Evidence for the calcium-dependent activation of phospholipase D in thrombin-stimulated human erythroleukaemia cells

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Human erythroleukaemia (HEL) cells were exposed to thrombin and other platelet-activating stimuli, and changes in radiolabelled phospholipid metabolism were measured. Thrombin caused a transient fall in $\text{PtdIns}P$ and $\text{PtdIns}P_2$ levels, accompanied by a rise in diacylglycerol and phosphatidic acid, indicative of a classical phospholipase C/diacylglycerol kinase pathway. However, the rise in phosphatidic acid preceded that of diacylglycerol, which is inconsistent with phospholipase C/diacylglycerol kinase being the sole source of phosphatidic acid. In the presence of ethanol, thrombin and other agonists (platelet-activating factor, adrenaline and ADP, as well as fetal-calf serum) stimulated the appearance of phosphatidylethanol, an indicator of phospholipase D activity. The Ca^{2+} ionophore A23187 and the protein kinase C activator phorbol myristate acetate (PMA) also elicited phosphatidylethanol formation, although A23187 was at least 5-fold more effective than PMA. Phosphatidylethanol production stimulated by agonists or A23187 was Ca^{2+} -dependent, whereas that with PMA was not. These results suggest that phosphatidic acid is generated in agonist-stimulated HEL cells by two routes: phospholipase C/diacylglycerol kinase and phospholipase D. Activation of the HEL-cell phospholipase D in response to agonists may be mediated by a rise in intracellular Ca^{2+} .

INTRODUCTION

Stimulation of mammalian cells with Ca^{2+} -mobilizing agonists causes the activation of intracellular phospholipases, resulting in the production of phospholipid-derived second messengers. Two major classes of signal-transducing phospholipases have received much attention, these being phospholipases A_2 and C. The phospholipases A_2 are thought to catalyse the first step leading to the formation of prostaglandins, leukotrienes and other eicosanoids by releasing the precursor arachidonic acid from phospholipids, whereas the phosphoinositide-specific phospholipases C hydrolyse $\text{PtdIns}(4,5)P_2$, thereby initiating a bifurcating signal pathway [1]. The products of the latter reaction are the Ca^{2+} -releasing second messenger $\text{Ins}(1,4,5)P_3$ and the protein kinase C activator 1,2-diacylglycerol. The subsequent phosphorylation of diacylglycerol by diacylglycerol kinase leads to production of an additional potential second messenger, phosphatidic acid. Although a physiological role for phosphatidic acid in signal transduction has not been conclusively established, this lipid when added exogenously has been shown to act both as a Ca^{2+} ionophore and as a stimulus itself for phosphoinositide hydrolysis, arachidonic acid release, intracellular Ca^{2+} mobilization and protein phosphorylation [2–6].

Aside from the well-known phospholipase C/diacylglycerol kinase route, novel pathways for generation of diacylglycerol and phosphatidic acid have recently been described [7–17]. These include phospholipases C, which hydrolyse phosphatidylcholine to yield diacylglycerol, and phospholipases of the D type, which yield phosphatidic acid directly and diacylglycerol indirectly, via phosphatidate phosphohydrolase. The relative contribution of phospholipase D versus the phospholipase C/diacylglycerol kinase pathway to the agonist-stimulated rise in phosphatidic acid may vary from one cell type to another. For example, in hepatocytes most of the phosphatidic acid produced in response to Ca^{2+} -mobilizing agonists seems to be derived from

phospholipase D activity [7], although in thrombin-stimulated human platelets the major route is probably through phospholipase C and diacylglycerol kinase [18,19].

The human erythroleukaemia (HEL) cell line expresses markers characteristic of the megakaryocyte/platelet lineage, such as the plasma-membrane glycoprotein IIb/IIIa complex, α_2 -adrenergic and thromboxane A_2 receptors, and α -granule proteins, including the platelet-derived growth factor [20–23]. HEL cells may thus provide a useful model for platelet biochemistry with the advantages of a cultured cell line. However, the transmembrane signalling pathways in HEL cells have not been extensively investigated. In this study we find that HEL cells respond to a variety of platelet agonists (thrombin, platelet-activating factor, ADP and adrenaline) with a rapid production of phosphatidic acid, a response which is usually associated with activation of phosphoinositide-specific phospholipase C. However, our results indicate that HEL cells possess an alternative pathway for phosphatidic acid production: direct hydrolysis of phospholipid(s) by phospholipase D. In contrast with some other systems in which phospholipase D activity has been reported, this enzyme in HEL cells appears to be activated primarily by a rise in $[\text{Ca}^{2+}]_i$.

EXPERIMENTAL

Materials

$[^3\text{H}]$ Arachidonic acid (~ 100 Ci/mmol) and $[^3\text{H}]$ myristic acid (~ 39 Ci/mmol) were purchased from Dupont/NEN, Wilmington, DE, U.S.A. Carrier-free $[^{32}\text{P}]\text{P}_i$ was from ICN, Irvine, CA, U.S.A. Platelet-activating factor and 1,2-diolein were from Avanti, Pelham, AL, U.S.A. Human thrombin (> 3000 units/mg) and ADP were from USB, Cleveland, OH, U.S.A. Cabbage phospholipase D, (–)-adrenaline, egg phosphatidylcholine and egg phosphatidic acid were from Sigma, St.

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; HEL cells, human erythroleukaemia cells; PMA, 4 β -phorbol 12-myristate 13-acetate.

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Louis, MO, U.S.A. Fetal-calf serum was from GIBCO, Grand Island, NY, U.S.A. All solvents for lipid extraction and t.l.c. were from Fisher, St. Louis, MO, U.S.A. 4β -Phorbol 12-myristate 13-acetate (PMA) was from LC Services, Woburn, MA, U.S.A. Ionophore A23187 (free acid) was from Calbiochem, La Jolla, CA, U.S.A. PMA and A23187 were dissolved in dimethyl sulphoxide and then diluted in incubation medium; the final [dimethyl sulphoxide] ($\leq 0.2\%$, v/v) was found to have no effect of its own on the parameters measured here.

Methods

HEL cells were purchased from the American Type Culture Collection (A.T.C.C. TIB 180), Rockville, MD, U.S.A., and maintained in suspension culture in RPMI 1640 medium supplemented with 10% (v/v) fetal-calf serum under a humidified atmosphere of air/CO₂ (19:1) at 37 °C. Before each experiment, cells were harvested by centrifugation (160 g for 10 min), washed, and resuspended to 5 ml in a modified Tyrode/Hepes medium composed of 134 mM-NaCl, 2.9 mM-KCl, 4 mM-NaHCO₃, 0.36 mM-NaH₂PO₄, 1 mM-CaCl₂, 1 mM-MgCl₂, 5 mM-dextrose, 0.2 mg of fatty acid-free BSA/ml and 10 mM-Hepes (pH 7.40 at 37 °C). The NaH₂PO₄ was omitted when cells were to be labelled with [³²P]P_i. Cells were then incubated with [³²P]P_i (0.2 mCi/ml), [³H]arachidonic acid (2 µCi/ml) or [³H]myristic acid (10 µCi/ml), as indicated in the Figure legends, for a period of 2 h at 37 °C to incorporate the radioisotope into cellular phospholipids. Labelled cells were then washed twice and resuspended in Tyrode/Hepes medium as above, except that the MgCl₂ concentration was lowered to 0.1 mM, as this was found to facilitate responses to the ionophore A23187. In some experiments (see Fig. 4) a portion of the HEL cells was incubated in Tyrode/Hepes medium containing 1 mM-EGTA and 0.608 mM-CaCl₂, yielding a calculated free Ca²⁺ concentration of 100 nM, as determined with the FREECAL program [24] for the Macintosh computer (kindly provided by Dr. Lawrence Brass, University of Pennsylvania) and verified by a Ca²⁺-selective electrode. All experiments were carried out at a cell density of 10⁶/ml. Samples (0.5 ml) of HEL-cell suspension were incubated at 37 °C with various treatments, and incubations were terminated by addition of 1.9 ml of chloroform/methanol (1:2, v/v) for extraction of phospholipids and neutral lipids, or chloroform/methanol/HCl (50:100:1, by vol.) for experiments in which polyphosphoinositide levels were measured. Lipids were extracted by the Bligh & Dyer method [25] and separated by t.l.c. as follows. Phosphatidic acid, 1,2-diacylglycerol and phosphatidylethanol were separated on Whatman LK-6D plates in a solvent system consisting of the upper phase of ethyl acetate/iso-octane/acetic acid/water (9:5:2:10, by vol.) [26]. Phosphatidylethanol production was verified with a different solvent system [8]: chloroform/methanol/acetic acid (13:3:1, by vol.), yielding identical results. Lipids were made visible under u.v. light after spraying with aq. 1% toluidinylnaphthalenesulphonate, identified by comigration with authentic standards, and scraped from the plate. Radioactivity was measured by liquid-scintillation counting in Biosafe II cocktail (RPI, Mount Prospect, IL, U.S.A.). [³²P]Phosphoinositides were separated on Merck silica-gel 60 plates developed in chloroform/methanol/20% (v/v) methylamine (30:18:5, by vol.) [27]. The [³²P]phospholipids were detected by autoradiography, scraped off, and quantified by Čerenkov counting. Unless noted otherwise, all data shown are from a single experiment which was repeated at least twice with similar results. Phosphatidylethanol standard was prepared by transphosphatidylation of egg phosphatidylcholine with cabbage phospholipase D [28].

RESULTS AND DISCUSSION

Phosphatidic acid production and phosphoinositide turnover in HEL cells stimulated by thrombin and other platelet agonists

In human platelets, thrombin elicits the activation of phosphoinositide-specific phospholipase C, resulting in a breakdown and resynthesis of phosphoinositides and generation of diacylglycerol, which is then converted into phosphatidic acid [29,30]. To assess the presence of this pathway for phosphatidic acid synthesis in HEL cells, we first examined the effect of the platelet agonist thrombin on HEL-cell [³²P]phosphoinositide and [³²P]phosphatidic acid metabolism. Fig. 1 shows the rapid increase in [³²P]phosphatidic acid evoked by thrombin. This was accompanied by a transient decline in [³²P]PtdInsP and [³²P]PtdInsP₂ levels. [³²P]PtdIns remained relatively stable over the 2 min time course. The stimulation of [³²P]phosphatidic acid production was half-maximal at approx. 0.2 NIH unit of thrombin/ml and reached a peak at 1–3 units/ml (results not shown). These results indicate that in HEL cells, as in platelets, the thrombin receptor is coupled to phosphoinositide-specific phospholipase C. This conclusion is in agreement with the recent finding by Michel *et al.* [23] that thrombin stimulates an increase in [³H]inositol phosphates in HEL cells.

Several other platelet agonists were also found to evoke the production of [³²P]phosphatidic acid in this cell line, though to a lesser extent than thrombin. Platelet-activating factor (100 nM) caused a 30% increase in phosphatidic acid at 1 min; ADP and adrenaline (10 µM) evoked a response of similar magnitude (results not shown). Aside from platelet agonists, addition of 10% (v/v) fetal-calf serum to HEL cells stimulated a 2-fold rise in [³²P]phosphatidic acid at 2 min (results not shown), presumably reflecting the action of growth factor(s) present in the serum.

Fig. 1. Effect of thrombin on [³²P]phosphoinositide and phosphatidic acid levels in HEL cells

Cells were prelabelled with [³²P]P_i in phosphate-free modified Tyrode/Hepes medium and then washed and resuspended as described under 'Methods'. Samples (0.5 ml) of cell suspension were stimulated at 37 °C for the indicated times with thrombin (2 units/ml). Reactions were stopped by addition of acidified chloroform/methanol; lipid extracts were then analysed for [³²P]PtdInsP₂ (□), [³²P]PtdInsP (◇), [³²P]PtdIns (○) and [³²P]phosphatidic acid (●; PA) by t.l.c. Phospholipid radioactivity in unstimulated samples did not change over the time course of the experiment. Data are means from duplicate determinations.

Fig. 2. Time course of production of [³H]arachidonyl-phosphatidic acid and [³H]1,2-diacylglycerol in response to thrombin

HEL cells prelabelled with [³H]arachidonic acid were stimulated for the indicated times with thrombin (2 units/ml). Samples were then analysed for [³H]phosphatidic acid (PA; ●) and 1,2-diacylglycerol (DG; ■). Data are means $\pm$ S.E.M. from four to five experiments, normalized as percentages of the maximal response for each experiment.

Kinetics of thrombin-stimulated phosphatidic acid and diacylglycerol production

To gain further insight into the pathway(s) by which activated HEL cells synthesize phosphatidic acid, we determined the time course of diacylglycerol and phosphatidic acid accumulation after stimulation by thrombin in cells prelabelled with [³H]arachidonic acid. If phosphatidic acid arises solely through the phospholipase C/diacylglycerol kinase pathway, then diacylglycerol production should precede, or at least coincide with, the rise in phosphatidic acid. In fact, near-maximal [³H]phosphatidic acid production was seen at a time when the major rise in [³H]diacylglycerol was just beginning (Fig. 2). This implies that much of the [³H]phosphatidic acid generated within the first 1 min of stimulation by thrombin is derived from a pathway other than, or in addition to, phospholipase C/diacylglycerol kinase. The simplest explanation for these results would appear to be a thrombin-induced activation of phospholipase D.

Agonist-stimulated production of phosphatidylethanol

To test the possibility that phosphatidic acid arises through the action of a phospholipase D, we prelabelled HEL cells with [³H]myristic acid, a saturated fatty acid preferentially incorporated into phosphatidylcholine [31]. Labelled cells were then incubated with ethanol and assayed for production of [³H]phosphatidylethanol. Since this unusual lipid results from phospholipase D-catalysed transphosphatidylation and is not formed via 'de novo' or base-exchange pathways, phosphatidylethanol synthesis may be taken as unequivocal evidence of phospholipase D activity [8,19,28]. Fig. 3 shows the time course of [³H]myristoyl phosphatidic acid and phosphatidylethanol accumulation in HEL cells stimulated by thrombin. Both products increased rapidly within the first 1 min after thrombin addition, after which phosphatidic acid levels slowly declined, whereas phosphatidylethanol continued to increase until 10–15 min. In longer-term experiments (results not shown), phosphatidylethanol reached a plateau by 15 min and remained at this level for at least 30 min in the continued presence of thrombin. The sustained increase in [³H]phosphatidylethanol levels might indicate that this unnatural phospholipid is relatively

Fig. 3. Time course of [³H]phosphatidic acid and [³H]phosphatidylethanol accumulation in thrombin-stimulated HEL cells

HEL cells were prelabelled with [³H]myristic acid, washed, and then incubated for 5 min with ethanol (1%, v/v; ○, ◇, □, △) or without ethanol (control; ●, ◆, ■). Samples were further incubated for the indicated times with thrombin (1 unit/ml) or no additional treatments and then analysed for [³H]phosphatidic acid and [³H]phosphatidylethanol. (a) [³H]Phosphatidic acid: ●, control; ◇, ethanol alone; ●, thrombin alone; ○, thrombin with ethanol. (b) [³H]Phosphatidylethanol: △, ethanol alone; ■, thrombin alone; □, thrombin with ethanol. The untreated control, omitted for clarity, was identical with ethanol alone. Data are means $\pm$ range from duplicate samples.

resistant to further metabolism, although this possibility remains to be tested directly. [³H]Phosphatidylethanol production was also detectable in response to other agonists, as shown in Table 1. Thus HEL cells appear to possess an agonist-activated phospholipase D pathway for phosphatidic acid production, in addition to the classical phospholipase C/diacylglycerol kinase pathway (see above). Thrombin-stimulated [³H]phosphatidic acid production was decreased in the presence of ethanol (Fig. 3), consistent with a diversion of [³H]phosphatidyl residues towards phosphatidylethanol synthesis [28]. However, the possibility that ethanol may inhibit phosphatidic acid accumulation through additional mechanisms should not be overlooked. The presence of phospholipase D was further supported by the glycerol-dependent formation of phosphatidylglycerol [32] in response to thrombin (results not shown).

Phosphatidylethanol accumulation in response to A23187 and phorbol ester

Ca²⁺-mobilizing agonists such as thrombin, which stimulate phosphoinositide turnover, cause both an increase in [Ca²⁺]_i and activation of protein kinase C. Previous studies in other cell types have indicated that phospholipase D activation may be secondary to the activation of protein kinase C [9,13–16]. In HEL cells we

Table 1. Ca^{2+} -dependence of [^3H]phosphatidylethanol production in response to different stimuli

[^3H]Myristic acid-labelled HEL cells were incubated for 10 min in medium containing 1% (v/v) ethanol and either 1 mM- CaCl_2 or 1 mM-EGTA along with the indicated stimuli. Reactions were then stopped and samples analysed for [^3H]phosphatidylethanol. Data are means $\pm$ range from duplicate determinations, with the fold increase over basal shown in parentheses. Abbreviations: PAF, platelet-activating factor; FCS, fetal-calf serum.

Stimulus	[^3H]Phosphatidylethanol (c.p.m.)	
	1 mM- CaCl_2	1 mM-EGTA
None (control)	65 $\pm$ 15 (1.0)	61 $\pm$ 20 (1.0)
A23187 (300 nM)	4434 $\pm$ 28 (68.2)	76 $\pm$ 0 (1.2)
PMA (100 nM)	804 $\pm$ 15 (12.4)	734 $\pm$ 16 (12.0)
Thrombin (1 unit/ml)	507 $\pm$ 20 (7.8)	138 $\pm$ 29 (2.3)
PAF (100 nM)	92 $\pm$ 7 (1.4)	58 $\pm$ 5 (1.0)
Adrenaline (10 μM)	158 $\pm$ 2 (2.4)	66 $\pm$ 1 (1.1)
ADP (10 μM)	109 $\pm$ 3 (1.7)	54 $\pm$ 5 (0.9)
FCS (10%, v/v)	193 $\pm$ 5 (3.0)	62 $\pm$ 0 (1.0)

find that the protein kinase C activator phorbol myristate acetate indeed elicits [^3H]phosphatidylethanol production, as shown in Table 1. The maximal effect of PMA was obtained at a concentration of 10–30 nM (results not shown). Thus an involvement of protein kinase C in phospholipase D activation is likely in this system as well. However, a potentially more significant role for Ca^{2+} mobilization in regulation of phospholipase D is indicated by the striking increase in [^3H]phosphatidylethanol seen in HEL cells stimulated by a low concentration (300 nM) of the Ca^{2+} ionophore A23187 (Table 1). This effect of A23187 was strictly Ca^{2+} -dependent and was not associated with cellular damage (as assessed by Trypan Blue exclusion).

Extracellular Ca^{2+} requirement for agonist-stimulated phosphatidylethanol production

Chelation of extracellular Ca^{2+} with EGTA was found to block [^3H]phosphatidylethanol formation in response to thrombin and other agonists as well as A23187, but not the response to phorbol ester (Table 1). Combined with the marked stimulatory action of A23187, these results suggest that a rise in $[\text{Ca}^{2+}]_i$ is sufficient stimulus for activation of phospholipase D in HEL cells, and that the agonist-linked pathway requires external Ca^{2+} . Furthermore, the lack of sensitivity of phorbol ester-stimulated phosphatidylethanol production to Ca^{2+} removal (as opposed to the agonist-induced response) suggests that protein kinase C activation is not the primary mediator of phospholipase D activation in agonist-stimulated HEL cells. Protein kinase C may, however, contribute to the agonist-induced activation of phospholipase D in a manner similar to the co-ordinate regulation of platelet phospholipase A_2 by Ca^{2+} and protein kinase C [33].

Chelation of extracellular Ca^{2+} as in the previous experiment (Table 1) could suppress agonist-stimulated phospholipase D activity by two potential mechanisms: (a) prevention of a rise in $[\text{Ca}^{2+}]_i$, which is the signal for activation of the enzyme, or (b) depletion of intracellular Ca^{2+} , which is an essential cofactor for the enzyme, but not itself an activator. The latter would be analogous to phosphoinositide-specific phospholipase C, which requires sub-micromolar Ca^{2+} for receptor-dependent activation, but usually is not directly Ca^{2+} -activated within the physiological

Fig. 4. Ca^{2+} -dependence of thrombin-stimulated [^3H]phosphatidylethanol production

[^3H]Myristic acid-labelled cells were preincubated for 5 min in the presence of 1% (v/v) ethanol in normal medium containing 1 mM- CaCl_2 ($\square$, $\triangle$) or in medium containing 100 nM free Ca^{2+} fixed with a Ca/EGTA buffer ($\blacksquare$, $\blacktriangle$) as described under 'Methods'. Samples were further incubated for the indicated times with no additional treatment ($\triangle$, $\blacktriangle$) or with thrombin (1 unit/ml) ($\square$, $\blacksquare$) and then analysed for [^3H]phosphatidylethanol. Data are means $\pm$ range from duplicate determinations.

range of $[\text{Ca}^{2+}]_i$. To distinguish between these possibilities, we compared [^3H]phosphatidylethanol formation in cells incubated in normal (1 mM total Ca^{2+}) medium or in a low- Ca^{2+} medium in which external free $[\text{Ca}^{2+}]$ was fixed at 100 nM (by a Ca/EGTA buffer), a value slightly higher than the resting $[\text{Ca}^{2+}]_i$ in HEL cells (40–70 nM) determined by fura-2 fluorescence [23]. This approach was taken to decrease the transmembrane Ca^{2+} gradient while avoiding the potential depletion of basal $[\text{Ca}^{2+}]_i$. Fig. 4 shows that in medium containing 100 nM free Ca^{2+} thrombin-stimulated phosphatidylethanol formation was abolished at 2 min and greatly inhibited at later times, suggesting that a net influx of extracellular Ca^{2+} is a prerequisite to this agonist.

In summary, the present study demonstrates that HEL cells respond to thrombin and other platelet agonists with a rapid turnover of polyphosphoinositides, 1,2-diacylglycerol production, and phosphatidic acid accumulation, indicating the presence of a phosphoinositide-hydrolysing phospholipase C. However, at least a portion of the phosphatidic acid is probably derived from agonist-induced activation of phospholipase D, as evidence by: (a) the more rapid rise in phosphatidic acid than in diacylglycerol, (b) agonist- and ethanol-dependent phosphatidylethanol production, and (c) inhibition of phosphatidic acid production by ethanol at the same time that phosphatidylethanol is accumulating. The Ca^{2+} -dependency of phospholipase D activation in this system suggests the following sequence of events: receptor occupancy $\rightarrow$ Ca^{2+} influx/mobilization $\rightarrow$ phospholipase D activation $\rightarrow$ phosphatidic acid production. Interestingly, recent reports indicate that phospholipase D activation in another leukaemic cell line, HL-60 cells, is also Ca^{2+} -requiring [10,34]. Previous work by Cockcroft & Allan [35] had suggested that the Ca^{2+} -dependent formation of phosphatidic acid in stimulated neutrophils is derived from a phospholipase D pathway. Further work is needed to determine the mechanism(s) which regulate the activity of phospholipase D, as well as the relationship of this novel enzyme to the other signal-transducing phospholipases.

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