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Inositol 1,4,5-trisphosphate-induced release of sequestered Ca^{2+} from highly purified human platelet intracellular membranes

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Evidence has accumulated in support of a role for intracellularly generated inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] in raising cytosol [Ca^{2+}] when various hormones, neurotransmitters, growth factors and other stimulants act on cell surfaces. The increase in [Ca^{2+}] that follows stimulant–receptor interaction is accompanied by rapid hydrolysis of phosphoinositides. One product, Ins(1,4,5)P_3, arising from the breakdown of phosphatidylinositol 4,5-bisphosphate was shown to promote the release of Ca^{2+} from non-mitochondrial stores in a variety of cells. Although platelet intracellular membranes have been implicated in the control of cytosol [Ca^{2+}] and we previously characterized a Ca^{2+}-sequestering mechanism associated with them, we have as yet no knowledge of how this Ca^{2+} store is mobilized after a stimulus–receptor interaction at the platelet surface. Using free-flow electrophoresis, we isolated and purified human platelet intracellular membranes. They show high enrichment and exclusive localization of the endoplasmic-reticulum marker NADH:cytochrome *c* reductase, and they sequester Ca^{2+} by an ATP-dependent process, reaching steady-state values in 10–12min. Saturation with Ca^{2+} occurs at around 10–30μM external Ca^{2+}. When Ins(1,4,5)P_3 is added to the ^{45}Ca-loaded vesicles, a rapid release of Ca^{2+} occurs (approx. 35% in 15–30s). The magnitude of the release depends upon external [Ca^{2+}], being maximum in the range 0.3–0.8μM and low at external [Ca^{2+}] > 1μM. After release there is a rapid re-uptake of Ca^{2+}, with restoration of the former steady-state values within 1min. Half-maximal release occurs at approx. 0.25μM-Ins(1,4,5)P_3. This release and re-uptake pattern is not observed with ionophore A23187 or arachidonic acid, both of which liberate Ca^{2+} irreversibly. Inositol 1,4-bisphosphate was ineffective in releasing Ca^{2+} from these intracellular membranes. The results support the role of Ins(1,4,5)P_3 as a specific intracellular mediator, transducing the action of excitatory agonists acting on the platelet surface into metabolic, mechanochemical and other functional events, known to occur during platelet activation.

Many cells which respond to stimulus–receptor interactions at their surface do so through a variety of intracellular metabolic and mechanochemical processes, which are regulated by changes in the cytosol [Ca^{2+}] in the submicromolar to micromolar range (Brattin *et al.*, 1982; Pozzan *et al.*, 1983; Biden *et al.*, 1984; Prentki *et al.*, 1984*a*). Although an increase in cytosol [Ca^{2+}] can occur through influx from the extracellular environment, it is believed that mobilization of intracellularly stored Ca^{2+} also contributes to the increase in cytosol [Ca^{2+}]. In many non-muscle cells the membranes of the endoplasmic reticulum have been implicated in both the storage and release process for Ca^{2+}, and in some tissues the uptake of Ca^{2+} into endoplasmic reticulum has been identified with a $Ca^{2+}+Mg^{2+}$-dependent ATPase activity present in the membranes (Brattin *et al.*, 1982). However,

Abbreviations used: PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; Ins(1,4)P_2, inositol 1,4-bisphosphate.

the exact manner by which Ca^{2+} is released from these membrane storage sites is at present unknown.

An additional feature of many cells which respond to surface stimuli is the rapid hydrolysis of PtdIns(4,5)P_2 in the first few seconds after receptor occupancy (Michell *et al.*, 1981; Berridge, 1981; Michell & Kirk, 1981). This breakdown of PtdIns(4,5)P_2 is believed to be due to a stimulus-induced increase in the activity of phospholipase C generating diacylglycerol and Ins(1,4,5)P_3, both of which have been implicated as second messengers (Michell & Kirk, 1981; Berridge, 1984; Nishizuka, 1984). Ins(1,4,5)P_3 has now been shown to mobilize Ca^{2+} in various tissues (Streb *et al.*, 1983; Joseph *et al.*, 1984; Hirata *et al.*, 1984; Burgess *et al.*, 1984; Prentki *et al.*, 1984*b*; see also reviews by Berridge, 1984; Berridge & Irvine, 1984; Fisher *et al.*, 1984), although no report relating to the platelet has yet appeared.

Most of the excitatory agonists for the platelet cause an elevation in cytosol [Ca^{2+}] (Rink & Hallam, 1984). The relative contributions to this increase in [Ca^{2+}] by the two processes, influx of extracellular Ca^{2+} and release of intracellular sequestered Ca^{2+}, have not been determined, and may of course vary with the different surface stimuli. The rapid formation of inositol phosphates, and particularly Ins(1,4,5)P_3, in thrombin-stimulated human (Graff *et al.*, 1984; Watson *et al.*, 1984; Siess & Binder, 1985) and rabbit platelets (Vickers *et al.*, 1984) has been reported, and such findings lend support to the view that hydrolysis of PtdIns(4,5)P_2 is the early event in platelet activation, which leads to the mobilization of intracellular Ca^{2+}.

We have developed a procedure, using density-gradient sedimentation followed by high-voltage free-flow electrophoresis, for the isolation of highly purified fractions of human platelet plasma and intracellular membranes (Menashi *et al.*, 1981). The basis for the differential identification depends on the exclusive localization of the endoplasmic-reticulum marker enzyme NADH:cytochrome *c* reductase (15–20-fold enriched with respect to homogenate specific activity) in the platelet intracellular-membrane fractions, and of adenylate cyclase (10–12-fold enriched) in the surface-membrane fraction (Menashi *et al.*, 1981). Other discriminating features are substantial differences in cholesterol/phospholipid ratios (Lagarde *et al.*, 1982), in polypeptide and glycopeptide profiles (Hack & Crawford, 1984), and the finding that the full complement of enzymes concerned with arachidonic acid release and prostanoid synthesis predominate in the intracellular membranes (Carey *et al.*, 1982; Authi *et al.*, 1985), where also the 72kDa polypeptide target for aspirin acetylation is specifically located (Hack *et al.*, 1984). In studies of Ca^{2+} uptake with these purified membrane fractions, the surface-membrane vesicles show no capacity to sequester Ca^{2+} in the presence of ATP, whereas the intracellular-membrane vesicles actively take up Ca^{2+} at a rate and to a final vesicle steady-state concentration which relate to the extra-vesicle [Ca^{2+}] to which they have been exposed (Menashi *et al.*, 1984). In the present study we report on the effects of the putative second messenger Ins(1,4,5)P_3 on human platelet intracellular-membrane vesicles preloaded with ^{45}Ca.

Materials and methods

All reagents used were of analytical grade. Proteinase-free neuraminidase (type X; 1 unit of activity = 1.0μmol of *N*-acetylneuraminic acid liberated/min) was obtained from Sigma Chemical Co., Poole, Dorset, U.K. $^{45}CaCl_2$ (10–40mCi/mg of Ca^{2+}) was purchased from Amersham International, Amersham, Bucks., U.K.

The procedure for the isolation of the platelets and for purification of membrane subfractions has been reported elsewhere (Menashi *et al.*, 1981). The significant modifications that we have made for the present study are the omission of EDTA from the sorbitol density gradient and recovery of the membranes by centrifugation on to a cushion of 3.5M-sorbitol buffered to pH7.2 with Hepes. The omission of EDTA results in the surface-membrane vesicles locating in the electrophoresis chamber as a single peak instead of as two surface-membrane subfractions. Recovery of the membranes on a 3.5M-sorbitol cushion facilitates their resuspension and maintains the vesicles sealed, which is a necessary requisite for the Ca^{2+}-uptake studies.

Fresh blood samples were obtained from the National Blood Transfusion Service Laboratories, Tooting, London S.W.17, U.K. and Brentwood, Essex, U.K. They were processed in the laboratory within 2–3h of donation. To isolate the platelets, the whole blood was centrifuged at 200*g* for 20min to prepare platelet-rich plasma. This was removed and, after acidification to pH6.4 by the dropwise addition of 0.15M-citric acid, was centrifuged at 1200*g* for 20min to obtain a platelet pellet. The platelet pellet was resuspended in a buffer containing 152mM-NaCl, 4mM-KCl, 3mM-EDTA, 10mM-Hepes, pH7.2, and re-centrifuged at 180*g* for 5min to remove any residual cells. After a further sedimentation (1200*g* for 20min), the cells were resuspended in the same buffer but adjusted to pH6.2. This suspension was treated with neuraminidase at a concentration of 0.03–0.05 unit/ml for 20min at 37°C and then washed in the

same medium, pH 7.2 at room temperature. Neuraminidase treatment decreases the surface-membrane electronegativity by removal of sialic acid moieties and improves resolution of the two membrane species in the electrophoresis chamber. The neuraminidase-treated platelets were carefully resuspended in cold sonication buffer [0.34M-sorbitol/10mM-Hepes/Aprotinin (0.1 unit/ml), pH 7.2 at 4°C] and sonicated for 10s (Dawes Sonifier; position 6 at maximum tuning) while kept at 4°C. The suspension was centrifuged (1200*g* for 15min at 4°C), the supernatant removed and kept, and the pellet of unbroken cells and large cell fragments was suspended in a further volume of cold sonication buffer and subjected to a further 10s sonication. After centrifugation, the two supernatants were pooled before application to the density gradients. A mixed membrane fraction, well separated from and uncontaminated by granular organelles, was then isolated on a linear sorbitol-density gradient (1.0–3.5M-sorbitol/Hepes, pH 7.2, centrifuged at 42000*g* for 90min). The mixed membrane fraction, containing elements of both surface and intracellular origin, was removed from the gradient and concentrated by centrifugation (100000*g* for 90min) on a cushion of 10mM-Hepes-buffered 3.5M-sorbitol, pH 7.2. This mixed membrane fraction was applied to the chamber of a Bender Hobein VAP 5 electrophoresis unit operating at 110V/cm and 140mA, with an injection flow rate of approx. 2ml/h and a chamber buffer flow rate of 2ml/h per fraction. Two discrete vesicle subfractions were resolved; the most electronegative peak, which had been unaffected by neuraminidase treatment at the whole-cell level, represented the intracellular membranes, and the least electronegative fraction represented the plasma-membrane vesicles modified by removal of sialic acid. Pools of the two fractions were concentrated by centrifuging on to a cushion of 3.5M-sorbitol/Hepes, pH 7.2, and the intracellular membranes were used to study Ca^{2+} uptake and release.

Ca^{2+} uptake and release were measured in an incubation mixture (1ml) containing 120mM-KCl, 5mM$MgCl_2$, 1mM-ATP, 20mM-Tris/HCl, pH 7.0, and approx. 1μCi of ^{45}Ca (10–40mCi/mg of Ca). Ca^{2+} concentrations were controlled in the range 0.01–10μM by Ca^{2+}–EGTA buffers as described by Portzehl *et al.* (1964) and Durham (1983). Membranes (20–60μg of protein) were added last to start the reaction, and the mixtures were incubated at 21–22°C for 15min or as indicated in the Results and discussion section. At the end of the incubation period, 0.9ml of the incubation mixture was removed and filtered rapidly through a Millipore membrane (pore size 0.45μm), followed by washing with 3×10ml of ice-cold buffer containing 120mM-KCl, 5mM-$MgCl_2$, 20mM-Tris/HCl, pH 7.0, and 50μM-$CaCl_2$ (unlabelled). The filter membranes holding the vesicles were then dried and the radioactivity was counted by liquid scintillation.

In experiments using the different agents, e.g. Ins(1,4,5)P_3, ionophore A23187 etc., agents were added usually after 15min incubation (steady-state values) or as stipulated in the legends of Fig. 3(*a*) and Table 2, and the incubation mixture was stopped at set times as described above.

Initially Ins(1,4,5)P_3 was supplied by Dr. R. F. Irvine. Further amounts on Ins(1,4,5)P_3 and Ins(1,4)P_2 were prepared from red cells by Dr. R. F. Irvine's modification (Irvine *et al.*, 1984*a*) of the procedure of Downes *et al.* (1982).

Results and discussion

We have previously shown that human platelet intracellular membranes prepared by free-flow electrophoresis are able to sequester Ca^{2+} in the presence of MgATP (Menashi *et al.*, 1984) and that this activity is not displayed by plasma membranes. Uptake of Ca^{2+} is rapid and reaches a steady state after 10min incubation. Fig. 1 shows the intravesicle steady-state content of Ca^{2+} reached with different external concentrations of the cation. The maximum difference in stored Ca^{2+} for change in external [Ca^{2+}] occurs in the range 0.1–1μM external Ca^{2+}, which is believed to be the operational range for platelet cytosol [Ca^{2+}] changes involved in the regulation of intracellular metabolic and cytoskeletal events. Fig. 2(*a*) shows the time scale of release after the addition of 5μM-Ins(1,4,5)P_3 to the intracellular-membrane vesicles preloaded to steady-state equilibrium by incubation with 0.5μM-Ca^{2+}. Some 30–50% of the sequested Ca^{2+} is released during the first 15–30s

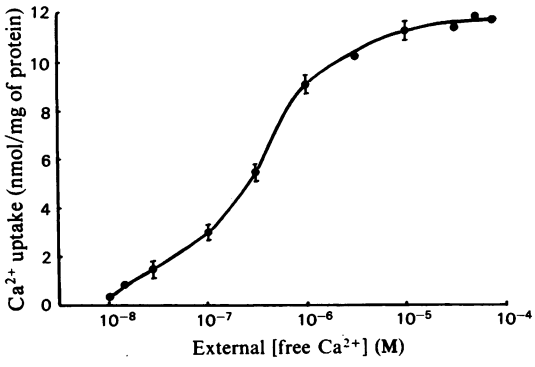

Fig. 1. *Relationship of sequestered Ca^{2+} contents in intracellular membranes to different external* [Ca^{2+}]
Ca^{2+} uptake was measured as described in the Materials and methods section. Incubations were stopped at 15min.

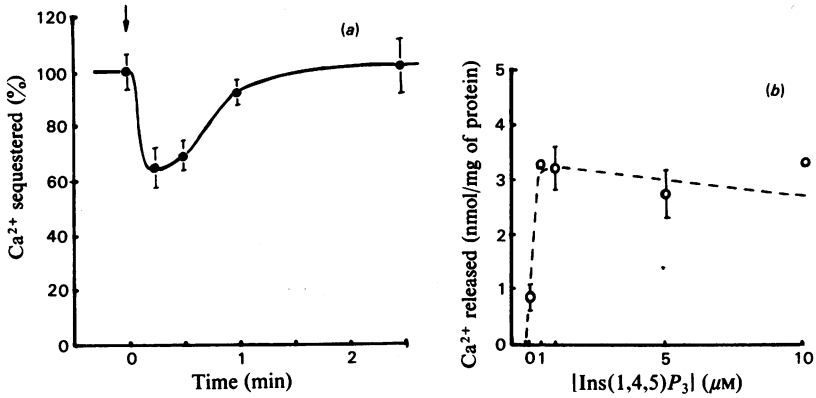

Fig. 2. (*a*) *Time course of the release of sequestered intracellular-membrane* Ca^{2+} *by Ins(1,4,5)*P_3*, and* (*b*) *dose–response relationship of Ins(1,4,5)*P_3*-induced* Ca^{2+} *release measured at 30s after addition to membranes preloaded with* Ca^{2+}. Ca^{2+} uptake in the presence of 0.5 μM-Ca^{2+} was allowed to proceed until steady state was reached after 15min incubation (see Materials and methods section), at which time 5 μM-Ins(1,4,5)P_3 was added. Reactions were stopped at the times indicated, by rapid filtrations through a 0.45 μm filter. Vertical bars represent S.D. ($n = 4$). '100% Ca^{2+}' in membranes represented 8.8 $\pm$ 0.5 nmol/mg of protein, the steady-state values reached in the presence of 0.5 μM external Ca^{2+}. Arrow indicates addition of Ins(1,4,5)P_3. Similar time courses of release were obtained at other concentrations of external Ca^{2+}, with maximal release occuring at either 15 or 30s after addition of Ins(1,4,5)P_3.

Table 1. *Release of* Ca^{2+} *from preloaded membrane vesicles at different external* Ca^{2+} *concentrations*
Results are means $\pm$ S.D. The numbers in parentheses are either numbers of determinations, or the actual values for duplicate observations.

External [Ca^{2+}] (μM)	Vesicle [Ca^{2+}] after 15min incubation (nmol/mg of protein)	Amount of Ca^{2+} released 30s after adding 5 μM-InsP_3	Ca^{2+} released (%)
0.1	3.0 $\pm$ 0.2 (5)	0.5 (0.4, 0.56)	16 (13, 18.7)
0.3	7.7 $\pm$ 0.3 (5)	2.2 $\pm$ 0.3 (4)	28 $\pm$ 4
0.5	8.8 $\pm$ 0.25 (5)	3.1 $\pm$ 0.6 (5)	35 $\pm$ 7
1.0	9.5 $\pm$ 0.3 (5)	1.9 $\pm$ 0.4 (3)	20 $\pm$ 4
3.0	10.7 $\pm$ 0.45 (5)	1.9 $\pm$ 0.5 (4)	18 $\pm$ 5
50	11.4 $\pm$ 0.25 (5)	0.9 (0.6, 1.2)	8 (5, 10)

after the addition of Ins(1,4,5)P_3 to the medium. The data presented in Fig. 2(*a*) are means $\pm$ S.D. for four different preparations of intracellular membranes; after this rapid Ins(1,4,5)P_3-induced release there is a rapid re-uptake of the Ca^{2+}, with full restoration of the former steady-state values 30–60s after the release event. Fig. 2(*b*) shows the dose–response relationship of Ins(1,4,5)P_3-induced release of Ca^{2+} from preloaded vesicles measured 30s after addition of the inositol phosphate. Half-maximal release occurs with about 0.25 μM-Ins(1,4,5)P_3, and concentrations of Ins(1,4,5)P_3 above approx. 1 μM showed no further increase in the releasing effect. These Ins(1,4,5)P_3 concentrations are well within the effective range for its reported action in releasing Ca^{2+} from internal stores in permeabilized whole-cell preparations, but the concentrations required for maximal release with the highly purified platelet membranes are substantially lower than those required for effects of similar magnitude with cardiac and insulinoma microsomal preparations (Prentki *et al.*, 1984*b*; Hirata *et al.*, 1984). We assume that impurities present in crude microsomal fractions contribute to a non-specific binding of Ins(1,4,5)P_3 unrelated to the Ca^{2+}-releasing effect. In our present studies with a range of external [Ca^{2+}] between 0.1 and 50 μM, the pattern of Ins(1,4,5)P_3-induced release was essentially similar at all concentrations, with a rapid initial release followed by re-uptake to former steady-state values. However, at the upper and lower limits of this [Ca^{2+}] range, i.e. 0.1 and 50 μM external Ca^{2+}, the magnitude of the release was considerably less than that shown by Ins(1,4,5)P_3 in the presence of 0.5 μM external Ca^{2+}, which in our experiments gave the maximal effect (Table 1). When Ins(1,4)P_2 was added to the membrane suspensions held at steady-state [Ca^{2+}], no release of the sequestered Ca^{2+} occurred (results not

shown), supporting a previously observed structural specificity for the release phenomenon (Irvine *et al.*, 1984*b*). It was also shown (Fig. 3*a*) that the course of the Ins(1,4,5)P_3-induced release of Ca^{2+} differs markedly from the release profiles observed with the calcium ionophore A23187 (2μM) or arachidonic acid (10 and 25μM) introduced to the intracellular membranes at steady-state [Ca^{2+}]. Only Ins(1,4,5)P_3 produced the re-uptake phenomenon, and both the ionophore and the fatty acid irreversibly released the sequestered cation, the fatty acid giving $40 \pm 6\%$ and $84 \pm 2\%$ release at 10 and 25μM respectively in 2.5min and the ionophore 90% release in 2.5min. Fig. 3(*b*) shows the data from a typical experiment using 5μM-Ins(1,4,5)P_3 added to the uptake medium at zero time (i.e. just before the addition of intracellular membranes); no effect was observed on either the rate or the extent of the Ca^{2+} uptake. However, both A23187 and arachidonic acid added at zero time inhibit the uptake of Ca^{2+} into the vesicle; see Fig. 3(*b*).

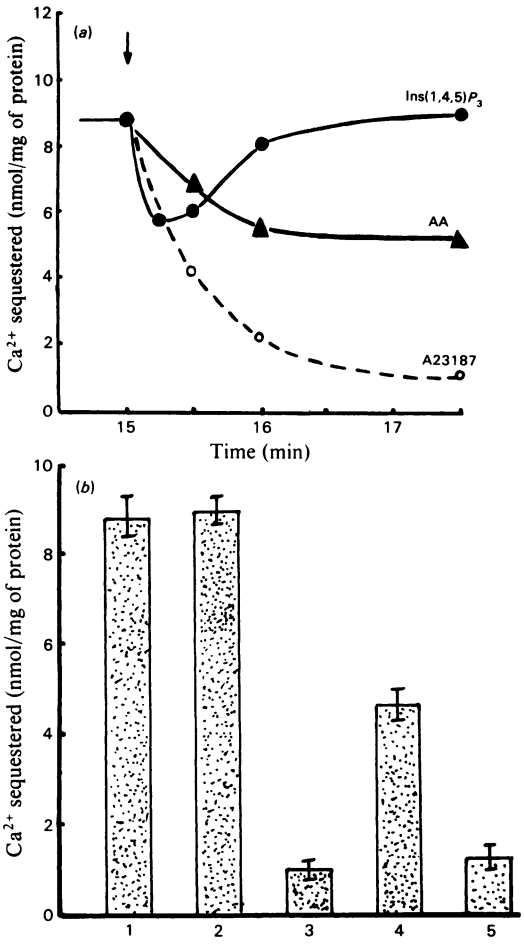

Fig. 3. (*a*) *Time course of Ins(1,4,5)P_3-induced release of sequestered Ca^{2+} compared with that induced by 2μM-A23187 and 10μM-arachidonic acid (AA), and (b) effect of Ins(1,4,5)P_3, A23187 and arachidonic acid on the extent of Ca^{2+} sequestration by intracellular membranes.*

(*a*) All agents were added at steady-state conditions (i.e. after 15min incubation) and reactions were stopped as in the legend to Fig. 2. A23187 and arachidonic acid were added as solutions in ethanol (maximum final concn. 0.5%). This concentration of ethanol does not affect the rate or extent of uptake. (*b*) Agents [5μM-Ins(1,4,5)P_3, 2μM-A23187 and 10μM-arachidonic acid] were added before addition of intracellular membranes. Reactions were terminated at 15min by rapid filtration. Column 1 represents Ca^{2+} uptake by control membranes, column 2 that in the presence of 5μM-Ins(1,4,5)P_3, column 3 that in the presence of 2μM-A23187, column 4 that in the presence of 10μM-arachidonic acid, and column 5 that by control membranes in the absence of added ATP. Vertical bars represent S.D. ($n = 3$).

At present we have no satisfactory explanation for the rapid re-accumulation of Ca^{2+} after Ins(1,4,5)P_3-induced release, although this phenomenon has been observed by others (Streb *et al.*, 1983; Dawson & Irvine, 1984). In the studies by Dawson & Irvine (1984), for example, it was proposed that vesicle heterogeneity may be the explanation, with Ca^{2+} released from Ins(1,4,5)P_3-responsive vesicles being taken up by insensitive vesicles. With the purified platelet intracellular membranes we have not been able to identify responsive and unresponsive membrane vesicles in our fractions. However, using a sample of ^{32}P-labelled Ins(1,4,5)P_3, we have identified in the intracellular membranes an active phosphomonoesterase. This enzyme has an apparent K_m for Ins(1,4,5)P_3 of approx. 10^{-5}M (N. Hack & N. Crawford, unpublished work), and we believe that the sequence of events that we observe with Ins(1,4,5)P_3 in which a rapid re-uptake follows the induced release may be accounted for by hydrolysis of the receptor-bound Ins(1,4,5)P_3 by phosphatase action. Fig. 4 shows the effect of adding a second portion of Ins(1,4,5)P_3 (1.6μM) to the intracellular membranes pre-loaded with $^{45}Ca^{2+}$ after they have passed through one complete uptake and release cycle. Although by the Millipore-membrane-filtration procedure it was not possible to record the [Ca^{2+}] changes continuously, as with Ca^{2+}-electrode studies, it was found that a second stimulus, given after the vesicles have returned to steady-state values, produces a release almost identical with that by the first application of Ins(1,4,5)P_3. Clearly, the membranes are not desensitized by Ins(1,4,5)P_3 addition, and the refilling of the vesicles during the re-uptake phase is probably related to Ins(1,4,5)P_3 degradation. Evidence for the degradation of Ins(1,4,5)P_3 associated with a re-uptake of Ca^{2+} in saponin-permeabilized

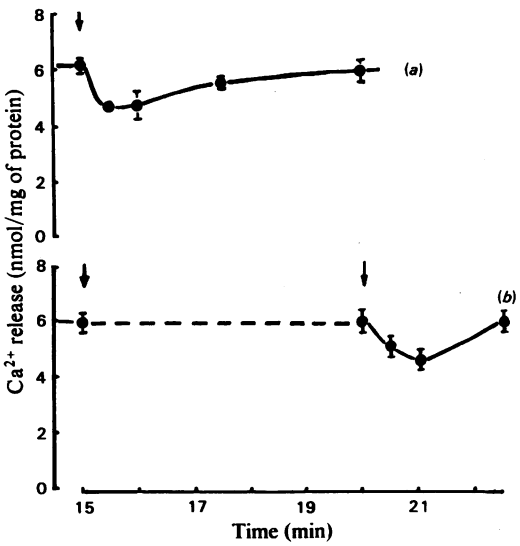

Fig. 4. *Effect of a second addition of Ins(1,4,5)P_3 on Ca^{2+} release from Ca^{2+}-loaded intracellular membranes in the presence of 0.3 μM external free Ca^{2+}*

(*a*) Single addition of Ins(1,4,5)P_3 (1.6 μM) after incubation to steady-state values (15 min). Identical results were obtained if the single challenge was given after 20 min incubation. (*b*) Second addition of 1.6 μM-Ins(1,4,5)P_3 given 5 min after first addition. Incubation conditions were as in Fig. 2. Arrows indicate times of Ins(1,4,5)P_3 additions.

hepatocytes has also been presented by Joseph *et al.* (1984), and our findings that a phosphomonoesterase is present in these platelet intracellular membranes which is active towards [^{32}P]Ins-(1,4,5)P_3 would also suggest that degradation occurs.

In conclusion, we believe that these studies are the first to demonstrate clearly a Ca^{2+}-releasing role for Ins(1,4,5)P_3 acting on highly purified and well-characterized platelet intracellular-membrane vesicles preloaded with Ca^{2+}. It has been well established previously that the platelet responds to certain excitatory agonists with both enhanced phosphoinositide turnover and an increase in cytosolic [Ca^{2+}]. The action of Ins(1,4,5)P_3 on these intracellular membranes from blood platelets, seen as a rapid release of sequestered Ca^{2+}, supports the concepts of Berridge (1984) that a specific binding site for Ins(1,4,5)P_3 may be a feature of the endoplasmic-reticulum membranes of responsive cells. The data presented here also strongly suggest that the reported phosphoinositide hydrolysis and increase in cytosol [Ca^{2+}] which occur in platelets after stimulus–receptor interaction at the surface membrane are linked through the second-messenger action of Ins(1,4,5)P_3 acting on the Ca^{2+}-storing intracellular-membrane complexes referred to by electron microscopists as the 'dense tubular membrane system' (DTS). Knowledge of the submolecular nature of a receptor for Ins (1,4,5)P_3 in these platelet membranes may well have some importance in the design of new anti-platelet drugs for use in clinical states such as thrombosis, transplant rejection, extracorporeal circuitry etc.

Since the submission of this manuscript, study by Feinstein and his colleagues (O'Rourke *et al.*, 1985) was drawn to our attention. These workers used Percoll density gradients to produce human platelet membrane fractions, and showed that membrane vesicle subfractions enriched (2–3-fold) in endoplasmic-reticulum marker enzymes sequestered Ca^{2+} by an ATP-dependent process. Such vesicles, preloaded with ^{45}Ca, rapidly released substantial amounts of the cation after the addition of 5 μM-Ins(1,4,5)P_3.

We are grateful to Dr. Robin F. Irvine for the initial supply of Ins(1,4,5)P_3 and for his guidance with subsequent preparations from red cells by following his modification of the procedure of Downes *et al.* (1982). The ^{32}P-labelled Ins(1,4,5)P_3 was also kindly provided by Dr. R. F. Irvine. We thank our colleague, Dr. N. Hack, for helpful discussions during the course of this study, and Mrs. B. J. Evenden for excellent technical assistance. We are also grateful for financial support from the British Heart Foundation.

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