Ionophore A23187 induces a refractory state in thrombin-activated release of inositol phosphates

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The phosphatidylinositol cycle has been proposed to be involved in the regulation of platelet functionality through the control of cytoplasmic Ca^{2+} levels. However, the requirements of phospholipase C for Ca^{2+} has not yet been elucidated in intact platelets. The primary purpose of the present study was to investigate the Ca^{2+} requirements of this enzyme in platelets from miniature swine by taking advantage of the permeabilizing properties of the ionophore A23187. Our results strongly suggest that the treatment of platelets with A23187 induces a refractory state in thrombin-stimulated release of inositol phosphates while 5-hydroxytryptamine (serotonin)-secretory capacity in response to thrombin remained constant. This refractory state seems to be dependent on some cytochalasin-inhibitable cytoskeletal phenomena.

INTRODUCTION

Platelets have been suggested to be actively involved in several pathological situations from atherosclerosis and severe infection complications to cancer (Schwartz, 1983; Lefer, 1983; Mehta, 1984). The synthesis and secretion of arachidonic acid active oxygenated metabolites are controlled by membrane phenomena in platelets and many other cellular systems (Irvine, 1982; Lapetina, 1982). In this regard, it is noteworthy that the inositide metabolism has been proposed to regulate both the platelet membrane changes that in turn activate aggregation and secretion, and the liberation of arachidonic acid (Irvine, 1982; Siess *et al.*, 1984; Watson *et al.*, 1984). Recent studies have established the phosphodiesteratic attack to PIP_2 as the foremost step in the chain of events leading to the inositide metabolism activation, which yields diacylglycerol and IP_3 (Berridge & Irvine, 1984). IP_3 acting as a second messenger would regulate intracellular Ca^{2+} levels through its release from intracellular stores. On the other hand the existence of a still-unidentified link with the Ca^{2+} influx from the extracellular milieu has been suggested (Beaven *et al.*, 1984).

One important question arises from a careful study of the regulation of the inositide metabolism: the still-unresolved problem of Ca^{2+} requirements for phospholipase C activation (Cockcroft, 1984). In this sense it is interesting to note that relatively high Ca^{2+} concentrations have been reported to activate phospholipase C both in cell-free systems (Wightman *et al.*, 1981; Siess & Lapetina, 1983; Creutz *et al.*, 1985) and ionophore-permeabilized macrophages (Moscat *et al.*, 1986). The confirmation of this fact in intact cells would make unclear the relationship between phosphoinositide hydrolysis and Ca^{2+} -mobilizing mechanisms. Even though previous studies have suggested the non-requirement of Ca^{2+} for thrombin-stimulated PIP_2 degradation, no data are presently available about the requirement for

this ion in thrombin-stimulated phospholipase C activation by using a more direct measurement, such as inositol phosphates (IPs) release (Billah & Lapetina, 1982). Thus the original purpose of the study reported here was concerned with the Ca^{2+} requirements for thrombin-stimulated phospholipase C activation in intact platelets. We hoped to take advantage of the ionophore A23187 permeabilizing properties for Ca^{2+} (Moscat *et al.*, 1986) and the fact that it does not activate the phosphatidylinositol cycle in platelets (Rittenhouse, 1984); this would allow us to study the activation by thrombin of the phosphoinositide metabolism at different Ca^{2+} concentrations. However, and surprisingly, A23187 induced a refractory state in the thrombin-stimulated IPs release while not affecting thrombin-stimulated 5-hydroxytryptamine secretion. These data strongly suggest that phospholipase C activation is not a necessary step for 5-hydroxytryptamine secretion. Our results also raise the possibility of Ca^{2+} -independent thrombin-stimulated 5-hydroxytryptamine secretion.

MATERIALS AND METHODS

Platelet-labelling procedure

Platelets were isolated from miniature swine as described by Siess *et al.* (1983). Platelet pellets were resuspended in Tyrode/Hepes buffer (134 mM-NaCl/12 mM- NaHCO_3 /2.9 mM-KCl/0.34 mM- NaH_2PO_4 /1 mM- MgCl_2 /1 mM-EGTA/5 mM-glucose/5 mM-Hepes, pH 7.4) at a concentration of 5×10^8 platelets/ml, containing either 10 μCi of [5,6,8,9,11,12,14,15- ^3H]-arachidonic acid (Amersham International; sp. radioactivity 120 Ci/mmol)/ml or 30 μCi of *myo*-[2- ^3H]-inositol (Amersham; sp. radioactivity 50 Ci/mmol)/ml or 5 μCi of [^3H]5-hydroxytryptamine ([^3H]serotonin; Amersham; sp. radioactivity 9 Ci/mmol)/ml. Platelet suspensions were incubated for 2 h at 37 °C. Afterwards, platelets were sedimented as described by Siess *et al.*

Abbreviations used: PI, phosphatidylinositol; PIP_2 , phosphatidylinositol 4,5-bisphosphate; IP_3 , inositol trisphosphate; IPs, inositol phosphates.

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(1983), but in the presence of prostacyclin ($1 \mu\text{g/ml}$). Finally cells were resuspended in the same buffer, without prostacyclin, at a concentration of 2×10^8 platelets/ml.

Measurement of 5-hydroxytryptamine secretion

Samples of [^3H]5-hydroxytryptamine-labelled platelets (0.5 ml) were stimulated at different times and the reaction was stopped by mixing with 0.5 ml of 6% (w/v) glutaraldehyde. Platelets were centrifuged at $10000 g$ for 1 min and supernatant radioactivity determined by liquid-scintillation counting.

Phospholipid fractionation

Lipids were extracted as described by Lapetina & Cuatrecasas (1979) and fractionated by t.l.c. using the upper phase of an ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (9:5:2:10, by vol.) solvent system. Phosphatidic acid and arachidonic acid were detected by autoradiography.

Extraction and fractionation of IPs

Water-soluble IPs were extracted as described by Downes & Michell (1981) and fractionated on a Dowex 1×8 (formate form) anion-exchange column as described by Berridge (1983). The elution pattern of IPs was previously assessed by using radioactive standards obtained from ^{32}P -labelled erythrocyte ghosts (Downes & Michell, 1981). [^3H]Inositol 1,4,5-trisphosphate was generously provided by Dr. R. F. Irvine.

Platelet permeabilization

Washed platelets were sedimented as described by Siess *et al.* (1983) and resuspended in cytosol-like buffer (5 mM-MgCl₂/120 mM-KCl/20 mM-Hepes, pH 7.4) in the presence or in the absence of MgATP (2 mM), according to the experiments, at a concentration of $(1-2) \times 10^8$ platelets/ml. Platelets were permeabilized by adding $10 \mu\text{g}$ of saponin (Sigma)/ml; this gave a ratio of 5–10 μg of saponin/ 10^8 platelets. At this ratio the release of cytosolic enzymes, such as lactic dehydrogenase, and of marker enzymes of intracellular organelles, was comparable with that observed with previously published permeabilization protocols (Brass & Joseph, 1985), indicating a successfully controlled permeabilization.

The Ca^{2+} concentration was adjusted by using Ca^{2+} /EGTA buffers and taking into account pH, ionic composition of the media and the association constants of EGTA and ATP, as described by Raaflaub (1956).

RESULTS

The results shown in Figs. 1 and 2 confirm previous results demonstrating the activation by thrombin of the PI cycle. The pattern of water-soluble IPs is compatible with a phosphodiesterase attack on PIP_2 yielding IP_3 as the fastest-released metabolite (Berridge & Irvine, 1984). Figs. 1 and 2 also indicate that the Ca^{2+} ionophore A23187 does not stimulate either IPs release or phosphatidic acid synthesis. On the other hand, A23187 does not stimulate IPs release either in the presence or in the absence of extracellular Ca^{2+} (Table 1). Interestingly, extracellular Ca^{2+} does not exert any effect on the thrombin-stimulated IPs release (Table 1). This could indicate that platelet phospholipase C is not a Ca^{2+} -activated enzyme. However, it is possible that the

Fig. 1. Time course of the effect of thrombin (4 units/ml) and A23187 ($10 \mu\text{M}$) on the accumulation of labelled water-soluble inositols

Platelets were labelled as described in the text. The Figure shows a typical experiment which was repeated four times with incubations in duplicate. —, Thrombin; ----, A23187. Abbreviations: IP, inositol phosphate; IP_2 , inositol bisphosphate.

Fig. 2. Time course of the effect of thrombin (4 units/ml) and A23187 ($10 \mu\text{M}$) on the synthesis of phosphatidic acid in [^{14}C]arachidonic acid-labelled platelets

The Figure shows a typical experiment which was repeated four times with incubations in duplicate.

presence or redistribution of intracellular Ca^{2+} could be a prerequisite for thrombin activation of IPs release. In order to test this possibility, platelet Ca^{2+} intracellular stores were depleted by incubating [^3H]inositol-labelled platelets with A23187 ($10 \mu\text{M}$) in a Ca^{2+} -free, EGTA-containing, medium, after which they were incubated in the presence of thrombin (4 units/ml) and total IPs release determined as previously described (Moscat *et al.*, 1986). Table 1 shows that, under these conditions, thrombin does not stimulate phospholipase C, which could be interpreted as a requirement for the presence of intracellular Ca^{2+} for thrombin-stimulated IPs release.

However, these data do not permit us to discern

Table 1. Effect of Ca^{2+} on A23187 and thrombin-stimulated water-soluble IPs production in platelets

Platelets were labelled as described in the text and incubated in the presence of extracellular Ca^{2+} or in a Ca^{2+} -free EGTA-containing medium. In depletion experiments cells were also incubated with A23187 (10 μM) for 2 min. After stimulation (3 min), total IPs were extracted and fractionated by anion-exchange chromatography. Results are means $\pm$ s.d. for four independent experiments with incubations in duplicate. Abbreviation used: ND, not determined.

Treatment	IPs production (d.p.m./0.5 ml of platelet suspension)		
	Incubations in the presence of extracellular Ca^{2+}	Incubations in the absence of extracellular Ca^{2+}	
		With intracellular Ca^{2+}	Intracellular Ca^{2+} depleted
None (basal)	4200 $\pm$ 50	4200 $\pm$ 100	4900 $\pm$ 200
Thrombin (4 units/ml)	24300 $\pm$ 680	23800 $\pm$ 870	5400 $\pm$ 900
A23187 (10 μM)	4100 $\pm$ 230	4000 $\pm$ 220	ND

Fig. 3. Effect of different Ca^{2+} concentrations on the thrombin-stimulated production of water-soluble inositols

[^3H]inositol-labelled platelets were incubated in a Ca^{2+} -free EGTA (1 mM)-containing medium in the presence of A23187 (10 μM) for 3 min. Afterwards Ca^{2+} at different concentrations was added in order to obtain different Ca^{2+} /EGTA ratios giving the required concentration for that cation. Either thrombin (4 units/ml) (●) or buffer (○) was added, and after 3 min the production of IPs determined. Controls were performed by treating labelled platelets with A23187 (10 μM) in the absence of Ca^{2+} (▲) and with thrombin (4 units/ml) without ionophore in the absence of Ca^{2+} (▲). Incubations without A23187 were performed in the presence of carrier vehicle whose amount never exceeded the 0.5% of the total volume. Each point is the mean $\pm$ s.d. for three different experiments with incubations in duplicate.

whether thrombin induces a previous increase in the level of cytosolic Ca^{2+} or whether resting levels are high enough to permit the stimulation by thrombin of IPs release. In order to explore further these possibilities, and taking into account the fact that A23187 seems to actually permeabilize the platelet membrane to ions, ionophore-treated [^3H]inositol-labelled platelets were incubated with different extracellular (and hence cytosolic) Ca^{2+} concentrations, after which thrombin was added and IPs

Table 2. 5-Hydroxytryptamine secretion in A23187- and thrombin-stimulated platelets

Cells were labelled as described in the text, after which they were incubated and stimulated in a Ca^{2+} -free EGTA-containing medium either with A23187 (10 μM) or thrombin (4 units/ml). Platelets were also incubated first in the presence of A23187 (3 min), after which thrombin was added and 5-hydroxytryptamine secretion measured 3 min after stimulus. The control experiment with A23187 alone was carried out for 6 min. Results are means $\pm$ s.d. for three independent experiments with incubations in duplicate.

Stimulus	5-Hydroxytryptamine secretion (% of total)
Basal	5 $\pm$ 3
Thrombin (4 units/ml)	89 $\pm$ 12
A23187	30 $\pm$ 8
A23187 + thrombin	92 $\pm$ 6

release measured over a wide range of Ca^{2+} concentrations. Fig. 3 clearly shows that, surprisingly, thrombin does not stimulate phospholipase C at any tested Ca^{2+} concentration, suggesting that ionophore pretreatment of platelets induces some sort of refractory state in the stimulation of the inositide metabolism to a further challenge by thrombin. This clearly contradicts previous results showing that the pretreatment of platelets with A23187 does not impair a further activation by thrombin of the PI cycle as measured by phosphatidic acid production (Lapetina *et al.*, 1981). These data appear to indicate that either phosphatidic acid synthesis is not a good measurement of the activation of the inositide metabolism or thrombin-stimulated phosphatidic acid synthesis is actually impaired in ionophore-treated platelets. The results shown in Table 4 (below) indicate that thrombin is unable to induce phosphatidic acid synthesis in [^{14}C]arachidonic acid-labelled ionophore-treated platelets. Our results, then, show that pretreating platelets with A23187 induces a refractory state in the activation of inositide metabolism to a further challenge by thrombin. This is established by measuring both

Fig. 4. Ionophore-induced 5-hydroxytryptamine secretion and refractoriness in the thrombin-stimulated IPs release

[^3H]5-Hydroxytryptamine-labelled platelets were incubated for 3 min in the presence of different ionophore concentrations, after which they were centrifuged and the radioactivity released into the supernatant was determined (Δ). Maximum (100%) release corresponds to the amount of [^3H]5-hydroxytryptamine released in response to the maximal dose of ionophore used (10^{-5} M). [^3H]Inositol-labelled platelets were incubated for 3 min in the presence of ionophore, after which thrombin (4 units/ml) was added and incubated for another 3 min and the IPs released determined ($\blacktriangle$). The amount of [^3H]IPs liberated in response to thrombin in the absence of ionophore was 26000 ± 1700 d.p.m./0.5 ml, whereas it was 3250 ± 280 d.p.m./0.5 ml in the presence of the ionophore maximal dose. In the absence of thrombin, 2500 ± 290 and 2150 ± 190 d.p.m./0.5 ml were produced with 0 and 10^{-5} M-ionophore respectively. A refractoriness of 100%, then, is defined as the amount of [^3H]IPs liberated by thrombin-treated platelets in the presence of the maximal dose of ionophore. Results are means $\pm$ S.D. for three different experiments with incubations in duplicate. Note that the 100% of A23187-induced 5-hydroxytryptamine secretion corresponds to $50 \pm 8\%$ of the total platelet labelling.

inositol phosphates release and phosphatidic acid synthesis.

This finding provides us with a valuable tool for inhibiting the thrombin activation of the inositide metabolism, which, in turn, will permit one to study the involvement of this pathway in several platelet functions. Therefore we investigated the relationship between IPs release and 5-hydroxytryptamine secretion in thrombin-stimulated platelets. Table 2 clearly shows that, although ionophore pretreatment impairs the release of IPs by thrombin, this agonist is, however, able to induce 5-hydroxytryptamine secretion under these conditions. Furthermore, Table 2 also shows that 5-hydroxytryptamine secretion is stimulated by thrombin in Ca^{2+} -depleted platelets. This further raises the possibility of the non-requirement of Ca^{2+} (neither intra- nor extracellular) in thrombin-stimulated 5-hydroxytryptamine secretion in platelets.

Taking into account that A23187 does activate

Table 3. (a) 5-Hydroxytryptamine secretion and (b) IPs release in saponin-permeabilized platelets

Either [^3H]5-hydroxytryptamine- or [^3H]inositol-labelled platelets were permeabilized with saponin (10 $\mu\text{g}/\text{ml}$) and incubated in the presence or in the absence of ATP (2 mM). Afterwards, platelets were stimulated with 4 units of thrombin/ml for 3 min and either 5-hydroxytryptamine secretion or IPs release determined. Free Ca^{2+} concentrations were obtained by using Ca^{2+} /EGTA buffers. Results are means $\pm$ S.D. for three different experiments with incubations in duplicate.

Conditions [Free Ca^{2+}] (M) . . .	(a) 5-Hydroxytryptamine secretion (% of total platelet labelling) or (b) IPs release (d.p.m.)	
	8×10^{-8}	5×10^{-6}
(a) - Thrombin	4 ± 3	5 ± 2
+ Thrombin	87 ± 9	88 ± 10
+ ATP	5 ± 3	30 ± 6
(b) - Thrombin	3500 ± 300	3800 ± 200
+ Thrombin	23700 ± 2700	23700 ± 2500
+ ATP*	23800 ± 2200	24500 ± 1900

* Incubated in the presence of thrombin (4 units/ml) for 3 min.

secretion, albeit to a much lesser extent than does thrombin (Table 2), we wondered whether the refractory state in thrombin-stimulated IPs release could in some way be related to the weak secretagogue properties of the ionophore. Fig. 4 shows the dose-response curves of the ionophore stimulation both of 5-hydroxytryptamine secretion and induction of the refractory state in thrombin-stimulated IPs release. These data clearly indicate that ionophore concentrations as low as 10^{-8} M originate refractoriness (80% of the maximum), whereas 5-hydroxytryptamine secretion is only stimulated by 10% of the maximum value. It appears unlikely, then, that the refractoriness in the thrombin-stimulated IPs release is due to some secretion-related event, although that possibility cannot be ruled out by these experiments. However, in order to provide some more definitive evidence, either [^3H]5-hydroxytryptamine- or [^3H]inositol-labelled platelets were permeabilized with saponin (10 $\mu\text{g}/\text{ml}$) in a Ca^{2+} -free EGTA-containing medium and 5-hydroxytryptamine secretion was induced by adding Ca^{2+} (5 μM) plus ATP (2 mM). Table 3 shows that, under these conditions, platelets secreted 5-hydroxytryptamine to an extent similar to that produced by 10^{-7} M-ionophore in intact platelets. Whereas ionophore at this concentration produces 90% of maximal refractoriness, saponin-permeabilized platelets in the presence of Ca^{2+} and ATP conserve their full thrombin-activatable phospholipase C activity (Table 3). It is worth noting that, in this experimental approach, we have been able to induce weak 5-hydroxytryptamine secretion in platelets by a thrombin-independent mechanism similar to that obtained with A23187; however, no refractoriness in inositide metabolism under these conditions was observed. Two main conclusions could be drawn from these experiments: (a) the small amount of A23187-induced 5-hydroxytryptamine secretion does not in itself account for the refractoriness in the inositide metabolism; (b) in saponin-permeabilized platelets some mechanism ac-

Fig. 5. Cytochalasin B inhibits the ionophore-induced refractory state in IP₁ release

[³H]5-Hydroxytryptamine-labelled platelets were pretreated with cytochalasin B for 3 min, after which they were incubated with A23187 (10 μ M) for 3 min and 5-hydroxytryptamine secretion determined ($\blacktriangle$). [³H]Inositol-labelled platelets were treated as described above, but were also stimulated for 3 min with thrombin (4 units/ml) and IP₁ release was determined ($\bullet$). An IP₁ release of 100% is defined as the amount of [³H]IP₁ released in response to thrombin in the absence of A23187. Results are means $\pm$ S.D. for four independent experiments with incubations in duplicate.

counting for the appearance of the refractory state could be impaired. Thus saponin might disrupt cytoskeletal structures essential for the appearance of the refractory state in inositide metabolism. This could explain the observation that, when saponin-permeabilized platelets are incubated in the presence of Ca²⁺ plus ATP, a similar situation to that produced by A23187, no refractory state is produced. In order to explore this further, platelets were treated with A23187 (10 μ M) after being preincubated with cytochalasin B, and both 5-hydroxytryptamine secretion and the thrombin-activated release of inositol phosphates were measured. Fig. 5 shows that although no effect of cytochalasin B on the ionophore-induced 5-hydroxytryptamine secretion can be detected, a finding in keeping with those previously published (Siess *et al.*, 1982), a clear inhibition of the refractory state is observed at a cytochalasin concentration of 10⁻⁷ M. This strongly suggests that the ionophore-induced refractory state in IP₁ could be explained, at least partially, by ionophore-produced changes in the platelet cytoskeletal structures. Since cytochalasin, through the inhibition of actin polymerization, inhibits the metabolism of the arachidonic acid released by platelet stimuli (Siess *et al.*, 1982), it could be argued that the refractory state could be due to the production by the ionophore of active oxygenated metabolites of arachidonic acid. However, the preincubation of platelets with indomethacin (20 μ M) (Table 4) does not inhibit the appearance of the refractory state, indicating that neither arachidonic acid nor its metabolites are the agents responsible for refractoriness and that cytochalasin B inhibition of the refractory state is not mediated by the inhibition of the arachidonic acid metabolism.

Table 4. Effect of inhibitors of arachidonic acid metabolism in the A23187-induced refractory state in thrombin-activated release of IP₁

Washed [¹⁴C]arachidonic acid-labelled platelets were incubated for 2 min with 20 μ M indomethacin (IND) in a Ca²⁺-free EGTA-containing medium, after which A23187 (10 μ M) was added and incubation continued for a further 3 min. Afterwards, thrombin (4 units/ml) was added for 3 min, after which lipids were extracted, and fractionated as described. Results are means $\pm$ S.D. for three independent experiments with incubations in duplicate.

Inhibitor	Refractoriness (% of basal value)		
	Arachidonic acid	Thromboxane B ₂	Phosphatidic acid
Thrombin	500 $\pm$ 50	800 $\pm$ 75	500 $\pm$ 35
IND + thrombin	460 $\pm$ 60	100 $\pm$ 6	440 $\pm$ 25
A23187	500 $\pm$ 50	870 $\pm$ 98	109 $\pm$ 6
A23187 + thrombin	620 $\pm$ 30	820 $\pm$ 59	105 $\pm$ 8
IND + A23187 + thrombin	680 $\pm$ 70	100 $\pm$ 12	102 $\pm$ 5

It is also interesting to note that thrombin is able to induce IP₁ release in permeabilized platelets at cytosolic Ca²⁺ concentrations (8 $\times$ 10⁻⁸ M; see Table 3) and in saponin-treated Ca²⁺-depleted platelets (results not shown). This is interesting, since we have shown that Ca²⁺ depletion in other cellular systems, such as macrophages, strongly impairs agonist-induced phospholipase C activation (Moscat *et al.*, 1986). The differing Ca²⁺-dependencies of phospholipase C from different cellular origins is a subject that deserves further research.

DISCUSSION

It is clear from these results that thrombin stimulates the release of water-soluble IP₁ in platelets from miniature swine, confirming previous data in other animal species (Watson *et al.*, 1984; Zavoico *et al.*, 1985).

When platelets were permeabilized with the Ca²⁺ ionophore A23187 and incubated in a wide range of Ca²⁺ concentrations, thrombin, surprisingly, was unable to induce IP₁ release at any tested Ca²⁺ concentration. Thus it appears that the pretreatment of platelets with A23187 induces some sort of refractoriness in thrombin-activated inositide metabolism. It is noteworthy that although thrombin-induced refractory states in 5-hydroxytryptamine secretion and thromboxane A₂ synthesis have previously been described (Shuman *et al.*, 1979; Reimers *et al.*, 1984), this is the first report of ionophore-induced refractory state in thrombin-stimulated inositide metabolism. In our opinion, this is in itself an interesting finding, whatever the mechanism by which the ionophore produces its effect, since it permits one to study the involvement of inositide metabolism in platelet functionality. Thus, interestingly, although ionophore pretreatment does actually impair thrombin-stimulated inositide metabolism, no alterations are observed in thrombin-activated 5-hydroxytryptamine secretion. Three main conclusions can be drawn from the results presented here: (a) the ionophore-induced refractoriness is neither at the level of thrombin receptors nor in the secretory machinery; (b) neither the stimulation of the inositide

metabolism nor the presence and/or the increase in Ca^{2+} levels appear to be required events for thrombin-induced 5-hydroxytryptamine secretion in platelets; (c) cytoskeletal structures appear to be involved in the appearance of the refractoriness, strengthening the links between cytoskeleton and phospholipid metabolism (Anderson & Marchesi, 1985; Burn *et al.*, 1985; Lassing & Lindberg, 1985).

The available data about the role of inositide metabolism in platelet 5-hydroxytryptamine secretion are, at present, controversial. Thus, Lapetina *et al.* (1985) and Ruggiero *et al.* (1985), using saponin-permeabilized platelets, suggested the absence of a link between both phenomena. It has been suggested that these studies should be considered with caution, because these authors failed to produce 5-hydroxytryptamine secretion in permeabilized platelets with Ca^{2+} concentrations as high as 5 mM (Brass & Joseph, 1985). However, it is noteworthy that we have succeeded in producing 5-hydroxytryptamine secretion in saponin-permeabilized platelets by adding Ca^{2+} with ATP present in the incubation media. Treatment with saponin, like electrical-discharge permeabilization, seems to induce the loss of some pool of ATP necessary for Ca^{2+} -activated 5-hydroxytryptamine secretion (Knight & Scrutton, 1985). Brass & Joseph (1985) and Authi *et al.* (1986), on the other hand, reported data strongly suggesting IP_3 -induced 5-hydroxytryptamine secretion in saponin-permeabilized platelets through what appeared to be a thromboxane A_2 -mediated mechanism. Our data, nevertheless, strongly suggest the existence of an inositide- and Ca^{2+} -independent thrombin-activated pathway for 5-hydroxytryptamine secretion in platelets. Our experimental model is not a consequence of ionophore-induced depletion of polyphosphoinositides (results not shown) but it rather seems to be associated with cytochalasin-inhibitable cytoskeletal changes. Concerning the other branch of the inositide metabolism, namely the diacylglycerol-induced activation of protein kinase C, our data also suggest the non-requirement of protein kinase C activation for thrombin-induced 5-hydroxytryptamine secretion. It is worth noting that recently published data strengthen the need for a still-unidentified second-messenger pathway that could explain the myosin phosphorylation evoked by thrombin at low Ca^{2+} concentrations (Hallam *et al.*, 1985). In this regard, the recent report of the presence of very high tyrosine kinase activity in platelet membrane permits one to speculate on still-undiscovered pathways for platelet activation (Golden *et al.*, 1986). Thrombin, however, does in fact strongly activate inositide metabolism; the role played by these phospholipids in platelet activation is still undoubtedly an attractive challenge for further research.

REFERENCES

- Anderson, R. A. & Marchesi, V. T. (1985) *Nature (London)* **318**, 295–298
- Authi, K. S., Evenden, B. J. & Crawford, N. (1986) *Biochem. J.* **233**, 709–718
- Beaven, M. A., Moore, J. P., Smith, G. A., Hesketh, T. B. & Metcalfe, J. C. (1984) *J. Biol. Chem.* **259**, 7137–7142
- Berridge, M. J. (1983) *Biochem. J.* **212**, 849–858
- Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312**, 315–321
- Billah, M. M. & Lapetina, E. G. (1982) *Biochem. Biophys. Res. Commun.* **109**, 217–223
- Brass, L. F. & Joseph, S. K. (1985) *J. Biol. Chem.* **260**, 15172–15179
- Burn, P., Rotman, A., Meyer, R. K. & Berger, M. M. (1985) *Nature (London)* **314**, 469–472
- Cockcroft, S. (1984) *Biochem. Soc. Trans.* **12**, 966–968
- Creutz, C. E., Dowling, L. G., Kyger, M. & Francson, R. C. (1985) *J. Biol. Chem.* **260**, 7171–7173
- Downes, C. P. & Michell, R. H. (1981) *Biochem. J.* **198**, 133–140
- Golden, A., Nemeth, S. P. & Brugge, J. S. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 852–856
- Hallam, T. J., Daniel, J. L., Kendrick-Jones, J. & Rink, T. J. (1985) *Biochem. J.* **232**, 373–377
- Irvine, R. F. (1982) *Biochem. J.* **204**, 3–16
- Knight, D. E. & Scrutton, M. C. (1984) *Biochem. Soc. Trans.* **12**, 969–972
- Lapetina, E. G. (1982) *Trends Pharm. Sci.* **2**, 115–119
- Lapetina, E. G. & Cuatrecasas, P. (1979) *Biochem. Biophys. Acta* **573**, 394–401
- Lapetina, E. G., Billah, M. M. & Cuatrecasas, P. (1981) *J. Biol. Chem.* **256**, 5037–5040
- Lapetina, E. G., Silio, J. & Ruggiero, M. (1985) *J. Biol. Chem.* **260**, 7078–7083
- Lassing, I. & Lindberg, U. (1985) *Nature (London)* **314**, 472–474
- Lefer, A. M. (1983) in *Handbook of Shock and Trauma* (Altura, B. M., ed.), vol. 1, pp. 355–368, Raven Press, New York
- Mehta, P. (1984) *Blood* **63**, 55–63
- Moscat, J., Aracil, M., Diez, E., Balsinde, J., Garcia-Barreno, P. & Municio, A. M. (1986) *Biochem. Biophys. Res. Commun.* **134**, 367–371
- Raaffaub, J. (1956) *Methods Biochem. Anal.* **3**, 301–305
- Reimers, H. J., Scharf, R. E. & Baker, R. K. (1984) *Blood* **63**, 858–865
- Rittenhouse, S. E. (1984) *Biochem. J.* **222**, 103–110
- Ruggiero, M., Zimmerman, T. P. & Lapetina, E. G. (1985) *Biochem. Biophys. Res. Commun.* **131**, 620–627
- Schwartz, S. M. (1983) *Proc. Soc. Exp. Biol. Med.* **173**, 1–14
- Shuman, M. A., Botney, M. & Fenton, J. W. (1979) *J. Clin. Invest.* **63**, 1211–1218
- Siess, W. & Lapetina, E. G. (1983) *Biochim. Biophys. Acta* **752**, 329–338
- Siess, W., Lapetina, E. G. & Cuatrecasas, P. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7709–7713
- Siess, W., Siegel, F. L. & Lapetina, E. G. (1983) *J. Biol. Chem.* **258**, 11236–11242
- Siess, W., Weber, P. C. & Lapetina, E. G. (1984) *J. Biol. Chem.* **259**, 8286–8292
- Watson, S. P., McConnell, R. T. & Lapetina, E. G. (1984) *J. Biol. Chem.* **259**, 13199–13203
- Wightman, P. D., Dahlgren, M. E., Hall, J. C., Davies, P. & Bonney, R. J. (1981) *Biochem. J.* **197**, 523–526
- Zavoico, G. P., Halenda, S. P., Sha'afi, R. & Feinstein, M. B. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3859–3862