Receptor- and phorbol-ester-mediated redistribution of protein kinase C in human platelets

Evidence that aggregation promotes degradation of protein kinase C

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Translocation of Ca^{2+} /phospholipid-dependent protein kinase (PKC) activity from cytosolic to membrane fractions was assessed in washed human platelet suspensions. Phorbol myristate acetate (PMA) induced a rapid loss of PKC activity from the cytosolic compartment in stirred platelets, which was not accompanied by measurable increases in membrane-associated activity, but was paralleled by a decrease in total cellular enzyme activity (cytosol plus membrane). When platelet aggregation was prevented by not stirring, (i) cytosolic activity was decreased by PMA, (ii) significant and maintained (1–15 min with PMA) increases in membrane-bound PKC were detected, and (iii) the decline in total enzyme activity was markedly slower. In stirred platelets, total and specific inhibition of PMA-induced aggregation by a fibrinogen-derived peptide (RGDS, i.e. Arg-Gly-Asp-Ser) promoted maximal increases in membrane-associated PKC in the presence of PMA and completely prevented the loss in cellular activity. Thrombin and collagen both induced a decrease in cytosolic PKC and a loss of total activity, but a significant rise in membrane activity was seen only with collagen; ADP had no detectable effect on enzyme distribution. These results demonstrate an agonist-induced redistribution of PKC and indicate that platelet aggregation may play an important role in the proteolysis, and hence persistence, of membrane-associated PKC. This observation has implications for the potency and duration of PKC-mediated responses induced by agonists and exogenous PKC activators.

INTRODUCTION

Receptor-mediated activation of phospholipase C with the subsequent hydrolysis of membrane phospholipids is known to be one of the initial events leading to platelet aggregation and dense-granule secretion [1,2]. Phospholipid hydrolysis produces at least two classes of intracellular messenger, these being inositol phosphates, which are important in regulating intracellular Ca^{2+} in platelets [3,4], and diacylglycerols (DAG), which can activate the family of Ca^{2+} /phospholipid-dependent protein kinases collectively known as protein kinase C (PKC) [5,6]. Tumour-promoting phorbol esters such as phorbol 12-myristate 13-acetate (PMA) substitute for DAG as activators of PKC [7], and accumulating evidence suggests that phorbol ester, in association with phospholipids, promotes the translocation of PKC to the membrane, and that rapid PKC degradation, or 'down-regulation', subsequently occurs via the action of a membrane-associated Ca^{2+} -activated protease [5]. At present, the importance of enzyme translocation and its relevance to receptor-mediated activation processes is unclear. Although membrane association of PKC has been observed in some cell types in response to agonists known to activate phospholipase C [8–10], limited [11] or undetectable changes [12] in agonist-induced PKC distribution have been noted in other systems. In platelets, PMA stimulates rapid association of enzyme activity with the particulate fraction [13,14], although there have

been no reports of receptor-mediated redistribution of cellular PKC activity.

In the present study we have assessed the distribution of PKC activity in cytosolic and membrane fractions prepared from thrombin-, collagen- and ADP-stimulated platelets. We have also addressed the possible role of platelet aggregation in PKC degradation by measuring the time course of PMA-induced PKC translocation under conditions promoting aggregation (stirred platelets) and those preventing the aggregatory response (unstirred platelets).

MATERIALS AND METHODS

Platelet preparation and measurement of platelet aggregation

Washed human platelets were prepared from citrated human blood as described previously [15]. Briefly, platelets were washed in buffer (pH 6.5) composed of 36 mM-citric acid, 5 mM-KCl, 0.5 mM- CaCl_2 , 103 mM-NaCl, 0.09% D-glucose, 30 nM-prostacyclin and 0.05 unit of hirudin (Bio-Pharm, Swansea, Wales, U.K.)/ml. After centrifugation (1500 g, 10 min) the platelet pellet was resuspended (pH 7.4) in a buffer containing 10 mM-Hepes, 5 mM-KCl, 1 mM- CaCl_2 , 103 mM-NaCl, 1 mM- MgSO_4 , 0.09% D-glucose and 0.05 unit of hirudin/ml. Platelet aggregation (320 μl incubations) was measured as an increase in light transmission by using a Payton dual-channel aggregometer linked to a chart recorder.

Abbreviations used: DAG, diacylglycerol; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

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Isolation and partial purification of membrane-bound and cytosolic PKC

Indomethacin-treated ($10 \mu\text{M}$) platelet suspensions [$(0.8\text{--}1.0) \times 10^9/320 \mu\text{l}$ of Hepes buffer] were incubated (37°C , $1\text{--}30 \text{ min}$) with or without stirring in the presence of PMA (160 nM), thrombin (2 units/ml ; Parke Davis, Morris Plains, NJ, U.S.A.), collagen ($40 \mu\text{g/ml}$; Hormon-Chemie, Munich, Germany), ADP ($100 \mu\text{M}$) or fibrinogen peptide [1 mM ; Nova Biochem. (U.K.) Ltd., Nottingham, U.K.] plus PMA. After incubation, platelets were collected by centrifugation ($1500 g$, 5 min ; 4°C), the supernatant was discarded and the pellet was resuspended in $100 \mu\text{l}$ of 20 mM-Tris/HCl ($\text{pH } 7.4$) containing 2 mM-EDTA , 0.5 mM-EGTA , $50 \mu\text{g}$ of leupeptin/ml, $1 \text{ mM-phenylmethanesulphonyl fluoride}$ and 1% β -mercaptoethanol (Buffer A). Platelets were disrupted by ultrasonication ($4\text{--}6 \times 10\text{s}$, setting 5; Ultrasonics Ltd.) and membranes were separated from cytoplasm by centrifugation at $35000 g$ for 60 min (2°C). Pellets were dispersed by brief sonication ($4 \times 5\text{s}$) in $200 \mu\text{l}$ of Buffer A and solubilized (30 min , 4°C) in the presence of 1% Nonidet P-40. Supernatants and solubilized membranes were partially purified by using DEAE-cellulose (DE52, Whatman). Columns ($200 \mu\text{l}$) were equilibrated with 1 ml of Buffer A, the fractions were loaded and the columns washed with a further 3 ml of Buffer A. Enzyme was eluted with $320 \mu\text{l}$ of Buffer A containing 0.12 M-NaCl , and the eluates were assayed immediately for PKC activity.

PKC assay

PKC activity in column eluates was measured in triplicate essentially as described by Persaud *et al.* [16]. The reaction mixture ($45 \mu\text{l}$) contained 20 mM-Tris/HCl ,

1.33 mM-CaCl_2 , 11.1 mM-MgCl_2 , $96 \mu\text{g}$ of phosphatidylserine/ml, $6.4 \mu\text{g}$ of diolein/ μl , 1.1 mg of histone IIIs/ml, $0.1 \text{ mM-}[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Amersham International; final sp. radioactivity 0.2 Ci/mmol) and $10 \mu\text{l}$ of column eluate ($2\text{--}10 \mu\text{g}$ of protein). The reaction was initiated by addition of label, carried out for 5 min (30°C) and terminated with 1 ml of 10% (v/v) trichloroacetic acid containing 1 mM-ATP and $2.5 \text{ mM-Na}_4\text{P}_2\text{O}_7$. After addition of $500 \mu\text{g}$ of bovine serum albumin (Cohn Fraction V) as a carrier, the precipitate was collected by filtration on to Whatman GF/C glass-fibre filters by using a Millipore filtration apparatus. Filters were washed (20 ml) with 5% trichloroacetic acid containing $10 \text{ mM-Na}_4\text{P}_2\text{O}_7$, and ^{32}P retention was measured by liquid-scintillation counting (LKB 1219 Rackbeta). PKC activity was calculated from the difference in ^{32}P incorporation in the presence and absence of added phospholipids. Protein was measured by the Bio-Rad assay (Bio-Rad, Munich, Germany). PKC activity was expressed as fmol of ^{32}P incorporated/ 5 min per μg of platelet protein, and data are shown as means $\pm$ S.E.M. Differences between means were assessed by Student's unpaired *t* test and considered significant when $P < 0.05$.

RESULTS

In unstimulated platelets most of the PKC activity was associated with the cytosolic fraction (stirred, $78 \pm 3\%$; unstirred, $84 \pm 3\%$ of total activity; $n = 6$). Exposure of stirred platelets to PMA (160 nM) caused a rapid disappearance of cytosolic activity, which was not accompanied by increases in membrane-associated activity (Table 1). Moreover, when compared with untreated platelets, the total cellular enzyme activity (cytosol plus membrane) had declined by approx. 50% after a 1.5 min

Table 1. Time-dependence of PMA-induced PKC translocation in intact platelets

Platelet suspensions [$(0.8\text{--}1.0) \times 10^9$] were incubated at 37°C with or without stirring in the absence (vehicle alone) or presence of PMA (160 nM) for the times indicated. Aggregation was only seen in stirred platelets. Vehicle was incubated for 6 and 30 min under stirred and unstirred conditions respectively. PKC activity in cytosolic and membrane fractions is expressed as mean $\pm$ S.E.M. of three (stirred) and four (unstirred) experiments with triplicate determinations per time point. The PKC (cytosol plus membrane) activity recovered in PMA-treated platelets is expressed as a percentage of the mean total PKC activity in resting platelets, which was taken as 100% . *Significant decrease versus vehicle control ($P < 0.01$); **significant increase versus vehicle control membrane ($P < 0.01$).

	PKC activity (fmol of ³² P incorporated/5 min per μg of protein)			
		PMA (160 nM) treatment for:		
Stirred platelets	Vehicle	1.5 min	3 min	6 min
Cytosol	24.8±0.8	11.9±0.7*	8.3±0.6*	7.4±0.5*
Membrane	6.1±1.1	4.1±1.4	5.2±0.3	4.3±0.7
Total PKC activity recovered (%)	100	52±6*	39±3*	38±4*

	PKC activity (fmol of ³² P incorporated/5 min per μg of protein)				
		PMA (160 nM) treatment for:			
Unstirred platelets	Vehicle	1 min	5 min	15 min	30 min
Cytosol	27.8±1.6	10.8±0.5*	11.6±0.7*	10.6±2.2*	5.3±0.5*
Membrane	4.1±0.3	10.0±0.3**	11.7±0.6**	12.2±2.1**	3.8±0.6
Total PKC activity recovered (%)	100	76±3*	82±6	66±7*	27±2*

Table 2. Agonist-induced changes in PKC distribution, PKC recovery and aggregation in stirred platelets

Platelets were challenged with thrombin (2 units/ml), collagen (40 $\mu\text{g/ml}$) or ADP (100 μM) for 1 and/or 5 min, and the PKC activity in cytosol and membrane fractions was compared with that in vehicle-treated (resting) incubations (5 min, stirred). In a separate set of experiments RGDS (1 mM) was incubated for 1 min before PMA (160 nM) addition for 1 or 5 min and the PKC activity in membrane and cytosol was measured. The extent of aggregation was assessed by measuring the percentage increase in light transmission after agonist addition. Total PKC activity recovered in the various incubations was calculated as described in the legend to Table 1. Results are given as means $\pm$ S.E.M. of 9 determinations in three separate experiments: * $P < 0.05$ versus resting cytosol; ** $P < 0.05$ versus resting membrane.

		PKC activity (fmol/5 min per μg of protein)		Total PKC activity recovered (% of resting)	Increase in light transmission (%)
		Cytosol	Membrane		
Resting		44.8 $\pm$ 0.9	11.0 $\pm$ 0.3	100	0
Thrombin	1 min	41.8 $\pm$ 0.7	13.0 $\pm$ 0.3	103 $\pm$ 2	27 $\pm$ 3
	5 min	29.5 $\pm$ 1.1*	10.7 $\pm$ 0.3	74 $\pm$ 5*	86 $\pm$ 3
Collagen	1 min	27.5 $\pm$ 0.9*	18.7 $\pm$ 0.9**	79 $\pm$ 4*	6 $\pm$ 1
	5 min	24.8 $\pm$ 1.7*	16.3 $\pm$ 0.6**	72 $\pm$ 3*	27 $\pm$ 3
ADP	5 min	43.7 $\pm$ 1.2	10.6 $\pm$ 0.7	98 $\pm$ 6	35 $\pm$ 4
Resting		57.2 $\pm$ 3.9	20.0 $\pm$ 1.2	100	0
PMA	1 min	20.9 $\pm$ 3.6*	19.5 $\pm$ 6.8	53 $\pm$ 12*	18 $\pm$ 4
	5 min	—	—	—	69 $\pm$ 6
RGDS + PMA	1 min	23.8 $\pm$ 6.9*	51.0 $\pm$ 8.1**	99 $\pm$ 3	0
	5 min	—	—	—	0

exposure to PMA, and was further decreased with extended incubation times (Table 1).

In parallel experiments, in which aggregation was prevented by not stirring the suspensions, similar decreases in cytosolic PKC were observed after a 1 min incubation with PMA (Table 1). Under these conditions, however, significant increases in membrane-bound PKC activity were detected after 1 min and persisted for up to 15 min in the presence of PMA. This effect was reversible, since membrane-associated PKC had returned to unstimulated values after 30 min treatment with PMA, whereas cytosolic activity was maximally decreased (to 21 $\pm$ 2 % of resting cytosolic activity; $P < 0.001$). Total PKC activity in unstirred platelets decreased with time of exposure to PMA, although the decline was much slower than that in aggregating platelets. In other experiments a 5 min incubation with the DAG analogue 1,2-dioctanoylglycerol ('DiC₈'; 300 μM) induced a similar translocation of PKC in unstirred platelets (cytosol 57 $\pm$ 2 % of resting; membrane 220 $\pm$ 8 % of resting; $n = 3$, $P < 0.0001$).

When stirred platelets were pre-treated (1 min) with a maximally effective concentration (1 mM) of RGDS (Arg-Gly-Asp-Ser), a fibrinogen-derived peptide which specifically inhibits the platelet aggregatory response [17,18], PMA (1 min)-induced aggregation was abolished, the decrease in cytosolic PKC activity was accompanied by increases in membrane-bound PKC, and the loss of total cellular PKC was completely prevented (Table 2).

The extent of aggregation induced by maximal doses of the physiological platelet activators thrombin (2 units/ml) and collagen (40 $\mu\text{g/ml}$) is included in Table 2. Thrombin caused a maximal aggregatory response after 5 min, whereas collagen promoted only about 25 % aggregation during the same time period. At the same concentrations, thrombin and collagen caused changes both in PKC distribution and in total PKC activity, albeit with different time courses (Table 2). Thus, after 1 min thrombin produced no changes in either total PKC activity or its distribution, whereas collagen decreased

cytosolic activity to 49 $\pm$ 9 % of resting values ($P < 0.0001$), with a concomitant increase in membrane-associated activity (178 $\pm$ 8 % of resting membrane; $P < 0.0001$). A loss of cytosolic PKC was also observed after a 5 min exposure to either agonist (collagen 39 $\pm$ 11 %; thrombin 60 $\pm$ 5 % of resting), although a significant increase in membrane-bound PKC was detected only in collagen-treated platelets (155 $\pm$ 3 % of control; $P < 0.05$). Incubation of stirred platelets with ADP (100 μM) had no measurable effect on either the distribution of PKC or total cellular PKC activity (Table 2).

DISCUSSION

Several lines of evidence suggest that PKC plays an important role in agonist-induced platelet activation. Phorbol esters or membrane-permeant DAG analogues can stimulate dense-granule secretion [19–21] and potentiate or inhibit aggregatory and secretory responses to physiological agonists [15, 22]. Furthermore, these effects can be antagonized by certain compounds (e.g. staurosporine), thought to act primarily as inhibitors of PKC [23].

Studies in a number of tissues, including platelets, suggest that PKC redistributes within cells during activation by exogenous phorbol esters and that rapid degradation by a Ca^{2+} -activated neutral protease follows its association with the membrane [24]. Results from the present study confirm previous observations [13,14] that PMA causes a relocation of platelet PKC activity, seen as a time-dependent decrease in cytosolic activity with, under certain circumstances, parallel increases in membrane activity. The PMA-induced decrease in total cellular PKC from stirred platelets perhaps reflects rapid degradation of membrane-bound enzyme, thus accounting for our inability to detect increases in membrane activity under these conditions at times when cytosolic activity was markedly decreased. The markedly slower loss of cellular PKC, coupled with the rapid and pro-

longed increases in membrane-associated PKC from unstirred suspensions, suggest that the degradation of PKC after translocation occurs more rapidly in aggregating platelets. In support of this hypothesis, total inhibition of aggregation by RGDS led to maximum translocation of PKC to the particulate fraction and complete prevention of the PMA-induced loss of enzyme activity. Extrapolating from these findings, it would appear that previous studies addressing the effect of PMA on the redistribution of platelet PKC activity [13,14] were performed in unstirred platelets, since neither group reported a loss of activity and detected significant increases in membrane-associated PKC. Although a link between calpain-mediated proteolysis of platelet contractile proteins and the aggregatory response has previously been reported [25], our results constitute the first demonstration of aggregation-dependent PKC proteolysis.

Although phorbol esters are useful for studying processes involved in the activation of PKC, they offer little information on the physiological relevance of its activation. Since recent reports [11,12] in some tissues suggest that PKC-mediated responses initiated by receptor-operating stimuli may occur without detectable changes in PKC distribution, we considered it essential to determine the effect of platelet agonists on cellular PKC localization. It is well documented that thrombin and collagen stimulate platelet aggregation and secretion in stirred platelets and lead to phosphatidate formation [26–28], a phosphorylated derivative of DAG, suggesting that they may act, at least in part, through the activation of PKC. In this study we have directly demonstrated that both agonists promote changes in PKC distribution in stirred platelets, implying that PKC translocation plays an important role in mediating the subsequent physiological responses. Although both agonists were used at maximal concentrations, we were able to demonstrate increased membrane-bound PKC activity only in collagen-stimulated platelets, perhaps suggesting differential PKC degradation after activation by these agonists. The maximal aggregatory response induced by thrombin compared with the partial collagen-induced aggregation may account for the apparently faster rate of PKC degradation after thrombin stimulation, and hence no measurable rise in membrane-associated activity. It is of interest that PMA (5 min) induced an aggregation which was 80 % of that caused by a 5 min exposure to thrombin; the high platelet count (10-fold higher than that usually employed for aggregation measurements) may be a factor contributing to the unusually potent effect of PMA. ADP had no effect on PKC distribution, providing further indirect evidence that this agonist does not activate phospholipase in indomethacin-treated platelets [29].

In conclusion, we have shown that platelet aggregation may play an important role in the persistence of membrane-associated PKC, and hence in its down-regulation. The aggregation-induced loss of membrane, and hence total, activity could represent an intrinsic 'switching-off' mechanism allowing termination of the effects of agonist- or phorbol-ester-mediated PKC activation. The relationship between platelet PKC proteolysis and the varied effects of PKC activators [16] remains to be determined.

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