Biochem. J. (1990) 271, 693–700 (Printed in Great Britain)

Phorbol ester treatment of intact rabbit platelets greatly enhances both the basal and guanosine 5′-[γ-thio]triphosphate-stimulated phospholipase D activities of isolated platelet membranes

Physiological activation of phospholipase D may be secondary to activation of phospholipase C

John VAN DER MEULEN* and Richard J. HASLAM†‡
Departments of *Pediatrics and †Pathology, McMaster University, Hamilton, Ontario, Canada L8N 3Z5

Rabbit platelets were labelled with [^{3}H]glycerol and incubated with or without phorbol 12-myristate 13-acetate (PMA). Membranes were then isolated and assayed for phospholipase D (PLD) activity by monitoring [^{3}H]phosphatidylethanol formation in the presence of 300 mM-ethanol. At a [Ca^{2+}_{free}] of 1 μM, PLD activity was detected in control membranes, but was 5.4±0.8-fold (mean±S.E.M.) greater in membranes from PMA-treated platelets. Under the same conditions, 10 μM-guanosine 5′-[γ-thio]triphosphate (GTP[S]) stimulated PLD by 18±3-fold in control membranes, whereas PMA treatment and GTP[S] interacted synergistically to increase PLD activity by 62±12-fold. GTP[S]-stimulated PLD activity was observed in the absence of Ca^{2+}, but was increased by 1 μM-Ca^{2+} (3.5±0.2-fold and 1.8±0.1-fold in membranes from control and PMA-treated platelets respectively). GTP exerted effects almost as great as those of GTP[S], but 20–30-fold higher concentrations were required. Guanosine 5′-[β-thio]diphosphate inhibited the effects of GTP[S] or GTP, suggesting a role for a GTP-binding protein in activation of PLD. Thrombin (2 units/ml) stimulated the PLD activity of platelet membranes only very weakly and in a GTP-independent manner. The actions of PMA and analogues on PLD activity correlated with their ability to stimulate protein kinase C in intact platelets. Staurosporine, a potent protein kinase inhibitor, had both inhibitory and, at higher concentrations, stimulatory effects on the activation of PLD by PMA. The results suggest that PMA not only stimulates PLD via activation of protein kinase C but can also activate the enzyme by a phosphorylation-independent mechanism in the presence of staurosporine. However, under physiological conditions, full activation of platelet PLD may require the interplay of protein kinase C, increased Ca^{2+} and a GTP-binding protein, and may occur as a secondary effect of the activation of phospholipase C.

INTRODUCTION

In contrast with the established roles for phospholipases C and A_2 in signal transduction, the function of PLD has been less clear. This was initially due to difficulties in detecting the activity of this membrane-associated enzyme in animal tissues [1]. However, improved methods including exploitation of the ability of PLD to catalyse the formation of PEt, an abnormal phospholipid generated by transphosphatidylation in the presence of a phospholipid substrate and ethanol [2–5], have demonstrated the presence of the enzyme in many cell types and have permitted studies on its regulation. As a result, it is now clear that PLD plays some role in signal transduction [6]. This view is based on the following observations. First, agonist-dependent activation of PLD occurs in intact cells [3,4,7–10], including thrombin-stimulated platelets [11]. Second, PLD activity can be stimulated by GTP[S] in isolated membrane systems and sonicated cells, implicating GTP-binding proteins in the activation of this enzyme [3,5,8,9]. Third, phorbol esters cause stimulation of PLD in intact cells, suggesting a role for protein kinase C in the control of PLD activity [5,10,12,13]. However, the relationship between the effects of guanine nucleotides and protein kinase C has not been clearly defined. In the present study, by using isolated rabbit platelet membranes containing labelled phospholipids and the formation of [^{3}H]PEt to monitor PLD activation, we have found that phorbol ester treatment of intact platelets greatly potentiates the ability of GTP[S] to activate PLD. Since thrombin had little effect on the PLD activity of isolated platelet membranes, the results suggest that agonists may activate PLD indirectly through stimulation of protein kinase C and that the action of the latter on PLD is facilitated by a GTP-binding protein.

EXPERIMENTAL

Materials

[1,2,3-^{3}H]Glycerol (200 mCi/mmol) and [^{32}P]P_i were from Du Pont Canada (Mississauga, Ont., Canada). PMA, PDD, α-PDD, diC_8, GTP and protein standard solution were from Sigma (St. Louis, MO, U.S.A.), and GTP[S], GDP[S] and staurosporine were from Boehringer Mannheim Canada (Laval, Que., Canada). α-PMA was supplied by LC Services Corp. (Woburn, MA, U.S.A.), and silica-gel t.l.c. plates (Si250) were from J. T. Baker Chemical Co. (Phillipsburg, NJ, U.S.A.). PA standard was supplied by Serdary Research Laboratories (London, Ont., Canada), and PEt standard was prepared from egg lecithin as described in [2]. ACS scintillant was from Amersham Canada (Oakville, Ont., Canada). Apyrase was prepared as described in [14]. Human α-thrombin (2700 units/mg) was kindly provided by Dr. J. W. Fenton II of New York State Department of Health (Albany, NY, U.S.A.).

Abbreviations used: PLD, phospholipase D; PEt, phosphatidylethanol; GTP[S], guanosine 5′-[γ-thio]triphosphate; GDP[S], guanosine 5′-[β-thio]diphosphate; PMA, phorbol 12-myristate 13-acetate; α-PMA, 4α-phorbol 12-myristate 13-acetate; PDD, phorbol 12,13-didecanoate; 4α-PDD, 4α-phorbol 12,13-didecanoate; diC_8, *sn*-1,2-dioctanoylglycerol; PA, phosphatidic acid; pCa, $-\log[Ca^{2+}_{free}]$.

‡ To whom correspondence should be addressed.

Isolation and labelling of platelets

Blood was collected from male New Zealand White rabbits into 0.15 vol. of ACD anticoagulant [15] and centrifuged at 160 $g_{av.}$ for 15 min at room temperature. The platelet-rich plasma was removed and centrifuged at 2240 $g_{av.}$ for 15 min, after which the platelet pellet was resuspended in plasma at 5×10^9 platelets/ml. [^{3}H]Glycerol was added to give a final concentration of 50 μCi/ml, and the platelet suspension was incubated for 2 h at 37 °C. The platelets were then isolated as above and washed at room temperature, essentially as described by Ardlie *et al.* [16], by resuspension in Ca^{2+}-free Tyrode's solution containing BSA (3.5 mg/ml), apyrase (30 μg/ml), 0.26 mM-EGTA and 10 mM-Pipes (adjusted to pH 6.5 with NaOH). After further centrifugation at 1550 $g_{av.}$ for 10 min, the platelets were finally resuspended at 5×10^9/ml in the same medium without BSA or EGTA.

In some experiments, the platelets were also labelled with [^{32}P]P_i to permit measurement of changes in protein phosphorylation. In this case, platelets were resuspended in phosphate-free medium after centrifugation from plasma, and after further centrifugation were resuspended at 5×10^9 platelets/ml in phosphate-free and Ca^{2+}-free Tyrode's solution containing BSA (3.5 mg/ml), apyrase (30 μg/ml) and 10 mM-Pipes, pH 6.5, but no EGTA. They were then incubated for 30 min at 37 °C with 0.1 mCi of [^{32}P]P_i/ml. Finally, the platelets were centrifuged again and resuspended at 5×10^9 platelets/ml in medium containing phosphate but without either BSA or EGTA.

Phorbol ester treatment

Samples of platelet suspension were warmed to 37 °C, and PMA was added at final concentrations of 0.01–1 μM. Incubations with PMA were usually for 2 min and were terminated by the addition of 5 mM-EDTA, followed by rapid freezing. When the effects of staurosporine were studied, platelets were incubated with the compound for 5 min before addition of PMA. Dimethyl sulphoxide was used as a vehicle for both staurosporine and PMA, and was added at the same final concentration (0.2%, v/v) to all samples, including controls. In experiments with ^{32}P-labelled platelets, 40 μl samples of suspension were mixed with 0.5 ml of 10% (w/v) trichloroacetic acid immediately before addition of EDTA and were used for determination of protein phosphorylation.

Membrane isolation

PMA-treated and control platelets were subjected to two cycles of freezing (solid CO_2/methanol) and thawing (37 °C water bath), and the resulting particulate fraction was harvested by centrifugation at 30000 $g_{av.}$ for 40 min at 4 °C. The pellet was resuspended in a hyperosmotic buffer containing 1 M-KCl, 5 mM-EDTA and 25 mM-Hepes (adjusted to pH 7.4 with KOH) at a concentration equivalent to 25×10^9 platelets/7.5 ml of buffer, by using a Dounce homogenizer with an A pestle. After isolation as above, the membranes were then homogenized in a hypo-osmotic buffer (the same but without KCl). Membranes were again isolated and finally resuspended at a protein concentration of 0.5–1 mg/ml in a medium (buffer A) containing 100 mM-KCl, 2.5 mM-EGTA and 25 mM-Hepes (adjusted to pH 7.4 with KOH). Protein was determined by the Lowry [17] method, with Sigma protein standard.

Incubations

Incubation mixtures (1 ml) contained 0.4 ml of membrane suspension in buffer A (0.2–0.4 mg of protein, unless otherwise stated), 0.4 ml of buffer A containing sufficient $MgCl_2$, $CaCl_2$ and KOH to give a final [Mg^{2+}_{free}] of 0.5 mM, the required pCa and a final pH of 7.4, and 0.2 ml of buffer A containing other additions. The amounts of $MgCl_2$ and $CaCl_2$ required were calculated as described in [18]. In samples containing 300 mM-ethanol, 18 μl of 95% (v/v) ethanol was included. Incubations were at 37 °C, usually for 10 min, and were terminated by addition of 3.75 ml of chloroform/methanol (1:2, v/v).

Extraction and measurement of [^{3}H]phospholipids

Phospholipids were extracted as described by Bligh & Dyer [19]. Solvent was removed by centrifugation under vacuum, and the lipid was dissolved in 50 μl of chloroform for analysis by t.l.c. Samples and standards were applied to Si250 plates under N_2. The chromatographic solvent contained ethyl acetate/2,2,4-trimethylpentane/acetic acid (9:5:2, by vol.), as described in [20]. After development, plates were allowed to dry for 30 min and then stained with Coomassie Brilliant Blue R [0.03% (w/v) in 20% methanol containing 100 mM-NaCl] for 10 min, followed by destaining (20% methanol/100 mM-NaCl) for 5 min [21]. PEt and PA were made visible directly (if present in sufficient amounts) or localized in relation to PA and PEt standards. In the later experiments, PA and PEt were mixed with all experimental samples before t.l.c. The plates were sprayed with a fine water mist, and appropriate areas were scraped into vials containing 0.50 ml of methanol and 50 μl of acetic acid. ACS scintillant (8 ml) was added for counting of ^{3}H radioactivity (efficiency about 20%). Results were corrected for background radioactivity and chemical quenching; there was no detectable colour quenching by the amounts of stain present. Negligible amounts of ^{32}P were found in PEt from the membranes of platelets that were labelled with both [^{3}H]glycerol and [^{32}P]P_i. In individual experiments, incubations were performed in triplicate. For [^{3}H]PEt, blank values for the ^{3}H found before addition of ethanol or after incubation in the absence of ethanol were subtracted (except in Table 1). All results were standardized with respect to membrane protein. Values given for [^{3}H]PA and [^{3}H]PEt in each experiment are means ± S.E.M. or means ± S.E. of the difference, as appropriate. Error bars are shown in the Figures only when they lie outside the symbols. The effects described were observed in at least three separate experiments (unless otherwise indicated); statistics on variation between experiments are given in the text. When required, the significance of changes was evaluated by two-sided paired or unpaired *t* tests, as appropriate. Concentrations of guanine nucleotides causing half-maximal stimulation of [^{3}H]PEt formation were calculated by using a computer programme (HYPMIC) giving a least-squares fit to a hyperbola [22].

Measurements of protein phosphorylation

Trichloroacetic acid-precipitated platelet protein was dissolved in electrophoresis sample buffer, and protein equivalent to 10^8 platelets was analysed by SDS/PAGE, as described previously [23]. After autoradiography, the region of the gel containing ^{32}P-labelled pleckstrin (the major protein kinase C substrate in platelets; apparent M_r 47000) was cut out and counted for ^{32}P, which was measured as Čerenkov radiation in 0.01% (w/v) 4-methylumbelliferone. The labelling of protein in membranes prepared from ^{32}P-labelled platelets was studied by the same methods.

RESULTS AND DISCUSSION

Synergistic effects of PMA treatment and GTP[S]

In preliminary experiments, staining of t.l.c. plates with Coomassie Blue R [21] was used to demonstrate the formation of PA and PEt in platelet membranes incubated at a pCa of 6 with various other additions (Fig. 1). GTP[S] (10 μM) caused an

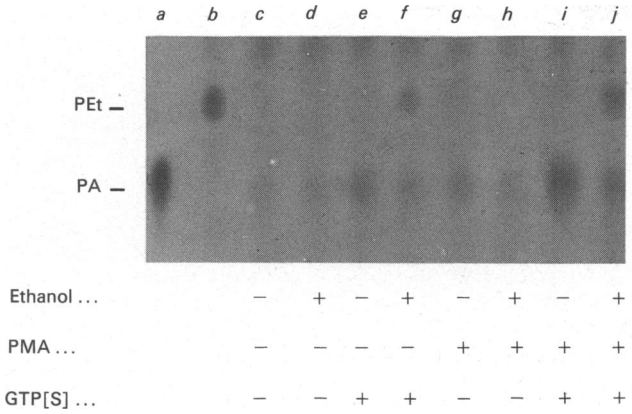

Fig. 1. Effects of treatment of platelets with PMA and incubation of membranes isolated therefrom with GTP[S] and/or ethanol on their content of PA and PEt, as detected by staining with Coomassie Brilliant Blue R after t.l.c.

Membranes isolated from control and PMA-treated platelets, as described in the Experimental section, were incubated for 10 min at 37 °C at pCa 6 with the additions indicated. Final concentrations of GTP[S] and ethanol were 10 μM and 300 mM respectively. Each sample contained 1 mg of membrane protein. Lipids were extracted, analysed by t.l.c. and made visible by staining with Coomassie Brilliant Blue R. Lanes *a* and *b* contain about 5 μg of standard PA ($R_F = 0.36$) and PEt ($R_F = 0.48$) respectively. Lanes *c–j* represent lipid extracts from the indicated incubation mixtures.

increase in PA in control membranes (lane *e*) and even more markedly in membranes derived from PMA-treated platelets (PMA-membranes; lane *i*). When ethanol (300 mM) was included in these incubation mixtures, decreases in this accumulation of PA were combined with the appearance of PEt (lanes *f* and *j*). Studies in several other cell types have indicated that the formation of PEt in the presence of phospholipid substrates and ethanol was attributable to PLD activity [2–5]. The formation of PA in the absence of added ATP was also consistent with the activation of PLD, as was the decrease in PA accumulation on addition of ethanol. These results indicated that GTP[S] may stimulate a PLD activity in platelet membranes, as previously reported in isolated rat hepatocyte membranes [3,8], and that prior PMA treatment of the platelets may enhance this effect.

Detection of the above increases in PA and PEt by staining showed that substantial changes in the masses of these phospholipids occurred. However, to study these phenomena in greater detail, we measured changes in [^{3}H]PA and [^{3}H]PEt in membranes derived from platelets labelled with [^{3}H]glycerol, which was possible with much smaller membrane samples (e.g. Table 1). The amounts of [^{3}H]PA present before incubation of control and PMA-membranes did not differ significantly and decreased to similar extents (22±2% and 24±3% respectively; means± S.E.M.., four expts.) during 10 min incubations at 37 °C without additions other than Mg^{2+} and Ca^{2+} ions (pCa 6). Incubation with 10 μM-GTP[S] prevented this decrease (control membranes) or caused a net increase in [^{3}H]PA (PMA-membranes) (Table 1). When comparison was made with the same membranes incubated for 10 min without GTP[S], this GTP analogue increased [^{3}H]PA by 21±2% in control membranes and by 67±8% in PMA-membranes (means±S.E.M., four expts.). Although addition of 300 mM-ethanol did not affect the decreases in [^{3}H]PA observed in the absence of GTP[S], it blocked (control membranes) or markedly diminished (PMA-membranes) the above increases in [^{3}H]PA caused by GTP[S] (Table 1).

[^{3}H]PEt formation during incubation of control membranes with 300 mM-ethanol alone was very low, but could be distinguished from blank values yielded by membranes extracted before incubation or after incubation without ethanol ($P < 0.05$, five expts.). However, with PMA-membranes, [^{3}H]PEt formation was much greater (Table 1), amounting to 5.4±0.8-fold that in paired control membranes (mean±S.E.M., five expts.). This result is consistent with the stimulation of PLD activity by phorbol esters reported in other systems [5,10,12,13,24]. Incubation of control platelet membranes with GTP[S] and ethanol caused an 18.2±3.1-fold stimulation of [^{3}H]PEt formation over that seen in the same membranes incubated with ethanol alone (mean±S.E.M., five expts.). Similar effects of GTP[S] on PLD activity have been observed in rat hepatocyte membranes [3,8], HL-60 cell lysates [5], permeabilized endothelial cells [9] and synaptosomes exposed to Triton X-100 [25]. A stimulatory effect of PMA was also described in HL-60 cell lysates, and the authors [5] suggested that PMA and GTP[S] increased PLD activity by

Table 1. Effects of treatment of [^{3}H]glycerol-labelled platelets with PMA and of incubation of membranes isolated therefrom with ethanol and/or GTP[S] on their contents of [^{3}H]PA and [^{3}H]PEt

Intact platelets were labelled with [^{3}H]glycerol and incubated with or without PMA before isolation of membranes, all as described in the Experimental section. Samples of membrane suspension were incubated for 0 or 10 min at 37 °C in medium adjusted to pCa 6 and containing the indicated additions. When present, the final concentrations of ethanol and GTP[S] were 300 mM and 10 μM respectively. [^{3}H]PA and [^{3}H]PEt were isolated and counted for ^{3}H radioactivity; the results were corrected for quenching and for the different protein contents of the membrane suspensions from control and PMA-treated platelets. Values are means±S.E.M. from triplicate incubations in the same experiment.

Platelet treatment	Incubation period (min)	Additions	10^{-3} × [^{3}H]PA (d.p.m./mg of protein)	10^{-3} × [^{3}H]PEt (d.p.m./mg of protein)
No PMA	0	None	10.20±0.22	0.40±0.06
	10	None	7.97±0.40	0.52±0.04
		Ethanol	7.99±0.31	0.72±0.05
		GTP[S]	10.03±0.10	0.69±0.04
		Ethanol+GTP[S]	7.75±0.25	5.35±0.24
PMA	0	None	10.38±0.14	0.22±0.03
	10	None	8.26±0.22	0.50±0.08
		Ethanol	8.02±0.11	2.26±0.04
		GTP[S]	14.90±0.09	0.85±0.09
		Ethanol+GTP[S]	9.56±0.41	22.08±0.19

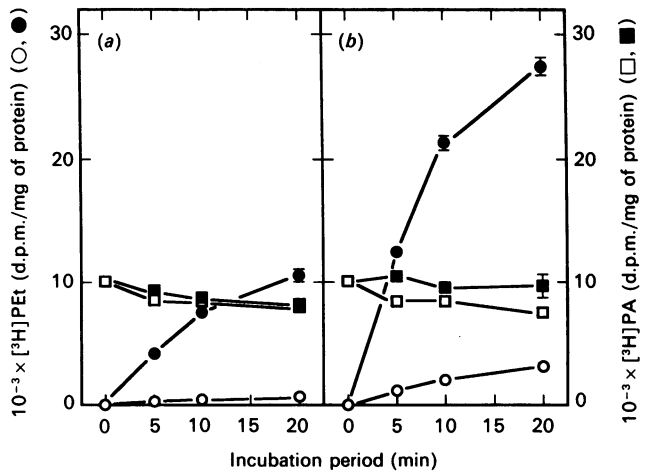

Fig. 2. Effects of GTP[S] on the time course of changes in [^{3}H]PA and [^{3}H]PEt in membranes from control and PMA-treated platelets

Membranes were isolated from control platelets (*a*) and PMA-treated platelets (*b*), that had previously been labelled with [^{3}H]glycerol. Samples containing ethanol (300 mM) and Ca^{2+} (pCa 6) were incubated for 0–20 min at 37 °C in the absence (○, □) or presence (●, ■) of 10 μM-GTP[S]. Membrane [^{3}H]PEt (○, ●) and [^{3}H]PA (□, ■) were then determined. For [^{3}H]PEt, blanks obtained from incubation of membranes in the absence of ethanol were subtracted. Values are means ± S.E.M. (or S.E. of the difference) from triplicate incubations in a single experiment.

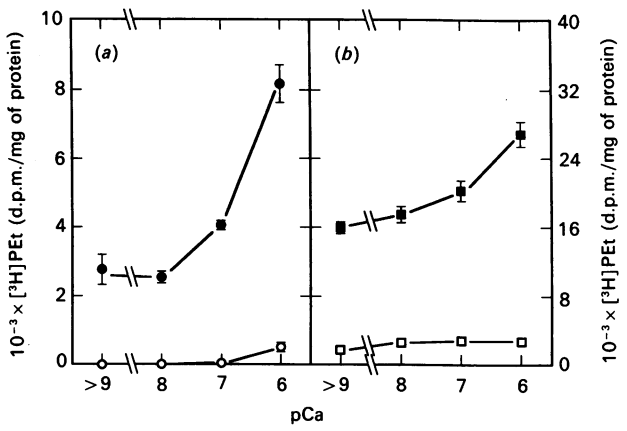

Fig. 3. Effect of the concentration of Ca^{2+} on [^{3}H]PEt formation in membranes from control and PMA-treated platelets

Membranes were isolated from control platelets (*a*) and PMA-treated platelets (*b*), that had previously been labelled with [^{3}H]glycerol. Samples were incubated for 10 min at 37 °C in the absence (○, □) or presence (●, ■) of 10 μM-GTP[S], in each case with and without ethanol (300 mM). Sufficient $CaCl_2$ to give the indicated pCa values was included. Membrane [^{3}H]PEt was then determined. Blanks obtained in the absence of ethanol were subtracted for each pCa value. Values are means ± S.E. of the difference from triplicate incubations in a single experiment.

independent mechanisms. However, when PMA-membranes from platelets were incubated with GTP[S] and ethanol, very large increases in [^{3}H]PEt formation were observed (Table 1), amounting to 62 ± 12-fold those seen in paired control membranes incubated with ethanol alone (mean ± S.E.M., five expts.). This stimulation of [^{3}H]PEt formation exceeded the sum of the separate effects of PMA treatment of platelets and of GTP[S] by a factor of 2.7 ± 0.2 (mean ± S.E.M., five expts.), indicating significant synergism between the actions of PMA and GTP[S] on PLD activity. In one experiment in which the effects of 75 mM- and 300 mM-ethanol were compared, [^{3}H]PEt formation was roughly halved at the lower ethanol concentration, though the pattern of results was qualitatively identical with that described above.

The decreases in [^{3}H]PA accumulation observed when 300 mM-ethanol was added to incubations of control or PMA-membranes with GTP[S] were much smaller than the corresponding increases in [^{3}H]PEt (e.g. Table 1), and accounted for only 25 ± 8% and 23 ± 2% of the [^{3}H]PEt formed in control and PMA-membranes respectively (means ± S.E.M., four expts.). Although this discrepancy could reflect an ability of PLD to catalyse transphosphatidylation more rapidly than hydrolysis of its phospholipid substrate, it seems more likely that the product PA is metabolized much more rapidly than PEt. The latter view is supported by the tendency of the membrane [^{3}H]PA content to decline during incubation of membranes in the absence of GTP[S] and by the continuous accumulation of [^{3}H]PEt, which approached linearity during the first 10 min of incubations (Fig. 2). Liscovitch [12] has proposed that PMA may selectively activate the transphosphatidylation reaction, at least in NG108-15 cells. This view is not supported by the present study, in which the discrepancies between the ethanol-induced decrease in [^{3}H]PA and increase in [^{3}H]PEt in control and PMA-membranes were not significantly different.

Role of Ca^{2+} ions

The dependence of [^{3}H]PEt formation on the concentration of Ca^{2+}_{free} is shown in Fig. 3. [^{3}H]PEt accumulation was significant in the absence of added Ca^{2+} ions (pCa > 9) or GTP[S] in PMA-membranes ($P < 0.05$ in each of three individual expts.) but not in control membranes. However, both membrane types formed substantial amounts of [^{3}H]PEt in the absence of Ca^{2+} ions when 10 μM-GTP[S] was present. These effects were not diminished when the EGTA concentration in the incubation mixtures was increased from 2.5 mM to 10 mM, indicating that they were truly Ca^{2+}-independent (results not shown). Addition of sufficient Ca^{2+} to give a pCa of 6 increased GTP[S]-stimulated [^{3}H]PEt formation by 3.5 ± 0.2-fold in control membranes and 1.8 ± 0.1-fold in PMA-membranes (means ± S.E.M., three expts.). The major part of these increases in PLD activity occurred between pCa values of 7 and 6 (Fig. 3), which correspond roughly to the values in intact resting and thrombin-stimulated platelets respectively [26]. These findings suggest that Ca^{2+} ions could play a role in the physiological regulation of PLD activity, particularly under conditions in which there is no activation of protein kinase C. In contrast, the GTP[S]-stimulated phospholipase C activity of rabbit platelet membranes was almost completely dependent on Ca^{2+} ions and was fully activated at pCa 7 [27].

Specificity of the effects of GTP[S]

The stimulation of [^{3}H]PEt formation by GTP[S] showed a clear dose-dependence in both control and PMA-membranes (Fig. 4). Maximum responses were seen with 10–30 μM-GTP[S]; higher concentrations were less effective. Analysis of the results obtained with GTP[S] concentrations below 30 μM indicated that half-maximal increases in [^{3}H]PEt required 0.25 ± 0.04 μM- and 0.12 ± 0.02 μM-GTP[S] in control and PMA-membranes respectively (means ± S.E.M., three expts.). Therefore PMA treatment appears to increase not only the maximum PLD activity achieved after GTP[S] addition, but also the affinity of the system for GTP[S]. The sensitivity of platelet membrane PLD to GTP[S] was much greater than observed in rat hepatocyte membranes [8], in which a half-maximal effect required 1.5 μM compound, but was comparable with that previously reported for the activation of phospholipase C in rabbit platelet membranes [27].

The specificity of the actions of GTP[S] on platelet PLD was

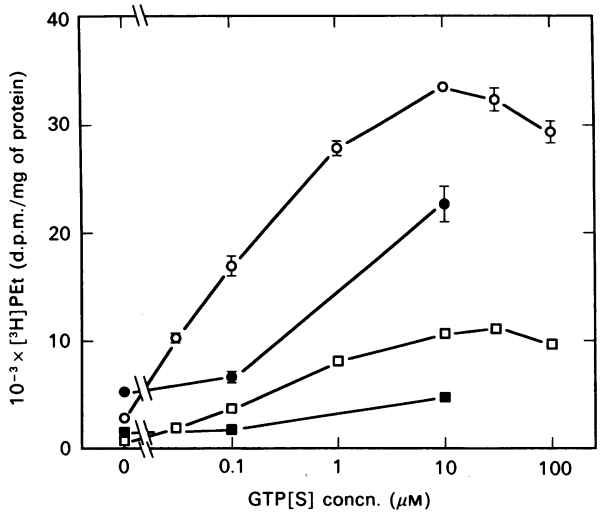

Fig. 4. Dose–response relationships for the stimulation of [^{3}H]PEt formation by GTP[S] in membranes from control and PMA-treated platelets; effects of GDP[S]

Membranes were isolated from control and PMA-treated platelets that had previously been labelled with [^{3}H]glycerol. Samples containing control membranes (□, ■) and PMA-membranes (○, ●) were incubated for 10 min at 37 °C in the presence of the indicated concentration of GTP[S], either without (○, □) or with (●, ■) 400 μM-GDP[S]. All incubation mixtures contained $CaCl_2$ giving pCa 6 and 300 mM-ethanol. Membrane [^{3}H]PEt was then determined; the ^{3}H present in the PEt region after t.l.c. of extracts of unincubated membranes was subtracted. Values represent the means ± S.E. of the difference from triplicate measurements in a single experiment.

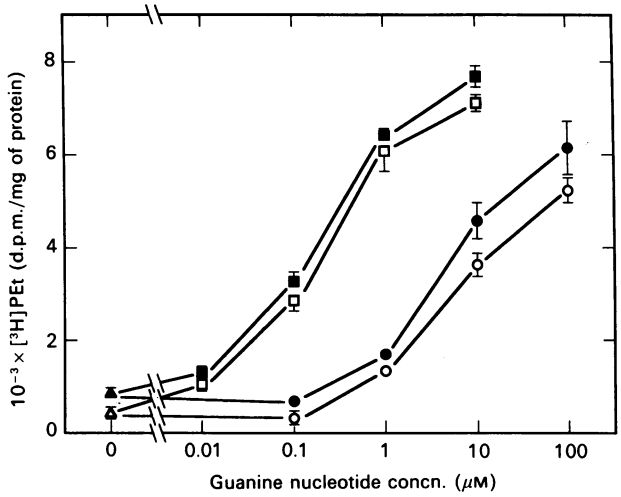

Fig. 5. Dose–response relationships for the stimulation of [^{3}H]PEt formation in control membranes by GTP[S] and GTP in the absence and presence of thrombin

Membranes were isolated from platelets that had been labelled with [^{3}H]glycerol but not incubated with PMA. Samples were incubated for 10 min at 37 °C with the indicated concentrations of GTP[S] (□, ■) or GTP (○, ●) in the absence (○, □) and presence (●, ■) of 2 units of thrombin/ml. All incubation mixtures contained $CaCl_2$ to give pCa 6 and 300 mM-ethanol. Membrane [^{3}H]PEt was then determined; the ^{3}H present in the PEt region after t.l.c. of extracts of unincubated membranes was subtracted. Values represent the means ± S.E. of the difference from triplicate measurements in a single experiment.

demonstrated by the ability of GDP[S] to inhibit GTP[S]-induced [^{3}H]PEt formation (Fig. 4). Although 400 μM-GDP[S] increased PLD activity by about 2-fold in the absence of GTP[S] in both control and PMA-membranes, it virtually prevented the additional stimulatory effects of 0.1 μM-GTP[S]. Added with 10 μM-GTP[S], 400 μM-GDP[S] caused less inhibition of [^{3}H]PEt formation, consistent with competition between GDP[S] and GTP[S] for a GTP-binding protein. The results suggest that GDP[S] behaved as a weak partial agonist in this system, though it is also possible that the GDP[S] contained trace amounts of a different stimulatory guanine nucleotide.

In addition to GTP[S], GTP itself enhanced [^{3}H]PEt formation (Fig. 5). This action of GTP was also partially inhibited by GDP[S] (results not shown). Analysis of dose–response curves indicated that half-maximal stimulation of [^{3}H]PEt formation required GTP concentrations of 6.0 ± 0.7 μM and 3.6 ± 0.4 μM in control and PMA-membranes respectively (means ± S.E.M., three expts.). In the experiments in which GTP and GTP[S] were directly compared (e.g. Fig. 5), the calculated maximum responses to GTP averaged 76% and 82% of those generated by GTP[S] in control and PMA-membranes respectively. Thus PMA treatment of platelets had effects on the response of PLD to GTP similar to those seen with GTP[S]. The results contrast with those obtained in previous work on the activation of phospholipase C in rabbit platelet membranes, in which, in the absence of an agonist such as thrombin, GTP had a maximum effect much smaller than that of GTP[S] [27]. This suggests that the GTP-binding protein involved in the activation of PLD may, like members of the *ras* superfamily or G_z, have an intrinsically low GTPase activity, or that a cofactor such as a GTPase-activating protein (GAP) may have been lost from the membrane fraction used [28]. Since the concentration of GTP in the platelet cytosol is likely to exceed that required for maximal activation of PLD by this compound, GTP may exert an essentially permissive role in the regulation of PLD by other factors.

Effects of thrombin

Addition of a high concentration of thrombin (2 units/ml) to platelet membranes incubated at pCa 6 caused small and variable increases in [^{3}H]PEt formation that were at the threshold of significance under individual incubation conditions. Similar effects were seen with both control and PMA-membranes in the presence and absence of GTP. For example, with 10 μM-GTP, thrombin increased [^{3}H]PEt formation by 16 ± 5% in control membranes (mean ± S.E.M., three expts.) and by 9 ± 2% in PMA-membranes (mean ± S.E.M., four expts.). Thrombin induced similar marginal increases in [^{3}H]PEt formation in control membranes over a wide range of concentrations of GTP or GTP[S] (Fig. 5). Thus, in contrast with previous studies on phospholipase C [27], our present experiments provided no convincing evidence of synergism between the effects of thrombin and of low concentrations of guanine nucleotides. Although the mechanism responsible for this weak effect of thrombin on membrane PLD activity is unclear and its biological significance is doubtful, we cannot exclude the possibility that our experimental conditions were inappropriate for detection of a more marked effect of thrombin. However, at present, the only evidence for a direct receptor-mediated guanine-nucleotide-dependent activation of PLD comes from experiments with hepatocyte membranes, in which P_2-purinergic agonists stimulated the enzyme in the presence of a low GTP[S] concentration [8], and with a synaptosomal preparation, in which muscarinic agonists were stimulatory in the presence of GTP[S] [25].

Mechanisms and possible significance of the activation of platelet PLD

The stimulation of [^{3}H]PEt formation in membranes isolated from platelets treated with PMA suggested that protein kinase C

Table 2. Effects of treatment of [^{3}H]glycerol-labelled platelets with various phorbol esters or diC_8 on the phosphorylation of pleckstrin and on the GTP[S]-stimulated formation of [^{3}H]PEt in isolated membranes

Platelets that had been labelled with both [^{3}H]glycerol and [^{32}P]P_i were incubated for 2 min with the indicated compounds. After samples were taken for measurement of pleckstrin phosphorylation, the remaining platelets were lysed and membranes prepared. Samples were incubated for 10 min at 37 °C at pCa 6 with 300 mM-ethanol and 10 μM-GTP[S]. [^{3}H]PEt was extracted and isolated by t.l.c.; the ^{3}H corresponding to PEt in extracts of unincubated membranes was subtracted. Values for [^{3}H]PEt are means ± S.E. of the difference from triplicate measurements in a single experiment.

Treatment of platelets	^{32}P in pleckstrin (c.p.m./10^8 platelets)	10^{-3} × [^{3}H]PEt in GTP[S]-stimulated membranes (d.p.m./mg of protein)
None	183	1.50 ± 0.12
PMA (1 μM)	1176	7.40 ± 0.15
α-PMA (1 μM)	177	1.45 ± 0.07
PDD (1 μM)	1034	4.79 ± 0.37
α-PDD (1 μM)	178	1.10 ± 0.08
DiC_8 (400 μM)	266	1.76 ± 0.03

may play a role in the activation of platelet PLD, as already proposed for several other cell types [5,12,13]. To verify the specificity of the action of PMA, platelets were also incubated with PDD and the biologically inactive isomers of these compounds, α-PMA and α-PDD (Table 2). Treatment of platelets with PDD was slightly less effective than PMA in stimulating [^{3}H]PEt formation in membranes, whereas both α-isomers were without effect. These results correlated perfectly with the phosphorylation of the protein kinase C substrate, pleckstrin, in the same platelets (Table 2). DiC_8, a potent activator of protein kinase C in human platelets [29], only had very weak effects on PLD activity and pleckstrin phosphorylation in rabbit platelets, presumably because the compound was unable to penetrate the platelets of the latter species. Studies on the concentration-dependence of the effect of PMA treatment of platelets on [^{3}H]PEt formation in isolated membranes showed that, although significant activation of PLD was detected with 0.01 μM-PMA, at least 1 μM-PMA was required for an optimal effect (Fig. 6). This requirement for a relatively high PMA concentration is largely explained by the high platelet concentration (5×10^9/ml) used. Whereas 0.1 μM-PMA caused complete phosphorylation of pleckstrin with 5×10^8 platelets/ml, 1 μM-PMA was required with 5×10^9 platelets/ml, presumably because most of the PMA was bound by the platelets (results not shown).

In addition, we attempted to establish the mechanism of action of PMA by experiments with staurosporine, which inhibits protein kinase C-mediated and other phosphorylation reactions in platelets [30]. Surprisingly, treatment of platelets with 10 μM-

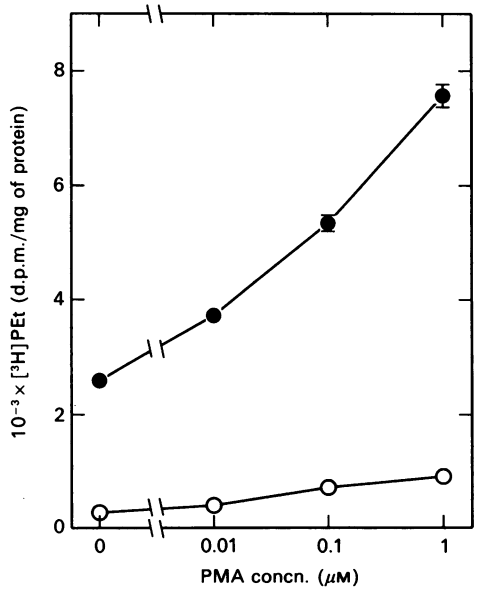

Fig. 6. Effects of treatment of intact [^{3}H]glycerol-labelled platelets with different concentrations of PMA on [^{3}H]PEt formation in membranes isolated from the platelets

Platelets labelled with [^{3}H]glycerol were incubated for 2 min with the indicated concentrations of PMA before preparation of membranes. [^{3}H]PEt was then measured in membranes incubated for 10 min at 37 °C in either the absence (○) or presence (●) of 10 μM-GTP[S]. All incubation mixtures contained $CaCl_2$ to give pCa 6 and 300 mM-ethanol. The ^{3}H corresponding to PEt in extracts of unincubated membranes was subtracted. Values for [^{3}H]PEt are means ± S.E. of the difference from triplicate measurements in a single experiment.

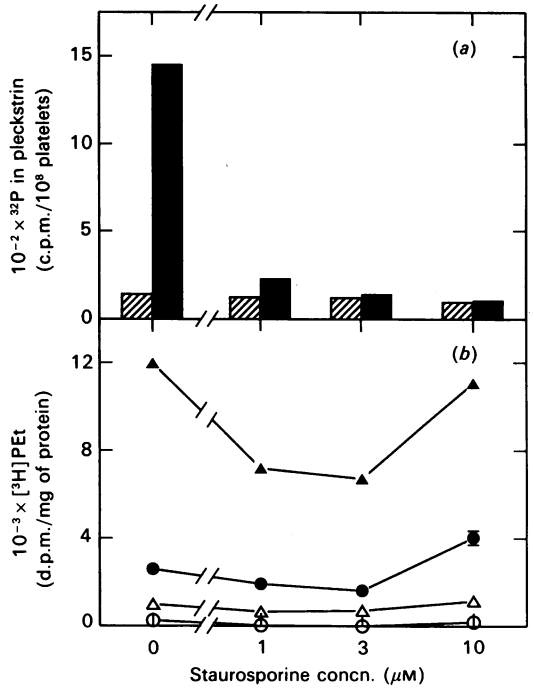

Fig. 7. Effects of treatment of [^{3}H]glycerol-labelled platelets with PMA and different concentrations of staurosporine on pleckstrin phosphorylation and on [^{3}H]PEt formation in membranes isolated from the platelets

Platelets that had been labelled with both [^{3}H]glycerol and [^{32}P]P_i were incubated for 5 min with the indicated concentrations of staurosporine, followed by a further 2 min without or with 0.2 μM-PMA. After samples were taken for measurement of pleckstrin phosphorylation (*a*) in the absence (▨) and presence (■) of PMA, the remaining platelets were lysed and membranes prepared. [^{3}H]PEt formation (*b*) was then measured in membranes from platelets that had been incubated without (○, ●) or with (△, ▲) PMA; these membranes were incubated for 10 min at 37 °C in the absence (○, △) or presence (●, ▲) of 10 μM-GTP[S]. All incubation mixtures contained $CaCl_2$ to give pCa 6 and 300 mM-ethanol. The ^{3}H corresponding to PEt in extracts of unincubated membranes was subtracted. Values for [^{3}H]PEt are means ± S.E. of the difference from triplicate measurements in a single experiment.

staurosporine did not block, and usually enhanced, the effects of PMA on PLD activity. Thus, with 1 μM-PMA, 10 μM-staurosporine increased [^{3}H]PEt formation by 54±20% in the absence of GTP[S] and by 23±11% in the presence of 10 μM-GTP[S] (means±S.E.M., seven expts.). Similar results were obtained with lower PMA concentrations (0.04 and 0.2 μM). These results were obtained despite complete inhibition by the staurosporine of the phosphorylation of pleckstrin in the intact platelets and suppression of all the PMA-stimulated ^{32}P incorporation into proteins in the membranes isolated from these platelets. These studies indicated that, at least in the presence of 10 μM-staurosporine, PMA can activate PLD by a mechanism that does not depend on increased protein kinase activity. However, further study showed that lower staurosporine concentrations could inhibit the action of PMA on PLD activity (Fig. 7), though the extent of inhibition varied between experiments. The largest inhibitory effects were seen with 3 μM-staurosporine, which was just sufficient to block completely the phosphorylation of pleckstrin induced by 0.2 μM-PMA (Fig. 7). Under these conditions, treatment of platelets with staurosporine decreased PMA-stimulated [^{3}H]PEt formation by 23±10% in membranes assayed in the presence of 10 μM-GTP[S] (mean±S.E.M., five expts.). These results indicate that staurosporine had two distinct actions; first, the compound inhibited the activation of PLD by PMA, probably by blocking protein kinase C activity, and second, in conjunction with PMA, it caused a phosphorylation-independent activation of PLD. The latter effect obscured the former to a variable degree. Experiments with H7, another inhibitor of protein kinase C [31], were uninformative, since this compound did not inhibit pleckstrin phosphorylation induced by PMA in rabbit platelets. We conclude from the close correlation between the effects of treatment of platelets with various phorbol esters on pleckstrin phosphorylation and PLD activation, together with the inhibitory action of low staurosporine concentrations, that much, if not all, of the action of PMA on PLD activity is usually mediated by protein kinase C. Further study will be required to determine whether the phosphorylation-independent action of PMA in the presence of staurosporine contributed significantly to the action of PMA alone, or whether this mechanism has a physiological parallel. Although some studies in other cell types have indicated that protein kinase C mediates the effects of phorbol esters on PLD activity [5,12,13], Billah *et al.* [24] have also reported evidence for a phosphorylation-independent mechanism in HL-60 cells and have suggested that PMA may directly activate PLD. To check the latter possibility, we incubated control platelet membranes with and without 1 μM-PMA in the presence of 10 μM-GTP[S]; no significant changes in [^{3}H]PEt formation were detected.

We have demonstrated that guanine nucleotides can activate PLD in platelet membranes and that there is a marked synergism between the effects of PMA treatment and GTP[S]. A comparable synergism has recently been observed in experiments with canine cerebral-cortex synaptosomes [25]. This interaction suggests that PMA treatment and GTP[S] act through a common component, rather than by two independent pathways, as proposed for HL-60 cells by Tettenborn & Mueller [5]. Possible mechanisms to explain the observed synergism include protein kinase C-mediated phosphorylation of PLD with a resultant increase in its catalytic activity and, perhaps, its affinity for a regulatory GTP-binding protein. Alternatively, the GTP-binding protein itself may be phosphorylated, leading to an enhanced affinity for PLD or GTP (as suggested by the decreases in the concentrations of GTP[S] and GTP required for half-maximal stimulation of enzyme activity after PMA treatment). A further possibility, not excluded by the present results, is that PMA treatment of platelets leads to a translocation of PLD and/or the relevant GTP-binding protein to the membrane. However, in this case, the translocated component would have to be firmly membrane-bound, in view of the rigorous washing conditions used in preparation of the membranes.

Rubin [11] has shown that thrombin can stimulate PEt formation in intact platelets and has suggested that it may activate PLD directly. However, our results suggest that PLD activation may not be part of primary plasma-membrane-based signal transduction in the platelet. First, thrombin caused minimal increases in membrane PLD activity, even in the presence of GTP. Second, enhanced PLD activity appeared to depend on prior activation of protein kinase C. Third, Ca^{2+} ions had effects on platelet PLD consistent with a regulatory mechanism in which full activation requires Ca^{2+} mobilization. These findings suggest that in intact platelets PLD activation may be secondary to the receptor-mediated activation of phospholipase C and may depend on the actions of the second messengers, diacylglycerol and inositol 1,4,5-trisphosphate. If this is the case, a physiological role for PLD should be sought in an effector mechanism, such as exocytosis, that is stimulated by PMA and Ca^{2+} ions in intact platelets [32–34]. In this context, it may be significant that PA, the normal product of PLD action, can promote phospholipid bilayer fusion in model systems [35]. A role in exocytosis for PA generated by activation of PLD, or for a metabolite of PA, has also been suggested in connection with studies on other cells [7,36]. Although it is well established that most of the PA formed in stimulated human platelets is derived from phosphoinositides through the actions of phospholipase C and diacylglycerol kinase [37,38], comparable studies have not been carried out with rabbit platelets. Moreover, if appropriately localized in the platelet, small amounts of PA generated by PLD activity might have effects distinct from those of PA formed from phosphoinositides. It is also conceivable that some of the effects of GTP[S] on secretion from permeabilized cells are mediated by the GTP-binding protein associated with PLD. Thus there is good evidence that GTP[S] can stimulate secretion from some permeabilized cells by a Ca^{2+}-independent mechanism that does not involve activation of phospholipase C [39–41]. However, in the permeabilized human platelet, this independence of Ca^{2+} is only partial [42,43], a fact that could be accounted for by an additional requirement for both Ca^{2+} ions and protein kinase C for optimal activation of PLD. It is also relevant that PMA inhibits the activation of platelet phospholipase C by physiological agonists or GTP[S] [44–47], but enhances guanine-nucleotide-induced 5-hydroxytryptamine secretion from permeabilized platelets at low Ca^{2+} concentrations ([46]; J. R. Coorssen, M. M. L. Davidson & R. J. Haslam,unpublished work). The latter effect has also been observed in permeabilized HL-60 cells [48] and could constitute a functional expression of the synergistic activation of PLD by PMA and GTP[S] described in the present paper.

This work was supported by a grant from the Medical Research Council of Canada (MT-5626). J. v. d. M. held an M.R.C. Research Fellowship. We thank Judy Sherwood for technical assistance.

REFERENCES

1. Chalifour, R. & Kanfer, J. N. (1982) J. Neurochem. **39**, 299–305
2. Kobayashi, M. & Kanfer, J. N. (1987) J. Neurochem. **48**, 1597–1603
3. Bocckino, S. B., Wilson, P. B. & Exton, J. H. (1987) FEBS Lett. **225**, 201–204
4. Pai, J.-K., Siegel, M. I., Egan, R. W. & Billah, M. M. (1988) J. Biol. Chem. **263**, 12472–12477
5. Tettenborn, C. S. & Mueller, G. C. (1988) Biochem. Biophys. Res. Commun. **155**, 249–255

6. Exton, J. H. (1990) J. Biol. Chem. **265**, 1–4
7. Cockcroft, S. (1984) Biochim. Biophys. Acta **795**, 37–46
8. Bocckino, S. B., Blackmore, P. F., Wilson, P. B. & Exton, J. H. (1987) J. Biol. Chem. **262**, 15309–15315
9. Martin, T. W. & Michaelis, K. (1989) J. Biol. Chem. **264**, 8847–8856
10. Ben-Av, P. & Liscovitch, M. (1989) FEBS Lett. **259**, 64–66
11. Rubin, R. (1988) Biochem. Biophys. Res. Commun. **156**, 1090–1096
12. Liscovitch, M. (1989) J. Biol Chem. **264**, 1450–1456
13. Cabot, M. C., Welsh, C. J., Zhang, Z.-C. & Cao, H.-T. (1989) FEBS Lett. **245**, 85–90
14. Molnar, J. & Lorand, L. (1961) Arch. Biochem. Biophys. **93**, 353–363
15. Aster, R. H. & Jandl, J. H. (1964) J. Clin. Invest. **43**, 843–855
16. Ardlie, N. G., Packham, M. A. & Mustard, J. F. (1970) Br. J. Haematol. **19**, 7–17
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
18. Fabiato, A. & Fabiato, F. (1979) J. Physiol. (Paris) **75**, 463–505
19. Bligh, E. G. & Dyer, W. J. (1959) Can. J. Biochem. Physiol. **37**, 911–917
20. Mallows, R. S. E. M. & Bolton, T. B. (1987) Biochem. J. **244**, 763–768
21. Nakamura, K. & Handa, S. (1984) Anal. Biochem. **142**, 405–410
22. Barlow, R. B. (1983) Biodata Handling with Microcomputers, pp. 116–121, Elsevier Science Publishers, Amsterdam
23. Imaoka, T., Lynham, J. A. & Haslam, R. J. (1983) J. Biol. Chem. **258**, 11404–11414
24. Billah, M. M., Pai, J.-K., Mullmann, T. J., Egan, R. W. & Siegel, M. I. (1989) J. Biol. Chem. **264**, 9069–9076
25. Qian, Z. & Drewes, L. R. (1989) J. Biol. Chem. **264**, 21720–21724
26. Rink, T. J. & Hallam, T. J. (1984) Trends Biochem. Sci. **9**, 215–219
27. Hrbolich, J. K., Culty, M. & Haslam, R. J. (1987) Biochem. J. **243**, 457–465
28. Santos, E. & Nebreda, A. R. (1989) FASEB J. **3**, 2151–2163
29. Lapetina, E. G., Reep, B., Ganong, B. R. & Bell, R. M. (1985) J. Biol. Chem. **260**, 1358–1361
30. Watson, S. P., McNally, J., Shipman, L. J. & Godfrey, P. P. (1988) Biochem. J. **249**, 345–350
31. Hidaka, H., Inagaki, M., Kawamoto, S. & Sasaki, Y. (1984) Biochemistry **23**, 5036–5041
32. Yamanishi, J., Takai, Y., Kaibuchi, K., Sano, K., Castagna, M. & Nishizuka, Y. (1983) Biochem. Biophys. Res. Commun. **112**, 778–786
33. Rink, T. J., Sanchez, A. & Hallam, T. J. (1983) Nature (London) **305**, 317–319
34. Krishnamurthi, S., Joseph, S. & Kakkar, V. V. (1986) Biochem. J. **238**, 193–199
35. Sundler, R. & Papahadjopoulos, D. (1981) Biochim. Biophys. Acta **649**, 743–750
36. Gruchalla, R. S., Dinh, T. T. & Kennerly, D. A. (1990) J. Immunol. **144**, 2334–2342
37. Mauco, G., Dangelmaier, C. A. & Smith, J. B. (1984) Biochem. J. **224**, 933–940
38. Tysnes, O.-B., Verhoeven, A. J. M. & Holmsen, H. (1988) Eur. J. Biochem. **174**, 75–79
39. Barrowman, M. M., Cockcroft, S. & Gomperts, B. D. (1986) Nature (London) **319**, 504–507
40. Cockcroft, S., Howell, T. W. & Gomperts, B. D. (1987) J. Cell Biol. **105**, 2745–2750
41. Vallar, L., Biden, T. J. & Wollheim, C. B. (1987) J. Biol. Chem. **262**, 5049–5056
42. Haslam, R. J. & Davidson, M. M. L. (1984) FEBS Lett. **174**, 90–95
43. Coorssen, J. R. & Haslam, R. J. (1990) FASEB J. **4**, A2310
44. MacIntyre, D. E., McNicol, A. & Drummond, A. H. (1985) FEBS Lett. **180**, 160–164
45. Rittenhouse, S. E. & Sasson, J. P. (1985) J. Biol. Chem. **260**, 8657–8660
46. Krishnamurthi, S. & Kakkar, V. V. (1988) Biochem. Biophys. Res. Commun. **156**, 1257–1264
47. Baldassare, J. J., Henderson, P. A. & Fisher, G. J. (1989) Biochemistry **28**, 56–60
48. Stutchfield, J. & Cockcroft, S. (1988) Biochem. J. **250**, 375–382

Received 18 June 1990; accepted 3 July 1990