Effect of phorbol ester treatment on receptor-mediated versus G-protein-activator-mediated responses in platelets

Evidence for a two-site action of phorbol ester at the level of G-protein function

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In platelets, and in several other cell systems, pre-treatment with protein kinase C activators such as phorbol 12-myristate 13-acetate (PMA) results in the inhibition of receptor-mediated responses, suggesting that protein kinase C may play an important role in the termination of signal transduction. In the present study, we have attempted to locate the site of action of phorbol ester by comparing thrombin-induced (i.e. receptor-mediated) platelet activation with that induced by guanosine 5'-[γ -thio]triphosphate (GTP[S]) and NaF, two agents which by-pass the receptor and initiate platelet responses by directly modulating G-protein function. After a 10 s pre-treatment with PMA (16 nM), dense-granule secretion induced by thrombin (0.2 unit/ml), GTP[S] (40 μ M) and NaF (30 mM) was potentiated, resulting in a greater than additive response to agent plus PMA. However, after a 5 min pre-treatment, thrombin-induced secretion alone was inhibited, whereas PMA plus GTP[S]/NaF-induced release remained greater than additive. [32 P]Phosphatidate formation in response to all three agents, in contrast, was inhibited by 50–70 % in PMA (5 min)-treated platelets. That secretion induced by these agents is a protein kinase C-dependent event was demonstrable by using staurosporine, a protein kinase C inhibitor which at concentrations of 1–10 nM inhibited (70–90 %) PMA-induced as well as thrombin- and NaF-induced secretion and protein phosphorylation. In membranes from PMA-treated platelets, thrombin-stimulated GTPase activity was significantly enhanced compared with that in untreated membranes (59 % versus 82 % increase over basal activity). The results suggest that inhibition of receptor-mediated responses by PMA may be directed towards two sites relating to G-protein activation: (i) receptor-stimulated GTPase activity and (ii) G-protein–phospholipase C coupling. Furthermore, the lack of inhibition of NaF- and GTP[S]-induced secretion by PMA suggests that different mechanisms may be involved in thrombin-induced and G-protein-activator-induced secretion.

INTRODUCTION

Several recent reports [1–6] have described inhibitory effects of protein kinase C activators such as phorbol 12-myristate 13-acetate (PMA) and 1,2-dioctanoylglycerol (diC_8) on receptor-mediated responses in platelets. These responses include phosphoinositide hydrolysis [1–4], intracellular Ca^{2+} elevations [1,2,5,6], arachidonate release [5,6] and dense-granule secretion [2,5,6], and, as the inhibitory effects of PMA and diC_8 are observed against most physiological agonists (thrombin, collagen, platelet-activating factor, the thromboxane mimetic U46619, adrenaline, vasopressin and ADP), the site of action is most probably post-receptor. As post-receptor events leading to nearly all activation responses in platelets are now believed to be dependent on G-protein function [7–10], we have examined the effect of PMA on G-protein function in the present work. Some previous work in this area has demonstrated that protein kinase C activation by PMA results in a suppression of the inhibitory (G_i) as well as stimulatory (G_s) effects on adenylate cyclase [11,12], and it has been suggested that phosphorylation of the α subunit of G_i by protein

kinase C [13] could be responsible for these effects. However, it is not yet known if the suppression of agonist-induced activation responses, e.g. inositol phospholipid breakdown and granule secretion, by PMA is also mediated via inhibitory effects at the level of G-protein function.

Our principal aims have been to examine this possibility, and also to locate the site of inhibitory action of PMA, i.e. to distinguish between inhibitory effects on receptor–G-protein interaction, and those on G-protein–GTP interaction. To achieve this, we have used two approaches. Firstly, we have examined the effect of PMA on phosphatidate formation (as an index of inositol phospholipid breakdown) and granule secretion induced by thrombin as well as by two agents that directly activate G-proteins and by-pass receptor-mediated events, namely the stable GTP analogue guanosine 5'-[γ -thio]triphosphate (GTP[S]) and NaF [7,10]. Secondly, since the present studies demonstrated that PMA treatment interferes with G-protein–phospholipase C coupling, we have focused on GTP hydrolysis as another possible site of action by measuring thrombin-stimulated GTPase activity in platelet membranes.

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; diC_8 , 1,2-dioctanoylglycerol; G-protein, GTP-binding protein; GTP[S], guanosine 5'-[γ -thio]triphosphate; 5HT, 5-hydroxytryptamine; PA, phosphatidate.

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MATERIALS AND METHODS

Materials

GTP[S] was purchased from Boehringer, Lewes, E. Sussex, U.K., PMA, GTP, NaF, indomethacin were from Sigma, Poole, Dorset, U.K., diC_8 was from Novabiochem, Lufelfingen, Switzerland, and thrombin (bovine) from Parke Davis, Morris Plains, NJ, U.S.A. All radiochemicals were obtained from Amersham International, Amersham, Bucks., U.K. Staurosporine was kindly given by Dr. H. Nakano, Kyowa Hakko Kogyo Co., Tokyo, Japan, and from Dr. R. Scartazzini and Dr. K. Scheibli, Ciba-Geigy, Basle, Switzerland.

Preparation of washed platelets

Blood collection from apparently healthy human (male) volunteers, preparation of platelet-rich plasma and washed platelets were as described previously [6]. Essentially, platelets were washed in a pH 6.5 buffer composed of 36 mM-citric acid, 5 mM-KCl, 103 mM-NaCl, 0.09 % glucose, 30 mM-prostacyclin and 0.05 unit of hirudin (Bio-Pharm, Swansea, U.K.)/ml, in which labelling with 0.5 μCi of 5-hydroxy[^{14}C]tryptamine (5HT) (57 Ci/mol)/ml or carrier-free [^{32}P]P_i was carried out. For the experiments involving [^{14}C]5HT labelling, 0.5 mM- Ca^{2+} was included in the incubation medium. After incubation (30 min at 37 °C) and centrifugation (1500 g, 10 min) the platelets were resuspended in a pH 7.4 buffer composed of 10 mM-Hepes, 103 mM-NaCl, 5 mM-KCl, 1 mM- MgSO_4 , 0.9 % glucose and 0.1 unit of hirudin/ml in the absence or presence of 1 mM- Ca^{2+} for experiments with permeabilized and intact platelets respectively. For experiments involving [^{32}P]P_i labelling, Ca^{2+} was omitted from the incubation medium; however, after the incubation period (90 min at 37 °C) the platelets were washed once more in the same pH 6.5 buffer plus 0.5 mM- Ca^{2+} by centrifugation, before final resuspension in the Hepes-based pH 7.4 buffer (with and without Ca^{2+} for experiments with intact and permeabilized platelets respectively). The platelet count in all experiments was $(2\text{--}3) \times 10^8/\text{ml}$. Platelets washed and resuspended in the absence of Ca^{2+} did not respond to additions of GTP[S] (10–100 μM) in the presence of saponin, but platelets prepared as described above, i.e. washed in the presence of 0.5 mM- Ca^{2+} , were responsive to GTP[S] in the presence of saponin. This could be because the latter washing procedure results in micromolar concentrations of residual free Ca^{2+} when the platelets are finally resuspended, which has previously been shown to be essential for GTP[S]-induced functions in platelets [7].

[^{14}C]5HT secretion

The release of [^{14}C]5HT from pre-labelled platelets was studied in stirred [^{14}C]5HT-labelled platelets (in the presence or absence of saponin) pre-treated with the cyclo-oxygenase inhibitor indomethacin (10 μM) to avoid effects of endogenously formed thromboxane A_2 as previously described [5].

[^{32}P]Phosphatidate (PA) formation

Indomethacin (10 μM)-treated ^{32}P -labelled platelets were incubated (30 s–5 min) with the agonist (in the presence or absence of saponin), terminated by the addition of 3.75 vol. of chloroform/methanol/HCl (10:20:0.3, by vol.) and extracted with 1 vol. each of 2 M-KCl and chloroform as described previously [14]. PA

was separated from the other phospholipids by t.l.c., by using the upper phase of ethyl acetate/acetic acid/isooctane/water (45:8:18:25, by vol.) as the solvent system.

Preparation of platelet membranes and assay of GTPase activity

Washed platelets resuspended in a Hepes-based buffer (pH 7.4) were prepared as described above, and were treated with PMA (16 nM), diC_8 (60 μM), 4 β -phorbol (16 nM) or dimethyl sulphoxide vehicle (0.04 %) for 10 min. At the end of the incubation, the platelet suspensions were diluted with 10 times their volume of 10 mM-Tris/HCl buffer containing 1 mM-EDTA (pH 7.4) and centrifuged at 30000 g for 15 min at 4 °C. The resulting pellet was resuspended by homogenization in ice-cold 10 mM-Tris/HCl buffer containing 1 mM-EDTA (pH 7.4), and the homogenate was centrifuged at 30000 g for 15 min at 4 °C. The pellet was washed once more in the same buffer by centrifugation, and the final pellet resuspended in 10 mM-Tris/HCl buffer (pH 7.4) and stored frozen in batches at –70 °C.

GTPase activity was measured by monitoring the liberation of [^{32}P]P_i from [$\gamma\text{--}^{32}\text{P}$]GTP as described by Houslay *et al.* [15] and following essentially the original method of Cassel & Selinger [16]. The reaction mixture (100 μl) contained 0.4 μM -[$\gamma\text{--}^{32}\text{P}$]GTP, 5 mM- MgCl_2 , 100 mM-NaCl, 1 mM-dithiothreitol, 0.5 mM-EGTA, 1 mM-ATP, 50 units of creatine kinase/ml and 12 mM-phosphocreatine in 50 mM-Tris/HCl buffer (pH 7.4), as well as the appropriate agonists/vehicle and platelet membranes (8–15 μg of protein).

Saponin concentrations

All experiments with GTP[S] were performed in saponin-permeabilized platelets. Some experiments with thrombin were also performed in permeabilized platelets for comparison, whereas most of those with thrombin and all those with NaF were carried out in intact platelets. The saponin concentrations used (10–15 $\mu\text{g}/\text{ml}$) caused 70–80 % release of [^3H]adenine metabolites from pre-labelled platelets [17], without causing significant release of lactate dehydrogenase. In all experiments with GTP[S]/thrombin and saponin, GTP[S] or thrombin was added 20 s before saponin and the reactions were terminated at set timed intervals after addition of saponin. PMA was added 10 s or 5 min before GTP[S]/thrombin.

PMA concentrations

In all experiments with intact platelets, a fixed concentration of PMA (16 nM) was chosen, which from our earlier studies [6] induces maximum protein phosphorylation and causes inhibition of receptor-mediated processes. With saponin-permeabilized platelets a 10-fold lower concentration was used (1.6 nM). This did not induce significant secretion on its own, but nevertheless inhibited thrombin-induced responses. Concentrations of PMA > 5 nM induced 70–80 % 5HT secretion in permeabilized platelets, probably owing to a synergistic effect with Ca^{2+} entering the cells, and hence could not be used.

RESULTS

Effect of PMA treatment on 5HT secretion induced by thrombin, NaF and GTP[S]

We have shown in earlier studies [5,6] that the time of

Fig. 1. Effect of PMA on agonist-induced secretion

The effect of preincubating [^{14}C]5HT-labelled platelets with PMA for 10 s or 5 min ($\square$) before addition of vehicle, thrombin (0.05 unit/ml) plus saponin, GTP[S] (40 μM) plus saponin or NaF (30 mM), which were incubated for a further 3 min, is shown. The effect of thrombin, GTP[S] and NaF alone in the absence of PMA is also shown ($\boxplus$). Experiments with thrombin and GTP[S] were performed in saponin-permeabilized platelets, and the concentration of PMA used here was 1.6 nM. Experiments with NaF in intact platelets employed a higher concentration of PMA, i.e. 16 nM. The values shown represent means $\pm$ S.E.M. for 12 determinations from four different experiments: * $P < 0.01$ representing greater-than-additive effect.

exposure to protein kinase C activators determines their effect on agonist-induced 5HT secretion. Thus with pretreatments of < 2 min and > 2 min with PMA, thrombin-induced secretion was significantly potentiated and inhibited respectively. In the present study, two pre-treatment times (10 s and 5 min) were selected to study the effect of PMA (16 nM) on secretion induced by sub-maximal concentrations of thrombin (0.05 unit/ml), NaF (30 mM) and GTP[S] (40 μM) (Fig. 1). The appropriate concentration of these agents was chosen from initial dose/response studies, which revealed sub-maximal concentrations of the different agents to be 0.05–0.08 unit/ml for thrombin, 10–30 mM for NaF and 20–50 μM for GTP[S].

A 10 s pre-treatment with PMA was found to enhance significantly secretion induced by all three agents, and the effect of agent plus PMA in each case was greater than additive. With a 5 min pre-treatment, however, a less than additive effect, indicating an inhibition of secretion, was only apparent with thrombin. The effect of PMA plus NaF/GTP[S] after a 5 min or even 10 min pre-treatment remained greater than additive. In a previous study [5] we demonstrated that a clear decrease in thrombin-induced secretion is only seen when the extent of secretion is maximal or near-maximal.

Effect of a protein kinase C inhibitor on 5HT secretion induced by PMA, thrombin and NaF

Fig. 2 summarizes the effect of staurosporine, the recently described inhibitor of protein kinase C [18], on secretion induced by thrombin and NaF in comparison with its effects on PMA. At concentrations of 5–10 nM, staurosporine totally inhibited PMA-induced phosphorylation of 45 and 20 kDa proteins and PMA-induced 5HT secretion. At similar or 10-fold higher concentrations higher respectively, staurosporine was found to inhibit NaF- and thrombin-induced secretion.

Effect of PMA on phosphatidate formation induced by thrombin, NaF and GTP[S]

Initial studies on the time course of [^{32}P]PA formation

Fig. 2. Effect of staurosporine on [^{14}C]5HT secretion induced by PMA, thrombin and NaF

[^{14}C]5HT-labelled platelets were incubated with various concentrations of staurosporine for 1 min before addition of PMA (16 nM), thrombin (0.2 unit/ml) or NaF (30 mM). The dashed line represents the effect of the drug on PMA (16 nM)-induced 45 kDa-protein phosphorylation, which was determined in ^{32}P -labelled platelets as described in [6]. The values shown represent means $\pm$ S.E.M. of 6–12 determinations from three or four different experiments.

with the different agents revealed that PA formation reached a peak at approx. 60 s, 120 s and 180 s after addition of thrombin, GTP[S] and NaF respectively (Fig. 3). As shown by earlier workers [1,3], PMA alone induced no significant PA formation over that in resting platelets during a 15 min incubation period. At the sub-maximal concentrations of the activators used, NaF seemed the most potent at inducing PA formation. Preincubation of platelets with PMA for 5 min decreased PA formation induced by all three agents by approx. 50–70 % (Table 1).

Thrombin-stimulated GTPase activity in membranes prepared from resting and PMA-treated platelets

Addition of thrombin (0.2 unit/ml) to membranes

Fig. 3. Time courses of thrombin, GTP[S]- and NaF-induced $[^{32}\text{P}]$ PA formation

Reactions were terminated at various times after addition of thrombin (0.05 unit/ml), GTP[S] (40 μM) and NaF (30 mM) and processed for determination of PA formation as described in the Materials and methods section. Experiments with thrombin and NaF were performed in intact platelets. Results are expressed as a percentage of the mean value in resting platelets. The values shown represent means $\pm$ S.E.M. of 12 determinations from four different experiments (* $P < 0.05$ versus resting).

prepared from resting platelets resulted in a 56 % increase in GTPase activity over basal (Table 2). In membranes from PMA-treated platelets, this increase was significantly higher (89 % increase over basal; $P < 0.005$). The basal GTPase activity in control and membranes from PMA-treated platelets was not significantly different ($P > 0.05$) (Table 2). Table 2 also shows that, compared with controls, the increase in thrombin-stimulated GTPase activity above basal was significantly greater in membranes from diC_8 (60 μM)-treated, but not in membranes from 4β -phorbol (16 nM)-treated, platelets.

DISCUSSION

Several recent reports [1–6,19–21] describing inhibitory effects of protein kinase C activators on agonist-induced responses in different cell systems have suggested a role for the enzyme in the termination of cellular responses. In platelets, a number of agonist-induced responses, including phosphoinositide hydrolysis, elevations of intracellular Ca^{2+} , arachidonate release and dense-granule secretion are inhibited by tumour-promoting

Table 1. Effect of PMA on agonist-induced $[^{32}\text{P}]$ PA formation

Thrombin- and NaF-induced responses were studied in intact platelets for this set of experiments. In permeabilized platelets, $[^{32}\text{P}]$ PA formation induced by 0.05 unit of thrombin/ml was 345 ± 15 % of resting (100 %). Platelets were preincubated with vehicle or PMA (1.6 nM and 16 nM) for the permeabilized and intact platelet experiments respectively) for 5 min before addition of the agonist and reactions were terminated 60 s, 120 s and 180 s after addition of thrombin, GTP[S] plus saponin and NaF respectively. The number of determinations for each incubation was 9–12 from three separate experiments. The stimulated values were expressed as a percentage of the mean resting value; * $P < 0.01$ compared with agonist control.

	$[^{32}\text{P}]$ PA formation (% of resting)
Resting	100
Thrombin (0.05 unit/ml)	380 ± 45
PMA + thrombin	$264 \pm 29^*$
GTP[S] (40 μM)	215 ± 11
PMA + GTP[S]	$135 \pm 11^*$
NaF (30 mM)	518 ± 49
PMA + NaF	$254 \pm 31^*$

phorbol esters and membrane-permeant diacylglycerols [1–6]. The mechanism of inhibitory action is unclear, although our own work [6] has demonstrated a correlation between the extent of phosphorylation of a 45 kDa protein and the degree of inhibition, suggesting that phosphorylation of a key protein(s) may directly or indirectly affect agonist-induced platelet activation. Since G-protein-dependent events play a major role in platelet stimulus–response coupling in various cell types [22,23], including platelets [7–10], we examined the effect of PMA on G-protein function.

Our results suggest that the inhibition of receptor-mediated activation processes in platelets by PMA is directed at least at two sites relating to G-protein function, namely G-protein-mediated phospholipase C activation and receptor-stimulated GTPase activity. Firstly, the inhibition of PA formation with all three agents tested (thrombin, GTP[S] and NaF) suggests that a common step in G-protein–phospholipase C coupling

Table 2. Thrombin-stimulated GTPase activity in membranes from resting, PMA (16 nM)-, diC_8 (60 μM)- and 4β -phorbol (16 nM)-treated platelets

The values represent means $\pm$ S.E.M. of 12 determinations from four experiments using different batches of membranes for each membrane treatment. The values in parentheses represent the percentage increases over basal in each case. * $P < 0.005$ compared with respective basal activity. † $P < 0.005$ compared with activity in control membranes.

Additions	GTPase activity (pmol/min per mg of protein)			
	Control	PMA-treated	diC_8 -treated	4β -phorbol-treated
Vehicle (basal)	11.08 ± 1.76	11.92 ± 2.75	10.94 ± 1.75	11.04 ± 2.94
Thrombin (0.2 unit/ml)	$17.28 \pm 1.89^*$ (56)	$22.56 \pm 3.4^{*\dagger}$ (89)	$19.45 \pm 1.14^{*\dagger}$ (78)	$16.74 \pm 2.45^*$ (52)

distal to receptor-G-protein coupling is affected. What this step may be, and whether phospholipase C itself is directly affected by PMA treatment, remain to be investigated. Secondly, the increased thrombin-stimulated GTPase activity in membranes from PMA-treated platelets suggests that another site through which PMA exerts its effects is GTP hydrolysis on the α subunit of the G-protein(s) involved. Enhanced GTPase activity is an effect which would effectively shorten the half-life of the α -subunit-GTP complex and decrease its efficiency in trans-membrane signalling. As this effect was also seen in membranes from diC_8 -treated platelets, but not in those prepared from platelets pre-treated with 4β -phorbol (the inactive parent compound that does not activate protein kinase C), a role for the enzyme in the potentiation of GTPase activity seems to be implicated. This effect of protein kinase C activators on GTPase activity, as well as the inhibitory effect on G-protein-phospholipase coupling discussed above (these are separate and distinct effects, as GTP hydrolysis is not applicable to the actions of GTP[S] and NaF), could well explain the inhibitory effects of PMA on receptor-stimulated phospholipase C activation and mobilization of intracellular Ca^{2+} in platelets [1-6].

Regarding 5HT secretion, our results suggest that different mechanisms may be involved in receptor-mediated versus G-protein-activator-mediated secretion, in that the former, but not the latter, is inhibited by PMA despite inhibition of PA formation with all three agents. One possibility is that GTP[S] and NaF can activate a G-protein distinct from that activated by thrombin-receptor occupancy, and perhaps one that is directly linked with granule exocytosis, and by-passes the effects of protein kinase C, as suggested previously in neutrophils [23]. However, this seems unlikely, because (1) staurosporine (which, apart from other kinases, also inhibits protein kinase C [18,24]) inhibited secretion induced by thrombin, NaF and GTP[S], and (2) GTP[S] does not induce 'Ca²⁺-independent' PA formation or secretion in platelets, as in neutrophils, where secretion in the absence of Ca²⁺ has been one of the main lines of evidence for the involvement of a distinct G-protein (G_e) in exocytosis.

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