Phosphorylation-dependent and -independent pathways of platelet aggregation

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We have used the non-specific inhibitor of protein kinases, staurosporine, to investigate the role of protein phosphorylation during aggregation, the mobilization of intracellular Ca^{2+} (Ca^{2+}_i) and intracellular pH (pH_i) in thrombin-stimulated platelets. The concentration of staurosporine chosen for these studies, $1\ \mu\text{M}$, was previously reported to inhibit protein phosphorylation completely but to have no effect on the activation of phospholipase C in thrombin-stimulated human platelets [Watson, McNally, Shipman & Godfrey (1988) *Biochem. J.* **249**, 345–350]. Aggregation induced by phorbol dibutyrate is slow (several minutes) and is inhibited completely by staurosporine. In contrast, aggregation induced by thrombin, platelet-activating factor or ionophore A23187 is rapid (occurs within 60 s), and is slowed, but not inhibited, in the presence of staurosporine. On the other hand, staurosporine causes a small potentiation of the peak $[\text{Ca}^{2+}]_i$ signal induced by thrombin and a marked increase in the half-life of decay of this signal, but has no effect on pH_i . Under conditions designed to prevent an increase in $[\text{Ca}^{2+}]_i$ (presence of Ni^{2+} to prevent Ca^{2+} entry, and depletion of the intracellular Ca^{2+} stores), aggregation induced by thrombin resembles that by phorbol dibutyrate and is now inhibited completely by staurosporine. Taken together, these results provide evidence for two signalling pathways for aggregation, a relatively rapid phosphorylation-independent route mediated by Ca^{2+} and a slower, phosphorylation-dependent, pathway mediated by protein kinase C. Since staurosporine slows aggregation induced by thrombin, it appears that under normal conditions these pathways interact synergistically.

INTRODUCTION

The activation of human platelets involves a series of biochemical changes which lead to shape change, granule secretion and aggregation. It is now generally accepted that, with thrombin, the initial event which triggers these responses is a hydrolysis of membrane inositol phospholipids, generating the two second messengers inositol 1,4,5-trisphosphate and 1,2-diacylglycerol (for review see Rink & Hallam, 1984). Diacylglycerol, in the presence of phospholipid and cytosolic Ca^{2+} , is believed to activate protein kinase C, leading to the phosphorylation of several cellular proteins, including a 40 kDa protein of unknown function (Nishizuka, 1984). Inositol trisphosphate stimulates Ca^{2+} release from intracellular stores, and, in addition, Ca^{2+} can also enter the cytoplasm across the plasma membrane through an unidentified pathway. This increase in $[\text{Ca}^{2+}]_i$ triggers a number of events, including the activation of myosin light-chain kinase and phospholipase A_2 ; the latter leads to the release of arachidonic acid, which is rapidly converted into cyclo-oxygenase and lipoxygenase products.

The precise role of these biochemical events in the initiation of shape change, granule secretion and aggregation responses, however, is still only poorly understood. For example, although the phosphorylation of myosin light chains correlates well with the onset of shape change, there is only a poor correlation with the secretion of dense granules (Daniel *et al.*, 1984). More recently, several studies on semi-permeabilized platelets have further highlighted a poor agreement between

protein phosphorylation and several of the responses associated with platelet activation; the relevance of many of these observations to the intact cell, however, is uncertain. Haslam & Davidson (1984) reported a poor correlation between protein phosphorylation and the secretion of 5-hydroxytryptamine from platelets permeabilized by electrical discharge and challenged with increasing concentrations of Ca^{2+} . Lapetina *et al.* (1985) demonstrated in saponin-permeabilized platelets that thrombin can stimulate both secretion and aggregation in the absence of protein phosphorylation. More recently still, we have been able to extend some of these observations to the intact cell, using a non-specific inhibitor of protein kinases, staurosporine (Watson *et al.*, 1988). We have observed thrombin-induced aggregation, albeit at a slower rate than in control tissues, in the absence of protein phosphorylation. On the other hand, staurosporine inhibited completely the secretion of 5-hydroxytryptamine and ATP, and whether this result reflects a difference between the permeabilized and intact cell preparations or an additional inhibitory action of staurosporine manifested against the secretory mechanism is not known.

The aim of the present study was to investigate the molecular basis of the staurosporine-resistant, phosphorylation-independent aggregation observed in intact platelets challenged with thrombin. Our results suggest that thrombin, and also other agonists, are able to induce platelet aggregation in the absence of phosphorylation through the mobilization of Ca^{2+} . This pathway is potentiated by protein phosphorylation.

METHODS

Platelets were obtained from two sources: either from healthy aspirin-free volunteers on the day of the experiment, or as platelet concentrates, donated within the previous 18 h, from the Blood Transfusion Unit, John Radcliffe Hospital, Oxford. Experiments were carried on blood obtained from both sources in all cases. Platelet-rich plasma was obtained from fresh blood by centrifugation at 200 *g* for 20 min. Platelets were loaded/labelled with indo1-AM (1.5 μ M), BCECF-AM (1.5 μ M) or [3 H]5-hydroxytryptamine (10 μ Ci) by incubation at 37 °C for 30 min (intracellular dyes) or 60 min (5-hydroxytryptamine). Platelets were subsequently isolated by centrifugation at 1000 *g* for 10 min in the presence of prostacyclin to raise intracellular cyclic AMP concentrations and thus prevent activation. The platelet pellet was resuspended in a modified Krebs bicarbonate buffer (composition in mM: NaCl 135, NaHCO₃ 12, KCl 2.8, NaH₂PO₄ 0.35, MgCl₂ 1, Hepes 5, glucose 5, EGTA 1, pH 7.3); EGTA was omitted for the aggregation studies. Labelling with [32 P]P_i (1 mCi/ml) was carried out at this stage by incubation at 37 °C for 60 min, followed by centrifugation at 800 *g* in the presence of prostacyclin and resuspension. Platelets were left at least 30 min before experimentation in order to allow the actions of prostacyclin to decay. Indomethacin (10 μ M) was included in the final resuspension buffer to remove any influence by cyclo-oxygenase products.

Experiments were performed in a shaking water bath, a spectrofluorimeter or, for studies involving measurements of aggregation, in a Born lumi-aggregometer. Platelet suspensions (0.5 ml) were prewarmed at 37 °C for 5 min before the appropriate additions. The experiment was stopped at various times by transfer to 1.88 ml of chloroform/methanol/HCl (50:100:1, by vol.) for analyses of phosphatidic acid, to 0.5 ml of 6% (v/v) glutaraldehyde in phosphate buffer (pH 7.3) for measurements of 5-hydroxytryptamine secretion, or to Laemmli (1970) buffer for estimates of protein phosphorylation. Protein phosphorylation, phosphatidic acid and 5-hydroxytryptamine secretion were quantified as previously described (Watson *et al.*, 1988) and radioactivity was measured by scintillation counting to a 5% level of significance. Each experiment was performed at least three times, usually with triplicate or quadruplicate determinations; statistical comparisons were made by Student's *t* test or by a Wilcoxon-Rank test. Results are shown in the text as means $\pm$ S.E.M.

[Ca²⁺]_i was measured by using indo1-loaded platelets, with a Perkin-Elmer LS-3 fluorescence spectrophotometer. The excitation wavelength was 355 nm, and emission was monitored at 500 nm. (Although the $F_{\max.}/F_{\min.}$ ratio is greater for indo1 at around 410 nm, staurosporine itself fluoresces strongly at this wavelength.) At the wavelengths used in this study, it should be noted that indo1 fluoresces more strongly in the absence of Ca²⁺ (Grynkiewicz *et al.*, 1985). [Ca²⁺]_i was estimated by using the equation:

$$[\text{Ca}^{2+}]_i = K_d \frac{(F_{\max.} - F_{\min.})}{(F - F_{\min.})}$$

where $F_{\max.}$, $F_{\min.}$ and F represent the fluorescence in the absence, in the presence and at intermediate concentration of Ca²⁺, and $K_d = 250$ nM (Grynkiewicz *et al.*,

1985). These values have been measured as outlined for the use of quin2 (Tsien *et al.*, 1982), with corrections for the presence of extracellular dye, estimated as follows: since only extracellular dye 'sees' a rapid change in external Ca²⁺, the difference in fluorescence made on chelating a formerly saturating concentration of Ca²⁺ is entirely attributable to extracellular dye going from maximal to minimal fluorescence states. Estimations of pH_i were made by essentially the same procedure as used for indo1, with excitation and emission wavelengths of 500 and 530 nm respectively (Rink *et al.*, 1982).

Phorbol dibutyrate, thrombin, indomethacin, phosphocreatine, creatine kinase, A23187, platelet-activating factor and ADP were obtained from Sigma, Poole, Dorset, U.K. Staurosporine was a gift from Dr. H. Nakano, Kyowa Hakko Kogyo Co., Tokyo, Japan, and was dissolved in dimethyl sulphoxide. Indo1-AM, BCECF-AM and ionomycin were from Calbiochem. EIPA was a gift from Merck, Sharp and Dohme, Rahway, NJ, U.S.A. Dowex anion-exchange resin was from Bio-Rad, Richmond, CA, U.S.A. [3 H]Inositol, [32 P]P_i and [3 H]5-hydroxytryptamine were from Amersham International, Amersham, Bucks., U.K.; [3 H]inositol was cleaned before use by passage through a Dowex column. All other reagents were of analytical grade.

RESULTS

Comparison of the effect of staurosporine on platelet activation by thrombin with other stimuli

We have previously reported that staurosporine inhibits completely thrombin-induced phosphorylation of a 40 kDa protein, a substrate for protein kinase C, and a 20 kDa protein, a substrate for myosin light-chain kinase, but that it has no effect on the formation of phosphatidic acid, indicating that this inhibitory action does not occur at the level of phospholipase C (Watson *et al.*, 1988). Essentially, identical results have been observed in the present study with a further platelet stimulant, platelet-activating factor (Table 1).

We also previously reported (Watson *et al.*, 1988), and have confirmed in the present study (see below), that

Table 1. Staurosporine inhibits protein phosphorylation but not phosphatidic acid formation induced by platelet-activating factor

Platelets were pre-labelled with [32 P]P_i and challenged with platelet-activating factor (PAF; 1 μ M) for 60 s in the absence or presence of staurosporine (St; 1 μ M). Phosphorylations of proteins of 47 and 20 kDa and formation of phosphatidic acid were measured as described in the Methods section. Results are shown as means $\pm$ S.E.M from four experiments performed in quadruplicate.

	³² P (d.p.m.)		
	47 kDa protein	20 kDa protein	Phosphatidic acid
Basal	123 $\pm$ 11	37.6 $\pm$ 3.8	105 $\pm$ 5
PAF	290 $\pm$ 27	123 $\pm$ 9.9	742 $\pm$ 69
PAF + St	119 $\pm$ 13	46.0 $\pm$ 4.0	673 $\pm$ 55

Fig. 1. Effect of staurosporine on platelet aggregation induced by various agonists

Experiments were carried out in a Born lumi-aggregometer, and aggregation is indicated by a decrease in optical density (OD); an increase in OD, which can be seen before the onset of aggregation in some instances, represents platelet shape change. Platelet-activating factor (PAF; 1 μ M), ADP (3 μ M), A23187 (A23; 500 nM) and phorbol dibutyrate (PDBu; 400 nM) were added as indicated by the white triangles, and solvent (Sol) or staurosporine (1 μ M; st) were added as indicated by the black triangles. The buffer contained indomethacin (10 μ M), phosphocreatine (2 mM) and creatine kinase (20 units/ml), and there was no added Ca^{2+} . Traces are representative from at least four separate experiments.

staurosporine slows, but does not prevent, thrombin-induced aggregation. Again, similar results were observed with platelet-activating factor (Fig. 1) and also with two further stimulants, ADP and ionophore A23187 (Fig. 1). In all cases, the shape-change response (reflected by the decrease in light transmission preceding aggregation in Fig. 1) was inhibited in the presence of staurosporine, consistent with the inhibition of myosin light-chain kinase (Daniel *et al.*, 1984). In contrast, aggregation induced by phorbol dibutyrate was inhibited completely by staurosporine (Fig. 1).

Role of the Na^+ - H^+ antiporter in the activation of human platelets by thrombin

In agreement with the results of Zavoico *et al.* (1986) and Siffert & Akkerman (1987), we found that activation

of human platelets by thrombin is associated with a rapid dose-dependent alkalinization of the platelet cytosol. For example, thrombin (0.3 unit/ml) increased the cytosolic pH from 7.03 ± 0.01 to a peak value of 7.11 ± 0.01 ($n = 5$) within 60 s. This response was not altered in the presence of 1 μ M-staurosporine; under these conditions thrombin (0.3 unit/ml) increased the pH to 7.12 ± 0.02 ($n = 5$) within 60 s. Similar results were observed for lower concentrations of thrombin (0.03–0.1 unit/ml). In contrast, a maximally effective concentration of phorbol dibutyrate (300 nM) produced a much smaller increase in cytosolic pH (usually < 0.03), which took up to 4 min to reach a plateau. Example traces of these results are shown in Fig. 2(b).

The increase in cytosolic pH induced by thrombin was reversed in the presence of the Na^+ - H^+ antiport inhibitor EIPA; for example, in the presence of EIPA, thrombin (0.3 unit/ml) decreased the cytosolic pH to 6.93 ± 0.01 ($n = 5$). Similar observations have been previously made by Zavoico *et al.* (1986) and Siffert & Akkerman (1987). This decrease in pH was significantly enhanced in the presence of 1 μ M-staurosporine to 6.89 ± 0.01 ($P < 0.05$; $n = 5$). On their own, EIPA and staurosporine induced a small decrease of cytosolic pH (< 0.03 in 120 s), or had no apparent effect, respectively. Example traces of these results are shown in Fig. 2(b).

The actions of EIPA on platelet aggregation and activation of phospholipase C by thrombin were also studied. EIPA had no significant effect on the concentration–response curve for aggregation induced by thrombin. In contrast, in the presence of staurosporine, EIPA inhibited aggregation induced by thrombin, although this effect was highly variable; in some cases complete inhibition was observed, whereas in others aggregation was either delayed or only partially inhibited; representative traces are shown in Fig. 2(a). EIPA had no effect on the formation of phosphatidic acid by thrombin in the absence or presence of staurosporine (Table 2).

Effect of staurosporine on Ca^{2+} mobilization by thrombin

Indo1 was successfully loaded into platelets to intracellular concentrations of $175 \pm 17 \mu\text{M}$. The resting $[\text{Ca}^{2+}]_i$ was similar in 1 mM external Ca^{2+} and in 'zero' external Ca^{2+} (1 mM-EGTA; external $[\text{Ca}^{2+}] < 1 \text{ nM}$); these values were $117 \pm 12 \text{ nM}$ and $117 \pm 7 \text{ nM}$ respectively. The addition of staurosporine to a sample of unloaded platelets produced a small but significant increase in the fluorescence signal. Having allowed for this autofluorescence, the effect of staurosporine on $[\text{Ca}^{2+}]_i$ in indo1-loaded platelets could be observed. The addition of staurosporine produced little departure from the resting $[\text{Ca}^{2+}]_i$ seen before drug treatment (Fig. 3).

Addition of thrombin (0.01–1.0 unit/ml) caused a rapid fall in fluorescence, followed by a slower recovery or partial recovery. This corresponds to a Ca^{2+} transient with rapidly rising and more slowly declining phases. The peak $[\text{Ca}^{2+}]_i$ was lower at lower concentrations of thrombin, and recovery was more often complete. At any particular dose of thrombin, the peak $[\text{Ca}^{2+}]_i$ reached was slightly greater in the presence of external Ca^{2+} than in its absence. This is in agreement with previous experiments suggesting the existence of at least two components in the thrombin-induced rise in $[\text{Ca}^{2+}]_i$ (e.g. Hallam & Rink, 1985a); part enters from the extracellular medium and part derives from intracellular stores.

Fig. 2. Effects of EIPA and staurosporine on pH_i and aggregation in platelets challenged with thrombin

(a) Aggregation. Platelets were challenged with thrombin (0.1 unit/ml) in the presence of EIPA (50 μ M) and/or staurosporine (St; 1 μ M). Thrombin (Thr) was added as indicated by the black triangles, whereas solvent, EIPA or staurosporine (St) was added as indicated by the white triangles. Traces are representative from at least four separate experiments. Other conditions are as described in Fig. 1 legend. (b) pH_i. Experiments were carried out in platelets loaded with the pH indicator BCECF (Rink *et al.*, 1982). The excitation and emission wavelengths were 500 and 530 nm respectively. Thrombin (0.1 unit/ml), staurosporine (1 μ M) and EIPA (50 μ M) were added as indicated by the adjacent triangle. The gap in the traces represents the time taken for addition of drugs and mixing. Traces are representative from at least four separate experiments.

Table 2. Effects of EIPA and staurosporine on formation of phosphatidic acid

Platelets were pre-labelled with [³²P]P_i and challenged with thrombin (Thr; 1 unit/ml) for 60 s in the presence of EIPA (50 μ M) and/or staurosporine (1 μ M; St). Results are shown as means $\pm$ S.E.M. from four experiments performed in quadruplicate.

	Phosphatidic acid (d.p.m.)
Basal	123 $\pm$ 12
Thr	3574 $\pm$ 308
Thr + EIPA	3509 $\pm$ 146
Thr + EIPA + St	3723 $\pm$ 198

When similar studies were performed in the presence of staurosporine, there appeared to be a delay in the recovery of the [Ca²⁺]_i transient after thrombin treatment in the absence or presence of external Ca²⁺. In addition, in the presence of 1 mM external Ca²⁺ staurosporine converted the monophasic rise in [Ca²⁺]_i seen in controls after thrombin treatment into a biphasic increase, with the second phase being slower than the first; no such

Fig. 3. Effect of staurosporine on the mobilization of Ca²⁺_i by thrombin and ionomycin

Experiments were carried out in platelets loaded with the Ca²⁺ indicator indo1 (Grynkiewicz *et al.*, 1985). The excitation and emission wavelengths were 355 and 500 nm respectively. Thrombin (0.02 unit/ml) and ionomycin (50 nM) were added as indicated by the black triangles, and solvent (S) or staurosporine (1 μ M; St) was added as indicated by the white triangles. The gap in the traces indicates the time of drug addition and mixing. The traces are representative from at least four separate experiments.

effect was seen in the absence of extracellular Ca^{2+} , suggesting that the second phase may represent the entry of external Ca^{2+} . The peak $[\text{Ca}^{2+}]_i$ signal induced by thrombin in the absence of external Ca^{2+} was unaltered in the presence of staurosporine, whereas that in the presence of 1 mM external Ca^{2+} was increased owing to the additional second phase. Two superimposed traces of these results from control and staurosporine-treated platelets challenged with thrombin are shown in Fig. 3.

The recovery of the $[\text{Ca}^{2+}]_i$ transient was analysed in terms of the half-life of decay. Owing to the non-linearity of the relationship between the fluorescence signal and $[\text{Ca}^{2+}]_i$, it should be noted that $t_{1/2}$ is not the half-life of decay of fluorescence. Since the half-life did not appear to vary greatly with the concentration of thrombin used, results obtained with a range of thrombin concentrations (0.02–1.0 unit/ml) were pooled. Staurosporine (1 μM) significantly slowed the time course of decay of $[\text{Ca}^{2+}]_i$ after thrombin challenge in the presence of 1 mM external Ca^{2+} ; the mean $t_{1/2}$ was increased from 46 ± 7 s to 78 ± 9 s ($P < 0.05$; $n = 5$). The $t_{1/2}$ in the absence of external Ca^{2+} was also increased by staurosporine (1 μM) from 50 ± 6 s to 68 ± 8 s, although this did not reach statistical significance.

The Ca^{2+} ionophore ionomycin produced a concentration-dependent increase in $[\text{Ca}^{2+}]_i$ when added in concentrations which were sub-threshold for protein phosphorylation (investigated in parallel experiments). The form of this transient differed from that produced by thrombin in that the rise in $[\text{Ca}^{2+}]_i$ was prolonged; recovery was slow. Staurosporine had no effect on the amplitude or time course of this transient. A representative trace is shown in Fig. 3.

Role of $[\text{Ca}^{2+}]_i$ in staurosporine-resistant aggregation

Having established that Ca^{2+} mobilization is not inhibited, and under some conditions is potentiated, in the presence of staurosporine, experiments were designed to investigate whether Ca^{2+} is the signal for the phosphorylation-independent aggregation. These studies were carried out in buffer containing either no added Ca^{2+} or EGTA (under these conditions the external $[\text{Ca}^{2+}]$ is in the micromolar range, Ca^{2+} being derived from the water used to make the buffer and from glassware etc.) or 2 mM- Ca^{2+} . It is essential for external $[\text{Ca}^{2+}]$ to be greater than 400 nM, since below this concentration the glycoprotein IIb-IIIa complex (i.e. the fibrinogen receptor) splits and platelets are unable to undergo aggregation (Brass *et al.*, 1985).

The importance of Ca^{2+} entry was investigated by carrying out experiments in the presence of Ni^{2+} , which has been shown to block the movement of Ca^{2+} into the platelet (Hallam & Rink, 1985a). Aggregation induced by thrombin in either the absence or the presence of staurosporine was not altered in the presence of Ni^{2+} . Thus it appears that Ca^{2+} entry from the external medium is not required for aggregation; a similar conclusion for thrombin-induced aggregation has been made by Ruda *et al.* (1988) through the use of peptide fragments of thrombin.

Experiments were also designed to investigate the importance of Ca^{2+} release from intracellular stores. Platelets were exposed to the Ca^{2+} ionophore A23187 in the presence of 1 mM-EGTA in order to deplete the intracellular Ca^{2+} store. Platelets treated in this manner underwent a transient shape change mediated by the

Fig. 4. Staurosporine inhibits thrombin-induced aggregation in the absence of Ca^{2+} mobilization

Experiments were carried out in a Born lumi-aggregometer as described in Fig. 2. Platelets were incubated in buffer containing EGTA (1 mM) and A23187 (300 nM) at 37 °C for 60 min. NiCl_2 (2 mM), CaCl_2 (2 mM) and, where indicated, staurosporine (1 μM ; St) were added as indicated by the black triangles, and thrombin (1 unit/ml; Thr) or phorbol dibutyrate (PDBu; 400 nM) was added as indicated by the white triangles. The traces are representative of three experiments.

mobilization of Ca^{2+} , but were unable to aggregate because of the low external Ca^{2+} concentration. After recovery from shape change and the return of $[\text{Ca}^{2+}]_i$ to resting values, 2 mM- Ca^{2+} was added and platelets were challenged with thrombin. The continued presence of the Ca^{2+} ionophore prevents the refilling of the intracellular Ca^{2+} stores (Rink & Sage, 1987). Platelets treated in this manner were still able to undergo aggregation in response to thrombin, albeit at a slower rate than controls. In the presence of staurosporine, aggregation was further slowed but still apparent. These results therefore demonstrate that aggregation can occur in the absence of Ca^{2+} release from intracellular stores.

In order to examine further the role of $[\text{Ca}^{2+}]_i$ in the onset of aggregation, however, it is necessary to eliminate Ca^{2+} mobilization from both intra- and extra-cellular sources within the same experiment. This was achieved by combining the above two approaches. As shown in Fig. 4, the rate of aggregation induced by thrombin was markedly inhibited under these conditions, and aggregation was reminiscent of that by phorbol dibutyrate. Moreover, the thrombin-induced aggregation was fully inhibited by staurosporine (Fig. 4).

DISCUSSION

Phosphorylation-independent pathway of aggregation

The present study was undertaken to examine the mechanism of aggregation in the presence of the non-specific inhibitor of protein kinases, staurosporine. Our previous observation (Watson *et al.*, 1988) that thrombin induces platelet aggregation, albeit at a lower rate, in the presence of staurosporine (and therefore in the absence of protein phosphorylation) has been extended to additional platelet stimuli, i.e. platelet activating factor, A23187 and ADP. Since these stimuli, unlike thrombin, have no proteolytic activity, it appears that this phosphorylation-independent route of aggregation is not mediated through the proteolytic pathway of platelet activation described by Lapetina *et al.* (1985) in saponin-permeabilized platelets (whether this pathway is also present in intact platelets is uncertain). The mechanism of platelet activation by platelet-activating factor and by the Ca^{2+} ionophore A23187 is through the metabolism of

phosphoinositides (Lapetina & Siegel, 1983) and the mobilization of Ca^{2+} (Rittenhouse, 1984) respectively. The mechanism of ADP-induced aggregation is more controversial. In the presence of cyclo-oxygenase inhibitors, platelet activation by ADP is associated with the mobilization of Ca^{2+} (Hallam & Rink, 1985b), but there are contradictory reports as to whether this is associated with the stimulation of phosphoinositide hydrolysis [cf. Fisher *et al.* (1985) and Sweatt *et al.* (1986) with Daniel *et al.* (1986)].

The present study has considered two potential signalling pathways for the staurosporine-resistant (phosphorylation-independent) aggregation. Siffert & Akkerman (1987) have suggested that low concentrations of thrombin stimulate platelet activation through increased Na^+-H^+ exchange, leading to alkalinization of the cytosol. In order to test whether this pathway is involved in the staurosporine-resistant aggregation, experiments were carried out in the presence of the Na^+-H^+ antiport inhibitor EIPA. EIPA decreased, but did not inhibit, aggregation induced by thrombin in the presence of staurosporine, and converted the alkalinization of the platelet cytosol induced by thrombin into an acidification, an effect potentiated by staurosporine. These data therefore demonstrate that aggregation in the presence of staurosporine can occur in the absence of alkalinization of the platelet cytosol, thus ruling out this pathway as the signal for the phosphorylation-independent aggregation. The decreased rate of aggregation observed in staurosporine-treated platelets in the presence of EIPA may be related to potentiation of the EIPA-induced acidification of the cytosol by staurosporine; platelet aggregation is known to be inhibited by a decrease in external pH (Lagarde *et al.*, 1980), possibly through a corresponding decrease in pH_i .

The present study has provided evidence, however, that it is an increase in $[\text{Ca}^{2+}]_i$, from both intra- and extracellular sources, which is the signal for the phosphorylation-independent aggregation. Under conditions in which Ca^{2+} mobilization was prevented, the aggregation response to thrombin resembled that to phorbol dibutyrate, and was not fully inhibited by staurosporine. Presumably the mechanism of aggregation by thrombin under these conditions is through the activation of protein kinase C.

Phosphorylation-dependent pathway of aggregation

Phorbol dibutyrate activates protein kinase C in human platelets and also stimulates secretion and aggregation, responses which occur in the absence of Ca^{2+} mobilization (Rink *et al.*, 1983). All of these responses, including aggregation, are inhibited by the non-selective protein kinase inhibitor staurosporine. This suggests that it is the activation of protein kinase C which is the initial signal that leads to the subsequent aggregation response induced by phorbol dibutyrate. The time course of aggregation by phorbol dibutyrate (minutes), however, is considerably slower than that induced by other platelet stimuli, e.g. platelet-activating factor, thrombin, ADP or A23187 (responses occur within 20–60 s).

Pathways of platelet aggregation: conclusions

Taken together, therefore, the above data suggest that there are at least two signalling mechanisms for aggregation. One appears to be through the activation of

protein kinase C and seems to be independent of Ca^{2+} mobilization. The other appears to be through the mobilization of Ca^{2+} and is independent of protein phosphorylation. Aggregation induced by either pathway alone, however, is slower than that which occurs in the presence of both Ca^{2+} mobilization and protein phosphorylation, suggesting that, under physiological conditions, these two pathways interact synergistically.

Role of protein kinase C in platelets

The present study has also provided information on the various roles of protein kinase C in platelets. Siffert *et al.* (1987) have suggested that thrombin stimulates Na^+-H^+ exchange in platelets through the activation of protein kinase C, since phorbol esters also increase pH_i . Zavoico *et al.* (1986) have emphasized, however, that the increase in pH_i induced by phorbol esters is both slower and smaller than that induced by thrombin, making it unlikely that the effects of thrombin are mediated by protein kinase C. We have made similar observations to those of Zavoico *et al.* (1986) and, in addition, observed staurosporine to have no effect on thrombin-induced alkalinization at concentrations which inhibit fully the activation of protein kinase C. These data strongly suggest that thrombin-induced alkalinization in platelets is not mediated through the stimulation of protein kinase C. A similar conclusion for thrombin-stimulated alkalinization of a smooth-muscle cell line was reached by Berk *et al.* (1987), who showed that the down-regulation of protein kinase C has no effect on alkalinization induced by thrombin.

The experiments with staurosporine have, however, provided further evidence that protein kinase C has an important physiological role in promoting Ca^{2+} removal from the platelet cytosol. Previously, Pollock *et al.* (1987) reported that phorbol esters promote Ca^{2+} extrusion from platelets, and Yoshida & Nachmias (1987) described phorbol ester-stimulated Ca^{2+} sequestration into intracellular organelles of platelets. The present study has shown that staurosporine, in concentrations which do not affect platelet Ca^{2+} -ATPase (K. Authi, personal communication) but which fully inhibit protein kinase C, potentiated the Ca^{2+} signal induced by thrombin. In contrast, staurosporine had no effect on the Ca^{2+} transient induced by concentrations of ionomycin which were sub-threshold for the activation of protein kinase C.

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