase inhibitor, did not inhibit the IP_3-induced release of ^{45}Ca from the permeabilised cells. In fact (as shown in table 1) the presence of indomethacin resulted in a consistent but barely significant increase (~4%) in ^{45}Ca released, above the levels recorded for IP_3 in the absence of indomethacin. Table 1 also shows that the IP_3-induced release of ^{45}Ca is unaffected by the presence of 80 nM free Ca^{2+} (Ca/EGTA buffer) in the external medium which of course equilibrates with the cytosol compartment in the permeabilised platelet. Additionally, the mitochondrial inhibitors antimycin (10 μM) and oligomycin (5 μM) were also without effect upon the release of ^{45}Ca suggesting that the stimulated release took place from a non-mitochondrial compartment and probably the endoplasmic reticulum-like membrane complex. Both thrombin (0.2 U/ml) and the Ca^{2+} ionophore A23187 (5 μM) released ^{45}Ca from a sequestered site with % release values of 46% and 48% respectively of the total cell associated ^{45}Ca.

4. DISCUSSION

The ability of IP_3 to mobilise Ca^{2+} from an intracellular storage site in blood platelets has been further substantiated in this present study. Using saponin-permeabilised human platelets, we have also identified (by the end product TXB_2) the major intracellular enzyme sequence that is activated immediately following IP_3 mobilisation of Ca^{2+}. This enzyme sequence is initiated by Ca^{2+} stimulation of phospholipase A_2 which results in the liberation of arachidonic acid. The fatty acid is rapidly converted to thromboxanes by the cyclooxygenase and thromboxane synthase, both of which are resident in the same intracellular membrane complex from which the Ca^{2+} is released by the IP_3. Release of TXA_2 from the cell mediates functional responses through binding to specific surface membrane receptors. The use of the

thromboxane receptor antagonist AH23848, applied at the intact cell level before permeabilisation, totally inhibited the IP_3-induced aggregation and release phenomena. This further emphasises the importance of thromboxane receptor occupancy in the full expression of these platelet properties. Release of arachidonic acid from diacylglycerol is not considered to be a Ca^{2+}-requiring process and in fact earlier observations from this laboratory indicated that diacylglycerol lipase activities (both the low-K_m and high-K_m forms of the enzyme) are substantially activated in the presence of EGTA and partially inhibited by elevated Ca^{2+} levels [19,20]. From our previous studies with saponin-permeabilised platelets and the present data, the amount of sequestered Ca^{2+} released by IP_3 appears to be sufficient to activate phospholipase-A_2 without the need for influx of external Ca^{2+} from the plasmatic environment. In the saponin-permeabilised platelet model using analytical grade water for all the reagents and no EGTA present, the environmental $[Ca^{2+}]$ is usually less than 3×10^{-6} M. This equilibrates with the platelet cytosol through the detergent-induced channels and it is the incremental change above this basal level, contributed to by IP_3 release of Ca^{2+} from an intracellular non-mitochondrial store, that triggers the liberation of the precursor arachidonic acid from membrane phospholipids. The minimal amount of TXA_2 believed to be required to produce platelet activation in aggregometry studies is generally thought to be around 25 ng/ml [21]. This amount is generated in a few seconds with 10 μM IP_3 and levels of IP_3 ~ 25 μM lead rapidly to TXB_2 concentrations in the external media over 100 ng/ml.

It should be noted, however, that in the present study less TXA_2 is produced by direct entry of IP_3 into the permeabilised platelet than when thrombin is used as the stimulus (140 and 220 ng/ml, respectively). Similarly IP_3 at optimum concentrations releases about 40–50% of the internally sequestered ^{45}Ca pool which can be released by 0.2 U/ml thrombin. One possible explanation for this is that in by-passing phospholipase C involvement initially, through the direct entry of IP_3 into the cytosol, other intracellular events initiated by thrombin receptor occupancy are not brought into interplay.

Two earlier reports presented the possibility that IP_3 may also have a direct effect on certain enzymes since it was shown that it stimulated protein kinases in brain cell lysates [22] and similarly in permeabilised platelets [23]. However, subsequent reports by both groups [24,25] indicated that the resulting phosphorylations observed in the earlier experiments from brain were not reproducible and in the latter case with platelets were due to secondary formation of TXA_2. It therefore appears that IP_3 acts predominantly, if not solely, through Ca^{2+} mobilisation from an internal store.

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