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The action of the protein kinase C inhibitor, staurosporine, on human platelets

Evidence against a regulatory role for protein kinase C in the formation of inositol trisphosphate by thrombin

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The ability of several putative inhibitors of protein kinase C (PKC) to block dioctanoylglycerol (DC8)-induced phosphorylation of a 47 kDa protein (a recognized substrate for PKC) in human platelets was investigated. Staurosporine (1 μM) caused complete inhibition of phosphorylation, whereas the other reagents were either inactive (polymyxin B) or gave only partial inhibition (C-1, H-7, tamoxifen). Staurosporine (1 μM) fully inhibited the phosphorylation of the 47 kDa protein in platelets challenged with thrombin, but also inhibited the phosphorylation of a 20 kDa protein which is a substrate for myosin light-chain kinase. The inhibition of both kinases by staurosporine was associated with the inhibition of thrombin-induced secretion of ATP and 5-hydroxytryptamine and a slowing of the aggregation response; staurosporine, however, had no effect on the formation of phosphatidic acid and inositol phosphates induced by thrombin. Staurosporine also reversed the inhibitory action of phorbol esters on thrombin-induced formation of phosphatidic acid. These data are consistent with a role for these two kinases in secretion and aggregation (although there must be additional control signals, since aggregation was only slowed, not inhibited), but suggest that neither kinase is involved in the regulation of phosphoinositide metabolism. This latter conclusion contradicts previous observations that the activation of PKC by phorbol esters or membrane-permeable diacylglycerols alters the apparent activity of both phospholipase C and inositol trisphosphatase. Possible explanations for this discrepancy are discussed.

INTRODUCTION

Activation of human platelets by agonists such as thrombin, collagen, platelet-activating factor, thromboxanes etc. is associated with the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), thus generating the two second messengers, 1,2-diacylglycerol (DG) and inositol trisphosphate (IP_3) (Lapetina & Watson, 1984; Rink & Hallam, 1984). DG remains in the plasma membrane and, in the presence of phosphatidylserine and Ca^{2+}, activates the normally cytosolic protein kinase C (PKC) (Nishizuka, 1984; Rink *et al.*, 1983). IP_3 diffuses into the cytoplasm and stimulates the release of Ca^{2+} from the dense tubular system, which is equivalent to the endoplasmic reticulum (O'Rourke *et al.*, 1985; Brass & Joseph, 1985). Both limbs of this pathway can be mimicked through the use of Ca^{2+} ionophores, e.g. A23187, to increase cytosolic Ca^{2+}, and with either membrane-permeable DGs (e.g. DC8) or phorbol esters to bring about the activation of PKC (Kaibuchi *et al.*, 1983; Lapetina *et al.*, 1985*a*). Using such pharmacological probes, Nishizuka and colleagues demonstrated that PKC and Ca^{2+} interact in a synergistic manner to produce secretion and aggregation (Kaibuchi *et al.*, 1983; Nishizuka, 1984).

The precise role played by PKC in platelet activation, however, is uncertain. Experiments by ourselves and others have shown that activation of PKC with phorbol esters or a membrane-permeable DG before challenge with agonists such as thrombin, collagen, platelet-activating factor etc. inhibits the subsequent hydrolysis of PIP_2 (Watson & Lapetina, 1985; Zavoico *et al.*, 1985; Rittenhouse & Sasson, 1985) and the mobilization of intracellular Ca^{2+} (MacIntyre *et al.*, 1985; Krishnamurthi *et al.*, 1986), and increases the metabolism of IP_3 (Molina y Vedia & Lapetina, 1986). PKC appears therefore also to have a negative-feedback influence over platelet activation. The hypothesis has therefore been proposed that PKC has a bidirectional role in platelet activation, i.e. it is involved in both secretion and aggregation, and also in limiting platelet activation by subsequently decreasing the hydrolysis of PIP_2 and promoting increased metabolism of IP_3.

The inhibitory action of PKC can be observed against a range of platelet agonists, e.g. thrombin, collagen and platelet-activating factor, indicating that the effect is post-receptor (Watson & Lapetina, 1985). The inhibition of PIP_2 hydrolysis is therefore most likely brought about either at the level of the G-protein which couples the receptor to phospholipase C, or through an inhibition of phospholipase C itself. The decreased mobilization of Ca^{2+} may be due to the inhibition of PIP_2 hydrolysis (Watson & Lapetina, 1985; Zavoico *et al.*, 1985; Rittenhouse & Sasson, 1985), to an increase in Ca^{2+}

Abbreviations used: PKC, protein kinase C; IP_3 inositol trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; DG, 1,2-diacylglycerol; DC8, dioctanoylglycerol; PA, phosphatidic acid; MLCK, myosin light-chain kinase; 5-HT, 5-hydroxytryptamine.

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extrusion from the platelet (Pollock *et al.*, 1987), or to increased metabolism of IP_3 (Molina y Vedia & Lapetina, 1986; Connolly *et al.*, 1986). The last hypothesis is of particular interest, since the predominant substrate for PKC in the platelet, a protein of 47 kDa (Imaoka *et al.*, 1983), is thought to be IP_3 phosphatase, and its phosphorylation by PKC leads to an increase in IP_3 metabolism (Connolly *et al.*, 1986).

From the above discussion it is clear that the role of PKC in the platelet is both complex and uncertain. The present study was undertaken, using newly developed inhibitors of PKC, to investigate further the role of this protein kinase in platelet activation. If PKC is involved in secretion and aggregation, these physiological responses should be blocked by these drugs. Similarly, if PKC has a negative-feedback role mediated at the level of PIP_2 hydrolysis and IP_3 metabolism, then inhibitors of this enzyme should increase both agonist-stimulated PIP_2 hydrolysis and IP_3 concentrations.

The study has used the reagents C-1 (Gerard *et al.*, 1986), tamoxifen (Su *et al.*, 1985; Horgan *et al.*, 1986) and polymyxin B (Naccache *et al.*, 1985), which have all been shown to inhibit phorbol ester-stimulated responses in neutrophils, staurosporine, which has been shown to inhibit a partially purified preparation of rat brain PKC (Tamaoki *et al.*, 1986), and H-7, which has been shown to block the inhibitory action of phorbol ester on phospholipase C activation in platelets (Poll *et al.*, 1986; Tohmatsu *et al.*, 1986).

METHODS

Platelets were obtained from two sources. Blood was either drawn on the day of the experiment from aspirin-free volunteers, with sterile 20 mM-sodium citrate as anticoagulant, and the platelet-rich plasma was obtained by centrifugation at 200 *g* for 20 min, or platelet concentrates were obtained from the Blood Transfusion Unit, John Radcliffe Hospital, and used within 20 h of donation. Platelets were isolated from platelet-rich plasma or the platelet concentrate by centrifugation at 1000 *g* for 10 min in the presence of prostacyclin to increase intracellular cyclic AMP. The platelet pellet was then resuspended in 1 ml of a modified Krebs bicarbonate buffer (composition, in mM: NaCl 135, $NaHCO_3$ 13, KCl 2.8, NaH_2PO_4 0.35, $MgCl_2$ 1, Hepes 5, glucose 5, EGTA 1; pH 7.3) and labelled with one of the following: [^{3}H]inositol (50 μCi/ml) for 3 h; [^{32}P]P_i (1 mCi/ml) for 1 h; or [^{3}H]5-HT (5 μCi/ml) also for 1 h. The EGTA was omitted in experiments involving the measurement of aggregation. The platelets were then centrifuged in the presence of prostacyclin at 1000 *g* for 10 min and resuspended at a concentration of approx. 4×10^8/ml in the above buffer. In some of the aggregation studies indomethacin (10 μM), phosphocreatine (5 mM) and creatine kinase (40 units/ml) were added to the buffer. Platelets labelled with [^{3}H]inositol were re-centrifuged and finally suspended in the above buffer containing LiCl (12 mM) substituted for an equimolar amount of NaCl. This concentration of Li^+ inhibits the conversion of inositol monophosphate into free inositol (Berridge *et al.*, 1982). The platelets were left at least 30 min before experimentation.

Experiments were performed in either a shaking water bath or, for studies involving measurements of ATP secretion and aggregation, in a Born lumi-aggregometer. Platelet suspensions (0.5 ml) were prewarmed at 37 °C for 5 min before the addition of inhibitors of PKC or phorbol dibutyrate, followed after a time interval of 60–300 s by thrombin or DC8. 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 inositol phophates or phosphatidic acid, to 0.5 ml of 6% (v/v) glutaraldehyde in phosphate buffer (pH 7.3) for analysis of 5-HT secretion, or to Laemmli (1970) buffer for analysis of protein phosphorylation. Inositol phosphates, protein phosphorylation, phosphatidic acid (PA) and 5-HT secretion were measured as previously described (Watson *et al.*, 1985; Nunn & Watson, 1987). Radioactivity was measured by scintillation counting to a 5% level of significance for each tube. Each experiment was performed at least three times, usually with triplicate or quadruplicate determinations. Statistical comparisons were made by Student's *t* test and by a Wilcoxon-Rank test.

Tamoxifen, polymyxin B, phorbol dibutyrate, thrombin, indomethacin, phosphocreatine, creatine kinase, A23187, platelet-activating factor and arachidonic acid were obtained from Sigma, Poole, Dorset, U.K. H-7 was purchased from Seikagaku Kogyo, Tokyo, Japan, and C-1 was kindly given by Dr. C. E. McCall, Bowman Gray, Wake Forest University, NC, U.S.A.; both reagents were dissolved in Krebs buffer and stored frozen. Staurosporine was a gift from Dr. H. Nakano, Kyowa Hakko Kogyo Co., Tokyo, Japan, and was dissolved in dimethyl sulphoxide. DC8 was from Nova, Läufefingen, Switzerland, and was dissolved in ethanol. Dowex anion-exchange resin was from Bio-Rad, Richmond, CA, U.S.A. [^{3}H]Inositol, [^{32}P]P_i and [^{3}H]5-HT were from Amersham International, Amersham, Bucks., U.K.; [^{3}H]inositol was cleaned before use by passage through a Dowex column.

RESULTS

Characterization of PKC inhibitors

Initial experiments were designed to investigate the ability of the putative PKC inhibitors to prevent phosphorylation of the 47 kDa protein by DC8 (10 μM) in intact platelets. The 47 kDa protein is the predominant substrate for PKC in the platelet (Fig. 1; Imaoka *et al.*, 1983). Polymyxin B had no effect on the phosphorylation of the 47 kDa protein by DC8 (Table 1). H-7 and C-1, at maximally effective concentrations, inhibited the phosphorylation of the 47 kDa protein by no more than 40% (Table 1). The similar actions and potencies of these two agents is consistent with their close structural similarity. The partial inhibition of 47 kDa phosphorylation by H-7 is in agreement with the results of Inagaki *et al.* (1984) on human platelets and Kawamoto & Hidaka (1984) on rabbit platelets. Tamoxifen (100 μM) inhibited the phosphorylation of the 47 kDa protein by more than 50% (Table 1), but also caused a general dephosphorylation of other proteins and a partial inhibition of thrombin-stimulated inositol phosphate formation (results not shown). Tamoxifen had no inhibitory action at a concentration of 10 μM (results not shown). Staurosporine (1 μM) decreased the phosphorylation of the 47 kDa protein by DC8 to below resting values (Table 1, Fig. 1). A concentration/response curve for staurosporine inhibition of 47 kDa phosphorylation is shown in Fig. 1; maximal inhibition was observed at a

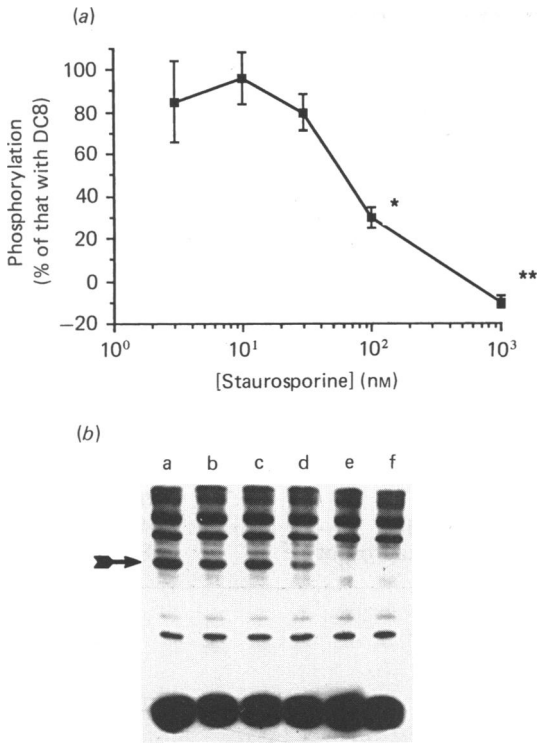

Fig. 1. Staurosporine inhibits the phosphorylation of a 47 kDa protein induced by DC8

(*a*) Dose/response curve for inhibition of 47 kDa-protein phosphorylation by staurosporine. Platelets, prelabelled with [^{32}P]P_i, were challenged with DC8 (10 μM) for 60 s in the presence of various concentrations of staurosporine. Each point represents the mean ± S.E.M. from four to eight experiments performed in triplicate: $*P < 0.05$; $**P < 0.01$. (*b*) An example autoradiogram of a one-dimensional gel of ^{32}P-labelled platelets challenged with DC8 (10 μM) in the presence of various concentrations of staurosporine. DC8 was included in lanes (a)–(e), and its solvent (ethanol) was added to lane (f). The concentration of staurosporine in each lane was as follows: (a) 3 nM; (b) 10 nM; (c) 30 nM; (d) 100 nM; (e) 1 μM; (f) solvent (dimethyl sulphoxide). The position of the 47 kDa protein is indicated by the arrow.

concentration of 1 μM, with approx. 50% inhibition at 100 nM.

Since staurosporine is the only agent which fully inhibited 47 kDa phosphorylation by DC8, it was used in most of the later experiments designed to study the role of PKC in platelet activation by thrombin. Agents which only partially inhibit PKC, such as C-1 or H-7, may have little effect on responses which require only minimal activity of PKC; for example, this may explain why H-7 does not inhibit phorbol-ester-induced secretion of 5-HT from human platelets (Inagaki *et al.*, 1984), but blocks the inhibitory action of phorbol ester on the activation of phospholipase C in platelets (Tohmatsu *et al.*, 1986; Poll *et al.*, 1986).

Staurosporine inhibits myosin light-chain kinase (MLCK), PKC and secretion of 5-HT and ATP in thrombin-stimulated platelets

The stimulation by thrombin of 20 kDa- and 47 kDa-protein phosphorylation, substrates for MLCK and PKC respectively, and the secretion of 5-HT were inhibited in parallel in the presence of increasing concentrations of staurosporine (Fig. 2). Maximal inhibition of 47 kDa-protein phosphorylation by staurosporine (1 μM) coincided with full inhibition of 20 kDa-protein phosphorylation and 5-HT secretion (Fig. 2). In a separate set of experiments, staurosporine (1 μM) inhibited completely the secretion of ATP by thrombin (Fig. 5).

Table 1. Action of putative inhibitors of PKC on phosphorylation of a 47 kDa protein induced by DC8

Platelets, prelabelled with [^{32}P]P_i, were challenged with DC8 for 60 s in the presence of various putative inhibitors of PKC. The phosphorylation of a 47 kDa protein, a known substrate for PKC, was analysed and compared with control values (i.e. phosphorylation by DC8 alone = 100%). Results are shown as means ± S.E.M. for three to eight experiments performed in triplicate; n.s., not significant. Incubation of DC8 (10 μM) with human platelets for 60 s increased the phosphorylation of the 47 kDa protein from 38.3 ± 7.5 to 158.6 ± 24.8 d.p.m.

Inhibitor	Concn.	Phosphorylation (%)	*P*
None (control)	–	100 ± 4.8	–
Polymyxin B	1 mM	100 ± 6.0	n.s.
H-7	60 μM	60.0 ± 7.7	< 0.001
	200 μM	71.4 ± 4.4	< 0.001
C-1	60 μM	65.7 ± 9.9	< 0.001
Tamoxifen	100 μM	44.7 ± 6.6	< 0.001
Staurosporine	1 μM	−10.0 ± 2.9	< 0.001

Staurosporine does not alter the activity of DG kinase

The conversion of DC8 into DC8-PA was not altered in the presence of either H-7 (60 μM) or a concentration of staurosporine (1 μM) which maximally inhibits 47 kDa-protein phosphorylation by DC8 (Table 2). This strongly suggests that DG kinase, the enzyme responsible for converting DC8 into DC8-PA, is not under regulatory control from PKC, and indicates that, under conditions of PKC inhibition, PA concentrations can be used to reflect the activation of phospholipase C by agonists such as thrombin (Lapetina & Cuatrecasas, 1979).

Staurosporine does not affect the activation of phospholipase C by thrombin

The formation of PA induced by thrombin was not altered in the presence of either 0.1 μM-staurosporine (Fig. 3) or 1 μM-staurosporine (Fig. 4), suggesting that the activation of phospholipase C by thrombin is not under regulatory control from PKC. Similarly, staurosporine (1 μM) had no apparent effect on the formation of inositol monophosphate, inositol bisphosphate and IP_3 by thrombin, after either 60 s or 600 s (Table 3). There was no measurable formation of inositol tetrakisphosphate, in either the absence or the presence of staurosporine after stimulation by thrombin (results not shown).

In agreement with the results of Watson & Lapetina (1985), phorbol dibutyrate (300 nM) inhibited the thrombin-induced formation of PA by approx. 45% (Fig. 4). This inhibitory action of the phorbol

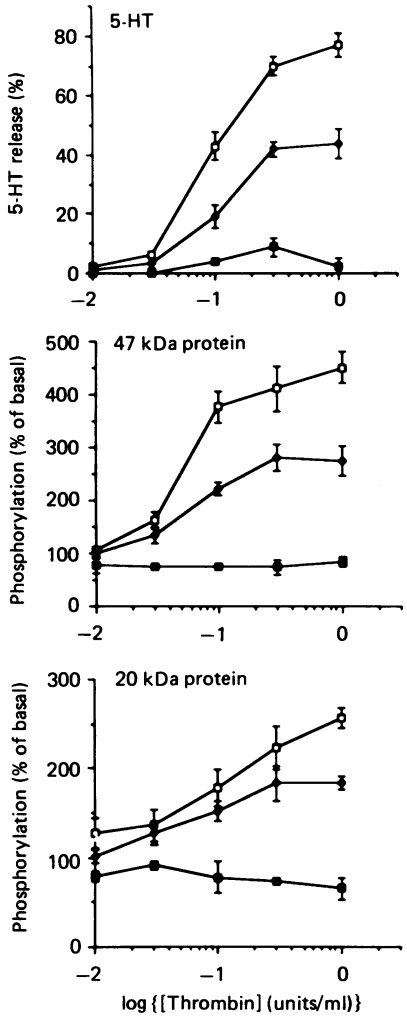

Fig. 2. Staurosporine inhibits 5-HT secretion and phosphorylation of 20 kDa and 47 kDa proteins induced by thrombin

Platelets prelabelled with [^{32}P]P_i and [^{3}H]5-HT were challenged with thrombin for 60 s in the presence of staurosporine (◆, 0.1 μM; ■, 1 μM) or its solvent (dimethyl sulphoxide; □, control). Each point represents the mean ± S.E.M. from three experiments performed in triplicate.

Table 2. Inhibitors of PKC have no apparent action on the metabolism of DC8 to DC8-PA

Platelets prelabelled with [^{32}P]P_i were challenged with DC8 (10 μM) for 60 s in the presence of H-7 (60 μM) or staurosporine (1 μM). The metabolism of DC8 or DC8-PA was measured by t.l.c. as described in the Methods section and compared with control values, i.e. metabolism of DC8 in the absence of PKC inhibitors = 100%. Results are shown as means ± S.E.M. for four experiments performed in triplicate. Incubation of DC8 (10 μM) with the platelets for 60 s increased the radioactivity in the portion of the t.l.c. plate scraped for DC8-PA from 532 ± 82 to 11657 ± 1052 d.p.m.

Additions	DC8-PH (%)
DC8	100 ± 3.6
DC8 + H-7	94 ± 13.0
DC8 + staurosporine	108 ± 13.7

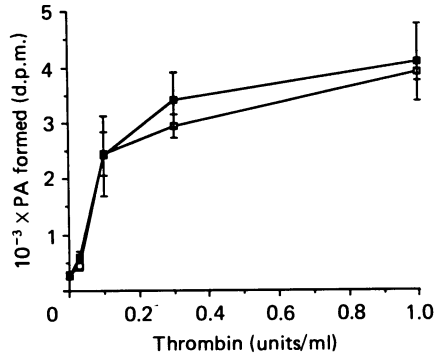

Fig. 3. Staurosporine does not inhibit formation of PA by thrombin

Platelets prelabelled with [^{32}P]P_i were challenged with thrombin for 60 s in the presence of staurosporine (■; 0.1 μM) or its solvent (dimethyl sulphoxide; □, control). Each point represents the mean ± S.E.M. of three experiments performed in triplicate.

dibutyrate was reversed in the presence of staurosporine to control values, i.e. the amount of PA formed in the presence of thrombin (Fig. 4).

Staurosporine inhibits shape change and slows aggregation by thrombin

Staurosporine (1 μM) inhibited completely the shape-change response induced by low concentrations of thrombin (results not shown), but only slowed the rate of aggregation induced by higher concentrations of thrombin (Fig. 5). Aggregation could still be observed in the presence of 10 μM-staurosporine, a concentration 10-fold in excess of that required to inhibit fully PKC (Fig. 2) and the secretion of ATP (Fig. 5). The slowing of the aggregation response by staurosporine could be observed over a range of thrombin concentrations with no apparent shift in the position of the dose/response curve (results not shown). The inhibitory action of staurosporine on thrombin aggregation could also be observed in conditions designed to remove the participation of secondary mediators, i.e. the inclusion of indomethacin, phosphocreatine and creatine kinase (results not shown).

Staurosporine also completely inhibited shape change and partially inhibited aggregation induced by 1–10 μM-arachidonic acid (indomethacin fully inhibited aggregation induced by arachidonic acid), 0.3–1 μM-A23187 (in the presence of indomethacin) and 10–100 nM-platelet-activating factor (results not shown). In contrast, staurosporine (1 μM) completely inhibited aggregation and shape change induced by 3–30 μg of collagen/ml (results not shown).

DISCUSSION

The present study has shown that staurosporine is a potent but non-selective inhibitor of protein kinases in human platelets. It is therefore of limited use for studying the role of PKC in responses in which MLCK is also

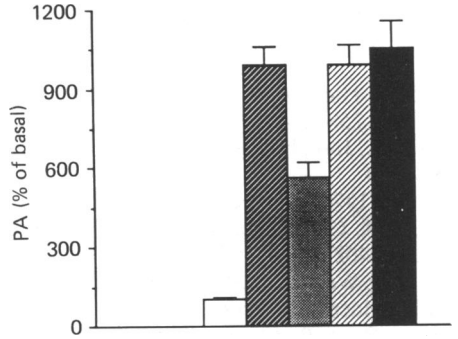

Fig. 4. Staurosporine reverses the inhibitory action of phorbol dibutyrate on thrombin-induced formation of PA

Platelets prelabelled with [^{32}P]P_i were challenged with thrombin (1 unit/ml) for 60 s in the absence or presence of staurosporine (1 μM) and/or phorbol dibutyrate (PDBu; 300 nM); PDBu and staurosporine were given 5 min before thrombin. Results are expressed as percentage of basal, and are shown as means ± S.E.M. for four experiments performed in triplicate. Key: □, basal; ▨, thrombin; ▩, PDBu + thrombin; ▨, staurosporine + thrombin; ■, staurosporine + PDBu + thrombin.

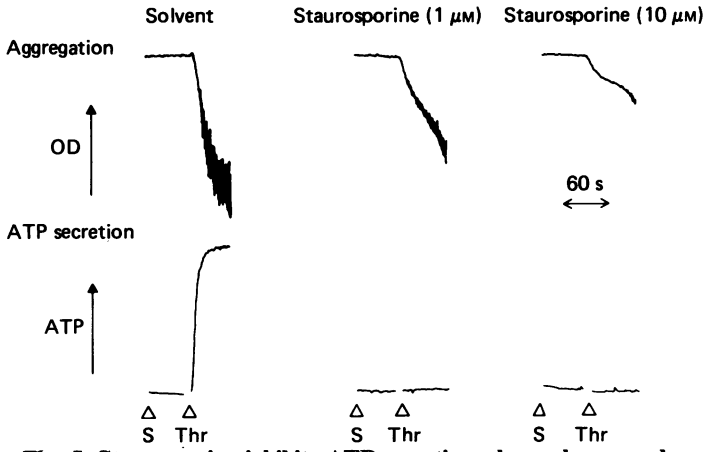

Fig. 5. Staurosporine inhibits ATP secretion, shape change and slows aggregation induced by thrombin

Platelets were challenged with thrombin (Thr; 1 unit/ml) for 60 s in the absence or presence of staurosporine (S; 1 μM) in a Born lumi-aggregometer. The secretion of ATP was measured with the luciferin–luciferase reagent, and shape change and aggregation were estimated by the optical density (OD) of the suspension. An increase in OD reflects shape change, and a decrease in OD indicates aggregation. This trace is representative of three experiments.

Table 3. Staurosporine does not inhibit thrombin-induced inositol phosphate formation

Platelets, prelabelled with [^{3}H]inositol and suspended in the presence of Li^+ (12 mM) were challenged with thrombin (1 unit/ml) in the presence or absence of staurosporine (1 μM) for 60 s or 600 s. Inositol phosphates were analysed by Dowex anion-exchange chromatography. There was no detectable formation of IP_3 after 10 min, possibly because of desensitization of the thrombin receptor (Huang & Detwiler, 1987). Results are shown as means ± S.E.M. for six experiments performed in quadruplicate and are shown as percentage increase above the basal values for each isomer (100 % represents basal). The resting radioactivities in inositol monophosphate (IP), inositol bisphosphate (IP_2) and IP_3 were 219.6 ± 15.5, 77.4 ± 2.1 and 66.8 ± 2.0 d.p.m. respectively.

	IP	IP_2	IP_3
Thrombin (60 s)	204 ± 13.4	277 ± 22.4	531 ± 103
Thrombin (60 s) + staurosporine	205 ± 16.3	240 ± 16.8	465 ± 62.5
Thrombin (600 s)	245 ± 16.7	476 ± 34.4	–
Thrombin (600 s) + staurosporine	249 ± 7.0	546 ± 46.3	–

involved. For example, although staurosporine completely inhibits the secretion of 5-HT by thrombin, there is insufficient evidence to rule out the involvement of MLCK in this response, and so nothing can be concluded about the role played by PKC in secretion.

Staurosporine can be used, however, in a negative manner to rule out the involvement of PKC and MLCK in a particular response. The slowing of the aggregation response to thrombin by staurosporine indicates that either MLCK and/or PKC participate in the onset of aggregation induced by thrombin. However, the observation that thrombin-induced aggregation can still proceed, albeit at a much slower rate, after complete inhibition of 47 kDa- and 20 kDa-protein phophorylation by staurosporine suggests that aggregation can proceed independently of these two kinases. The existence of a pathway of aggregation which is independent of protein phosphorylation has been previously hypothesized by Haslam & Davidson (1984) and Lapetina *et al.* (1985*b*). This pathway also appears to be utilized by arachidonic acid, platelet-activating factor and A23187 (in the presence of indomethacin). The inhibition of collagen-induced aggregation by staurosporine may indicate another site of action for this antibiotic in the platelet.

Staurosporine is clearly a very useful agent to study the role of PKC in responses not under regulation by MLCK. For example, full inhibition of DC8- or thrombin-stimulated 47 kDa-protein phosphorylation by staurosporine produced no apparent alteration in the formation of PA and inositol phosphates. These results suggest that the metabolism of DG by DG kinase, the hydrolysis of inositol phospholipids by phospholipase C and the metabolism of IP_3 by IP_3 phosphatase are not under regulatory control by PKC after agonist activation. Thus the previously reported observations that membrane-permeable DGs or phorbol esters inhibit phospholipase C activation (Watson & Lapetina, 1985; Zavoico *et al.*, 1985; Rittenhouse & Sasson, 1985) and also increase IP_3 metabolism (Molina y Vedia & Lapetina, 1986) do not appear to be of physiological significance.

The explanation for the contrasting results observed after PKC inhibition or activation is not known, but there are several explanations worthy of consideration. For example, both phorbol esters and membrane-permeable DGs have the potential to diffuse to intracellular sites which are inaccessible to the more lipophilic endogenous DGs formed as a result of PIP_2 hydrolysis. This may thus lead to the phosphorylation by PKC of proteins which are not normally substrates for the

enzyme, after stimulation by agonists such as thrombin. Alternatively, the identification of at least three forms of PKC (Coussens *et al.*, 1986; Ohne *et al.*, 1987), and the knowledge that PKC can be activated in either a DG-dependent or DG-independent manner after proteolysis (Murray *et al.*, 1987), raise the possibility that not all of these forms of the enzyme may be equally susceptible to inhibition by staurosporine. It is possible that the phosphorylation of the 47 kDa protein, from which the activity of staurosporine was estimated, is mediated predominantly by only one or two of the above active species of PKC.

Neither of these arguments, however, can explain the inability of staurosporine to alter the metabolism of IP_3 after the complete inhibition of 47 kDa-protein phosphorylation. Connolly *et al.* (1986) have suggested that the 47 kDa protein is IP_3 phosphatase and that its phosphorylation by PKC should lead to increased metabolism of IP_3. The present results clearly argue against such a role for PKC.

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