Guanine-nucleotide- and adenine-nucleotide-dependent regulation of phospholipase D in electroporabilized HL-60 granulocytes

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We have characterized the regulation of phospholipase D (PLD) in electroporabilized HL-60 granulocytes in which endogenous phospholipids were pre-labelled with [³H]oleic acid. Treatment of these permeabilized cells with the non-hydrolysable GTP analogues guanosine 5'-[γ-thio]triphosphate (GTP[S]) and guanosine 5'-[βγ-imido]triphosphate induced a sustained (near-linear for up to 60 min) accumulation of phosphatidic acid (PA). In the presence of ethanol a sustained production of phosphatidylethanol (PEt) was also observed. With increasing concentrations of ethanol, PEt formation increased, whereas PA formation declined; this indicated involvement of a PLD-type effector enzyme. The ability of GTP[S] to stimulate this PLD activity was Mg²⁺-dependent and was inhibited by GDP and its non-hydrolysable β-thio analogue. Ca²⁺, at concentrations ≤ nM, had no effect on the GTP[S]-dependent PLD activity. However, higher concentrations of Ca²⁺ produced a significant potentiation of this activity. Inclusion of MgATP (≥ 0.1 mM), but not other nucleoside triphosphates, also induced a large potentiation of GTP[S]-dependent PLD activation. In the absence of guanine nucleotides, MgATP elicited no significant activation of PLD. Significantly, this effect of ATP was not mimicked by adenosine 5'-[βγ-methylene]triphosphate, a non-hydrolysable ATP analogue. Rather, this analogue inhibited both basal and ATP-potentiated GTP[S]-dependent PLD activity. This suggests that the ability of ATP to potentiate GTP[S]-dependent PLD activity involves phosphotransferase action rather than simple allosteric effects induced by adenine nucleotide binding. The absolute magnitude of the GTP[S]-dependent PLD activity which could be potentiated by MgATP was decreased by 90 % when the permeabilized cells were preincubated for various times before addition of these stimulatory agents. This time-dependent loss of MgATP-induced potentiation was prevented when the permeabilized cells were preincubated in the presence of GTP[S]. These results demonstrate that electroporabilized HL-60 granulocytes can be used to discriminate synergistic roles for a GTP-binding protein(s) and an ATP-dependent process (kinase?) in the regulation of phospholipase D activity.

INTRODUCTION

The actions of phospholipase D (PLD)-type phosphodiesterases have been extensively characterized in plant tissues (reviewed in [1]). Similar enzyme activities have been implicated in the formation of phosphatidic acid (PA) in animal cells stimulated by a broad variety of physiological and pharmacological agents [2–8]. There is now substantial evidence which indicates that PLD-type enzymes represent a major effector system underlying receptor-stimulated hydrolysis of phosphatidylcholine (reviewed in [9–12]). PLD enzymes catalyse transphosphatidyl reactions in which the phosphatidyl moiety of the substrate phospholipid is transferred to appropriate nucleophilic acceptors. Under physiological conditions, with water as the nucleophile, such reactions yield PA. However, short-chain alcohols can also be utilized as nucleophiles, thereby allowing PLD-catalysed production of the corresponding phosphatidyl alcohol [1,11,13]. Accumulation of such unique transphosphatidyl products has been used to detect PLD activity unambiguously in diverse cell types [14–30].

In many cell types, PLD is activated by agonists for receptors which also activate phosphatidylinositol-specific phospholipase C (PI-PLC) effectors via G-protein-mediated mechanisms. Phorbol esters have also been shown to activate PLD in a wide range of cell types; this indicates that PLD activation may involve a critical regulatory role for protein kinase C. Moreover, down-regulation of protein kinase C in certain cell types greatly

attenuates the ability of Ca²⁺-mobilizing receptor agonists to stimulate PLD [29]. Such observations have led to the hypothesis that such receptor agonists may indirectly activate PLD subsequent to primary stimulation of a PI-PLC/protein kinase C cascade. However, there is additional evidence which suggests that PLD effector enzymes can be directly activated by G-proteins. Such studies require the use of broken-cell or purified membrane preparations. The PLD activity associated with purified hepatocyte plasma membranes can be stimulated *in vitro* by guanine nucleotides and P₂-purinergic agonists, but not by other receptor agonists (e.g. vasopressin and angiotensin II) [5,6,20]. In this membrane system PLD activation mediated by guanosine 5'-[γ-thio]triphosphate (GTP[S]) is independent of Ca²⁺ when [Ca²⁺] < 0.1 mM; higher Ca²⁺ concentrations are inhibitory. Cell lysates derived from HL-60 cells or rat brain have also been used to demonstrate activation *in vitro* of PLD by GTP[S] [7,16,19]. In homogenates from HL-60 granulocytes, the combined presence of GTP[S] and Ca²⁺ was found to be obligatory for maximal activation of PLD. Neither GTP[S] nor Ca²⁺ alone can support the activity [16]. GTP[S]-stimulated PLD activity has also been observed in saponin-permeabilized endothelial cells [18]. Van Der Meulen & Haslam [31] have recently reported that membranes prepared from phorbol 12-myristate 13-acetate (PMA)-treated platelets contain 18-fold more GTP[S]-dependent PLD activity than that measured in membranes from untreated platelets.

The most straightforward approach to characterizing G-

Abbreviations used: PLD, phospholipase D; PLC, phospholipase C; PI-PLC, phosphatidylinositol-specific PLC; PA, phosphatidic acid; PEt, phosphatidylethanol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; GTP[S], guanosine 5'-[γ-thio]triphosphate; ATP[S], adenosine 5'-[γ-thio]triphosphate; GDP[S], guanosine 5'-[β-thio]diphosphate; p[NH]ppG, guanosine 5'-[βγ-imido]triphosphate; p[CH₂]ppA, adenosine 5'-[βγ-methylene]triphosphate; PMA, phorbol 12-myristate 13-acetate.

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protein regulation of PLD under conditions defined *in vitro* is to perform subcellular fractionation and then test for the presence of both PLD catalytic activity and the relevant regulatory mechanisms in given cell fractions (e.g. cytoplasm versus membranes). However, by analogy to the family of PI-PLC isoenzymes [32], the PLD activities in mammalian cells may represent a family of related isoenzymic species. Moreover, PLD subtypes may be either soluble [33] or membrane-associated [13,30,31]. Precise regulation of such lipases *in situ* by regulatory proteins (e.g. G-proteins) may depend on: (1) the localization of the lipases in micro-environments easily disrupted by lysis of intact cells; or (2) the presence of a high cytosolic concentration (diluted upon lysis) of the lipase. To offset these potentially complicating factors, we have studied PLD regulation in radio-labelled HL-60 cells permeabilized by electroporation [34–36]. Electroporabilized neutrophils and HL-60 cells have been extensively used to characterize the role of guanine nucleotides and other ligands in the regulation of superoxide formation [37–40]. This method permits systematic, but independent, manipulation of the intracellular concentrations of small ($M_r < 1000$) ligands (guanine nucleotides, adenine nucleotides, bivalent cations) which may play roles in the regulation of PLD activity. At the same time, the use of such cells minimizes the loss/dilution of any PLD enzymes and relevant regulatory proteins. In this study, we demonstrate that the regulation of PLD activity in electroporabilized HL-60 granulocytes is strictly dependent on the presence of guanine nucleotides. However, this guanine-nucleotide-dependent activation can be further potentiated by an ATP-driven process.

MATERIALS AND METHODS

Cells

HL-60 cells (obtained from the American Tissue Type Culture Collection) were routinely cultured in Iscove's medium (GIBCO Laboratories, Grand Island, NY, U.S.A.) supplemented with 25 mM-Hepes and 10% (v/v) newborn-calf serum (Hyclone Laboratories, Logan, UT, U.S.A.). The cells were maintained in a humidified atmosphere of air/CO₂ (37:3).

Differentiation of HL-60 cells into neutrophil-like granulocytes

HL-60 cells were transferred to serum-free Iscove's medium supplemented with 25 mM-Hepes, 5 µg of transferrin/ml, 5 µg of insulin/ml and 5 ng of sodium selenite/ml; 500 µM dibutyryl cyclic AMP was included in this medium as differentiating agent [41,42]. The cells were used after a 72 h differentiation period.

Labelling of cellular phospholipids

Approx. 15 h before experimental measurements, [³H]oleic acid (0.3–0.5 µCi/ml) was added directly to the cultured, differentiated cells. Most of the added [³H]oleic acid was incorporated into cellular lipids, with approx. (1–2) × 10⁵ ³H c.p.m. being associated with the total phospholipid pool in 10⁶ cells. The major labelled phospholipids are phosphatidylcholine (PC) and phosphatidylethanolamine (PE). This is consistent with the fact that oleic acid is a predominant fatty acid in the *sn*-2 position of 1,2-diacyl-*sn*-glycero-3-phosphocholine [11].

Preparation of phosphatidylethanol (PEt) standards

PEt standard was prepared by using cabbage PLD and synthetic dipalmitoyl PC by modification of a previously described procedure [13]. In brief, 1.3 mM-dipalmitoyl PC, 0.1 M-sodium acetate buffer, pH 5.6, 37.5 mM-CaCl₂, 0.5 mM-SDS, 3 M-ethanol and 1.5 mg of cabbage PLD were incubated in a total

volume of 5 ml. After a 3 h incubation at 36 °C, the reaction was terminated with 25 ml of chloroform/methanol (2:1, v/v). The tube was vortex-mixed and centrifuged at 500 g for 10 min. The upper and middle phases were re-extracted with an equal volume of chloroform/methanol (2:1, v/v). The two lower phases were combined and dried in a Speed Vac Concentrator. The dried lipids were resuspended in 300 µl of chloroform/methanol (2:1, v/v) and stored at –70 °C. The product of this reaction was primarily PEt and a minor amount of PA.

Separation of PLD reaction products

PEt and PA, the products of PLD-catalysed reactions, were separated from each other and from other lipids by chromatography on 20 cm × 20 cm silica gel 60 glass plates (Universal Scientific, Atlanta, GA, U.S.A.). The developing solvent system was ethyl acetate/iso-octane/acetic acid (9:5:2, by vol.) [20]; this facilitated good separation of PA ($R_f = 0.24$) and PEt ($R_f = 0.36$) from each other, and from neutral lipids, fatty acids ($R_f = 0.75$ –1) and phospholipids ($R_f = 0$).

Electroporabilization of HL-60 cells

Labelled cells were washed twice with ice-cold Ca²⁺-free basal salt solution (BSS) (125 mM-NaCl, 5 mM-KCl, 1 mM-MgCl₂, 25 mM-Hepes, pH 7.5). The cells were then resuspended [(2–3) × 10⁷/ml] in ice-cold basic assay medium (120 mM-potassium glutamate, 20 mM-potassium acetate, 20 mM-NaHepes (pH 7.4), 1 mM-EGTA, 5 mM-glucose, 1 mg of BSA/ml). The cells were permeabilized by subjecting the suspension to nine successive discharges of an electric field (2.5 kV/cm) by using a 4 µF capacitor to provide a time constant of approx. 100 µs [34–36]. The suspension was mixed after every three discharges to ensure uniform permeabilization. Unless otherwise indicated, the permeabilized cells were immediately distributed into reaction tubes for assay of PLD activity. Permeabilization of the cells was validated by several methods, including: (1) uptake of Trypan Blue; (2) staining by the fluorescent probes ethidium bromide and Lucifer Yellow; and (3) release of K⁺ and [³²P]ATP. Studies with ethidium bromide (which fails to stain intact cells) revealed that > 95% of the cells were rendered permeable and remained so throughout a 1 h incubation at 37 °C (results not shown).

PLD assay

PEt is a unique phospholipid generated solely by PLD activity. Thus [³H]oleoyl-PEt, formed in response to agonist stimulation in the presence of ethanol, was measured as the index of PLD activation. Production of [³H]oleoyl-PA, the physiological product of the PLD catalysed reaction, was also determined. However, it should be stressed that net PA accumulation may also reflect production via a PLC/diacylglycerol kinase reaction series and/or degradation via phosphatidate phosphohydrolases. PLD activity in these permeabilized cells was assayed as follows. Immediately after permeabilization, 0.1 ml [approx. (2–3) × 10⁶ cells] of cell suspension was added to 0.9 ml of basic assay medium previously supplemented with appropriate agents and pre-warmed to 37 °C. Free Ca²⁺ concentrations were buffered as previously described [43]. Other additions are indicated in the Figure legends. After the indicated incubation times (generally 60 min), the reaction was stopped by adding 5 ml of chloroform/methanol (2:1, v/v). PA and PEt standards were added to the tubes, and the lipids were extracted and dried as described above. The dried chloroform extracts were resuspended in 50 µl of chloroform/methanol (2:1, v/v), spotted on silica-gel-coated glass plates, and developed in the previously described solvent system. The resolved lipids were located by staining with Coomassie Blue dye (0.03 g of Coomassie Blue/100 ml

of 100 mM-NaCl in 30 % methanol), followed by destaining with 100 mM-NaCl in 30 % methanol [44]. The areas of silica gel containing PEt and PA were scraped and mixed with 3 ml of Bio-Safe II (Research Products International) scintillation fluid. The radioactivity in [^3H]PEt and [^3H]PA was quantitated by liquid-scintillation spectrophotometry.

Materials

Adenosine 5'-[γ -thio]triphosphate (ATP[S]), GTP[S], guanosine 5'-[β -thio]diphosphate (GDP[S]) and guanosine 5'-[β - γ -imido]triphosphate (p[NH]ppG) were from Boehringer Mannheim; all other nucleotides were from Sigma. [9,10(n)- ^3H]oleic acid was from Amersham. L- α -PC (type IX-A) was from Sigma; PA (Na^+) was from Matreya (Pleasant Gap, PA, U.S.A.). All organic reagents were from Fisher. Cell-culture medium was from GIBCO.

Data presentation

In individual experiments, each incubation condition was assayed in duplicate or triplicate. Each experiment was performed two or three times. Unless otherwise indicated, the data presented are from representative experiments.

RESULTS

Guanine-nucleotide-dependent regulation of PLD activity

When electropermeabilized HL-60 granulocytes were incubated in a 'cytosol-like' medium (high [K^+], 1 mM-MgATP, 1 mM free Mg^{2+} and 200 nM free Ca^{2+}) supplemented with 0.4 % ethanol, a 2.5-fold increase in net PA accumulation was observed; this accumulation reached a plateau after 20 min of incubation at 37 °C (Fig. 1). Only very minor PEt formation was measured at various times during the 1 h incubation. In contrast, when the incubation mixture was additionally supplemented with 100 μM -GTP[S], both PEt and PA were produced at a near-linear rate. Under these incubation conditions, the GTP[S]-stimulated rate of PEt accumulation was at least 20-fold greater than the control rate. This kinetic profile is consistent with the possibility that a sustained stimulation of PLD effector enzyme was driven by a G-protein irreversibly activated by this phosphatase-resistant GTP analogue. GTP[S] (100 μM) had no effect on PEt and PA accumulation in cells which were not subjected to high-voltage discharge (results not shown).

In the absence of ethanol, GTP[S]-dependent [^3H]PA formation was increased 4-fold over control during a 60 min incubation (Fig. 2); no [^3H]PEt accumulation was observed. With increasing concentrations of ethanol in the reaction medium, progressively larger amounts of PEt were produced in the GTP[S]-treated cells. In contrast, [^3H]PA formation declined. In the control reactions lacking GTP[S], only minor [^3H]PEt accumulation could be measured in the presence of ethanol at concentrations up to 1.5 %. These results demonstrate that GTP[S] induced stimulation of a PLD-type activity which can catalyse transphosphatidylolation of either water or alcohol. It should be noted that, at any given concentration of ethanol, the summed radioactivity associated with both GTP[S]-stimulated products ([^3H]PEt and [^3H]PA) was considerably greater than that observed in the absence of ethanol. Presumably this is due to the greater metabolic stability of PEt than of PA.

Fig. 3 illustrates the concentration-response relationship characterizing GTP[S]-induced activation of PLD. Modest accumulation of both PEt and PA was observed in the presence of 1 μM -GTP[S]. The accumulation of both products continued to increase as GTP[S] concentration was raised to 100 μM (the

Fig. 1. Time course characterizing GTP[S]-stimulated PLD activity in electropermeabilized HL-60 granulocytes

Permeabilized cells were incubated for the indicated times at 37 °C in basic assay medium, supplemented with 0.4 % ethanol, 200 nM- Ca^{2+} , 2 mM- MgCl_2 , and 1 mM-ATP. Incubations were performed in either the absence (Δ , $\square$) or presence ($\blacktriangle$, $\blacksquare$) of 100 μM -GTP[S]. PEt ($\blacktriangle$, Δ) and PA ($\blacksquare$, $\square$) were assayed and quantified as described in the Materials and methods section. Each point represents the average ($\pm$ range) of duplicate determinations; in most cases, the range bars are smaller than the symbols. In this experiment, each incubation vial contained 2.5×10^6 cells which had incorporated 223 400 c.p.m. of [^3H]oleic acid into the bulk phospholipid pool.

Fig. 2. Differential effects of ethanol on the GTP[S]-dependent formation of PEt and PA by electropermeabilized HL-60 granulocytes

Permeabilized cells were incubated for 60 min at 37 °C in basic assay medium, supplemented with 200 nM- Ca^{2+} , 2 mM- MgCl_2 , and 1 mM-ATP. Incubations were performed in either the absence (Δ , $\square$) or presence ($\blacktriangle$, $\blacksquare$) of 100 μM -GTP[S]. The assay media also contained the indicated concentrations of ethanol. PEt ($\blacktriangle$, Δ) and PA ($\blacksquare$, $\square$) were assayed and quantified as described in the Materials and methods section. Each point represents the average ($\pm$ range) of duplicate determinations; in most cases, the range bars are smaller than the symbols. In this experiment, each incubation vial contained 2.1×10^6 cells which had incorporated 165 000 c.p.m. of [^3H]oleic acid into the bulk phospholipid pool.

highest value tested). We also tested the effects of other guanine nucleotides on the regulation of PLD activity in these electropermeabilized cells (Table 1). Only minor PEt formation was

Fig. 3. Concentration-response relationship characterizing the stimulation of PLD activity in electroporabilized HL-60 granulocytes by GTP[S]

Permeabilized cells were incubated for 60 min at 37 °C in basic assay medium, supplemented with 0.4% ethanol, 200 nM- Ca^{2+} , 2 mM- MgCl_2 and the indicated concentrations of GTP[S]. Incubations were performed in either the absence (Δ , $\square$) or presence ($\blacktriangle$, $\blacksquare$) of 1 mM-ATP. PEt ($\blacktriangle$, Δ) and PA ($\blacksquare$, $\square$) were assayed and quantified as described in the Materials and methods section. Each point represents the average ($\pm$ range) of duplicate determinations; in most cases, the range bars are smaller than the symbols. In this experiment, each incubation vial contained 2.5×10^6 cells which had incorporated 205 400 c.p.m. of [^3H]oleic acid into the bulk phospholipid pool.

Fig. 4. Concentration-response relationship characterizing the ATP-induced potentiation of GTP[S]-dependent PLD activity in electroporabilized HL-60 granulocytes

Before permeabilization, cells were depleted of endogenous ATP by treatment with 5 μM -antimycin A and 6 mM-deoxyglucose for 5 min at 37 °C. Permeabilized cells were incubated for 60 min at 37 °C in basic assay medium, supplemented with 0.5% ethanol, 200 nM- Ca^{2+} , 2 mM- MgCl_2 and the indicated concentrations of MgATP. Incubations were performed in either the absence (Δ , $\square$) or presence ($\blacktriangle$, $\blacksquare$) of 100 μM -GTP[S]. PEt ($\blacktriangle$, Δ) and PA ($\blacksquare$, $\square$) were assayed and quantified as described in the Materials and methods section. Each point represents the average ($\pm$ range) of duplicate determinations; in most cases, the range bars are smaller than the symbols. In this experiment, each incubation vial contained 2.5×10^6 cells which had incorporated 215 600 c.p.m. of [^3H]oleic acid into the bulk phospholipid pool.

Table 1. Effects of guanine nucleotides on PLD activity

Permeabilized cells were incubated for 60 min at 37 °C in basic assay medium, supplemented with 0.4% ethanol, 200 nM free Ca^{2+} , 2 mM- MgCl_2 , 1 mM-ATP and the indicated concentrations of guanine nucleotides. PEt and PA were assayed and quantified as described in the Materials and methods section. Each value represents the average ($\pm$ range) of duplicate determinations. In Expt. 1, each incubation vial contained 2.5×10^6 cells which had incorporated 205 400 c.p.m. of [^3H]oleic acid into the bulk phospholipid pool; in Expt. 2, each incubation was performed with 2.5×10^6 cells which had incorporated 242 000 c.p.m. of [^3H]oleic acid into the bulk phospholipid pool.

Expt.	Additions	PLD activity (^3H c.p.m./60 min per sample)	
		PEt	PA
1	Control	338 $\pm$ 9	556 $\pm$ 8
	GTP[S] (100 μM)	4270 $\pm$ 283	2401 $\pm$ 227
	p[NH]ppG (100 μM)	1280 $\pm$ 43	1174 $\pm$ 22
	GTP (100 μM)	444 $\pm$ 10	802 $\pm$ 15
	GTP (1 mM)	465 $\pm$ 7	734 $\pm$ 9
2	Control	390 $\pm$ 2	859 $\pm$ 5
	GDP[S] (1 mM)	499 $\pm$ 10	660 $\pm$ 21
	GTP[S] (10 μM)	4651 $\pm$ 121	4896 $\pm$ 203
	GTP[S] (10 μM) + GDP[S] (1 mM)	1174 $\pm$ 58	2515 $\pm$ 195
	GTP[S] (100 μM)	6306 $\pm$ 210	4468 $\pm$ 156
	GTP[S] (100 μM) + GDP[S] (1 mM)	2942 $\pm$ 59	3809 $\pm$ 71

elicited in response to either 0.1 mM- or 1 mM-GTP. However, a 50% increase in PA was observed. p[NH]ppG (0.1 mM) stimulated both PEt and PA formation, but was only 30% as efficacious as an equimolar concentration of GTP[S]. GDP[S] at 1 mM, though ineffective by itself, substantially decreased

GTP[S]-activated PEt formation. Inclusion of 1 mM-GTP and GDP in the assay medium also induced a decrease in the GTP[S]-stimulated PLD (results not shown).

Role of ATP in the regulation of guanine-nucleotide-dependent PLD activity

It is evident from the concentration-response relationship illustrated in Fig. 3 that the ability of GTP[S] to stimulate PLD activity was greatly potentiated when 1 mM-MgATP was included in the assay medium. The effects of ATP on GTP[S]-dependent PLD activity was characterized more completely in the experiment shown in Fig. 4. In these studies, the endogenous ATP derived from the electroporabilized cells was first decreased by pre-treating the cells with antimycin A and 2-deoxyglucose in glucose-free assay medium. Lu & Grinstein [40] have previously demonstrated that this protocol greatly decreases the residual ATP in electroporabilized neutrophils, by >95%. In these ATP-depleted cells, 100 μM -GTP[S] consistently produced a 5–10-fold increase in the net rate of PEt accumulation, as well as a smaller 2-fold increase in PA synthesis (Figs. 3 and 6, Table 1). Addition of 10 μM -MgATP did not further potentiate such stimulation. However [MgATP] $\geq$ 100 μM induced a significant potentiation of the GTP[S]-dependent accumulation of both PEt and PA. PEt, but not PA, synthesis was near-maximal with 0.5 mM-MgATP. In the absence of GTP[S], no significant amount of PEt was formed even when 1–5 mM-MgATP was included in the incubation medium; under these same conditions, a low amount of PA was synthesized. As noted above, the PA may also be derived via the PI-PLC/diacylglycerol kinase pathway. Millimolar concentrations of pyrimidine nucleoside triphosphates (UTP, CTP) could not substitute for ATP in potentiating GTP[S]-dependent PLD activity (results not shown).

Table 2. Effects of adenine nucleotides on PLD activity

Permeabilized cells were incubated for 60 min at 37 °C in basic assay medium, supplemented with 0.5 % ethanol, 200 nM free Ca^{2+} , 2 mM- MgCl_2 and the indicated concentrations of adenine or guanine nucleotides. PET was assayed and quantified as described in the Materials and methods section. Each value represents the average ($\pm$ range) of duplicate determinations; in Expt. 1, each incubation vial contained 2.5×10^6 cells which had incorporated 215 600 c.p.m. of [^3H]oleic acid into the bulk phospholipid pool; in Expt. 2, each incubation was performed with 2.5×10^6 cells which had incorporated 124 300 c.p.m. into the bulk phospholipid pool.

Expt.	Additions	PLD activity ([^3H] c.p.m.-PET/ 60 min per sample)
1	Control	660 $\pm$ 1
	GTP[S] (100 μM)	1632 $\pm$ 46
	ATP (1 mM)	700 $\pm$ 20
	ATP (1 mM)	13966 $\pm$ 145
	+ GTP[S] (100 μM)	
	p[CH ₂]ppA (1 mM)	865 $\pm$ 2
	p[CH ₂]ppA (1 mM)	1084 $\pm$ 15
	+ GTP[S] (100 μM)	
	p[CH ₂]ppA (1 mM)	4976 $\pm$ 30
	+ ATP (1 mM) + GTP[S] (100 μM)	
	ATP[S] (100 μM)	2066 $\pm$ 13
2	Control	456 $\pm$ 2
	GTP[S] (100 μM)	1494 $\pm$ 25
	ATP[S] (1 mM)	2702 $\pm$ 271
	ATP[S] (1 mM) + GTP[S] (100 μM)	3657 $\pm$ 204

Fig. 5. Effects of Mg^{2+} on GTP[S]-dependent PLD activity in electroporated HL-60 granulocytes

Permeabilized cells were incubated for 60 min in basic assay medium, supplemented with 0.5 % ethanol, 200 nM- Ca^{2+} , 1 mM- MgATP and the indicated concentrations of MgCl_2 . For the '0 Mg^{2+} ' incubations, the assay medium contained 1 mM-EDTA, 1 mM-ATP and no added MgCl_2 . Incubations were performed in either the absence (Δ , $\square$) or presence ($\blacktriangle$, $\blacksquare$) of 100 μM -GTP[S]. PET ($\blacktriangle$, Δ) and PA ($\blacksquare$, $\square$) were assayed and quantified as described in the Materials and methods section. Each point represents the average ($\pm$ range) of duplicate determinations; in most cases, the range bars are smaller than the symbols. In this experiment, each incubation vial contained 2.5×10^6 cells which had incorporated 203 300 c.p.m. of [^3H]oleic acid into the bulk phospholipid pool.

We also tested (Table 2) the effect of two ATP analogues: (1) adenosine 5'-[β -methylene]triphosphate (p[CH₂]ppA) which is non-hydrolysable, and (2) ATP[S], which, although phosphatase-resistant, can serve as a substrate for certain kinases. At 1 mM, p[CH₂]ppA decreased, by about 50 %, the GTP[S]-stimulated PET accumulation measured in the absence of MgATP . More significantly, the potentiated GTP[S]-dependent PLD activity observed in the presence of 1 mM- MgATP was inhibited by > 70 % when the assay medium also contained 1 mM-p[CH₂]ppA. The effects of ATP[S] were more complicated. ATP[S], in the absence of added GTP[S], stimulated PET formation in a dose-dependent manner. However, ATP[S] did not potentiate GTP[S]-dependent PLD activity. Rather, the PET formation stimulated by ATP[S] was additive to that stimulated by GTP[S]. Although ATP showed dramatic potentiation of PLD activation by GTP[S] (Fig. 4), 1 mM-ATP totally blocked PLD activation by 0.1 mM-ATP[S] (Table 1). These various observations suggest that the mechanism underlying ATP[S]-stimulated PLD activation is distinct from that underlying GTP[S]-dependent activity. Thus it is unlikely that the effects of ATP[S] are due solely to trans-thiophosphorylation of endogenous GDP to GTP[S].

Role of bivalent cations in the regulation of guanine-nucleotide-dependent PLD activity

Since Mg^{2+} is critical for stable GTP[S]/G-protein interaction [45], we tested the effect of this cation on GTP[S]-induced stimulation of PLD (Fig. 5). In the presence of 1 mM-EDTA and 1 mM-ATP (but in the absence of added Mg^{2+}), GTP[S] induced formation of a small amount of PET; PA formation was negligible. The GTP[S]-dependent formation of both PET and PA was greatly increased at $[\text{Mg}^{2+}] \geq 500 \mu\text{M}$. PET formation was maximal at 5 mM- Mg^{2+} . In the absence of GTP[S], Mg^{2+} (in the 0–10 mM range) had no effect on PET accumulation. Conversely, GTP[S]-independent PA formation was increased when cells were incubated in the presence of millimolar Mg^{2+} .

Ca^{2+} is known to exert significant effects on activities of many phospholipase effector enzymes both *in vitro* and *in vivo*. All data presented thus far were obtained from assays in which the free $[\text{Ca}^{2+}]$ was maintained at 200 nM, the approximate basal $[\text{Ca}^{2+}]$ in intact HL-60 cells [46]. The effects of Ca^{2+} on GTP[S]-dependent and GTP[S]-independent PLD activity in electroporated HL-60 cells are detailed in Fig. 6; 1 mM-EGTA was used to buffer Ca^{2+} . In the presence of 1 mM-EGTA and no added Ca^{2+} ($[\text{Ca}^{2+}] < 1 \text{ nM}$), GTP[S] was still able to produce a 10-fold stimulation of PET accumulation. Moreover, at concentrations $\leq 200 \text{ nM}$, Ca^{2+} also had no effect on GTP[S]-stimulated PET formation. Conversely, $[\text{Ca}^{2+}] \geq 1 \mu\text{M}$ produced a substantial potentiation of the GTP[S]-dependent accumulation of both PET and PA. PET accumulation appeared to show a biphasic dependence on Ca^{2+} in this concentration range, with an initial plateau in the physiological range (10 μM) and a second plateau in the millimolar range. Significantly, in the absence of GTP[S], there was only minor accumulation of either PET or PA by permeabilized cells exposed to $[\text{Ca}^{2+}]$ ranging from 1 nM to 5 mM.

Time-dependent decrease in the magnitude of guanine-nucleotide-regulated PLD activity after electroporation of HL-60 cells

In all experiments described thus far, the permeabilized cells were diluted into assay medium (containing the various activators) within 1 min after exposure to high-voltage discharge. In the course of developing this experimental protocol, we noted that the absolute amount of GTP[S]-stimulated PET (or PA) accumulation was invariably lower when permeabilized cells

Fig. 6. Effects of Ca^{2+} on GTP[S]-dependent PLD activity in electropermeabilized HL-60 granulocytes

Permeabilized cells were incubated for 60 min in basic assay medium, supplemented with 0.5% ethanol, 1 mM-EGTA, 2 mM- MgCl_2 , 1 mM-MgATP and sufficient CaCl_2 to yield the indicated concentrations of free Ca^{2+} . Incubations were performed in either the absence (Δ , $\square$) or presence ($\blacktriangle$, $\blacksquare$) of 100 μM -GTP[S]. PEt ($\blacktriangle$, Δ) and PA ($\blacksquare$, $\square$) were assayed and quantified as described in the Materials and methods section. Each point represents the average ($\pm$ range) of duplicate determinations; in most cases, the range bars are smaller than the symbols. In this experiment, each incubation vial contained 2.5×10^6 cells which had incorporated 229 900 c.p.m. of [^3H]oleic acid into the bulk phospholipid pool.

were stored for various times before samples were transferred into assay medium. This suggested a time-dependent decrease or 'inactivation' of the GTP[S]-dependent PLD activity. Accordingly, experiments were designed to characterize this phenomenon in greater detail. HL-60 cells were permeabilized, diluted into the basic assay medium (lacking GTP[S], ATP and Ca^{2+}), and then incubated at 37 °C for various times before the simultaneous addition of 100 μM -GTP[S], 1 mM-MgATP and sufficient CaCl_2 to yield 1 μM free Ca^{2+} . The cells were then incubated for an additional 60 min at 37 °C. Fig. 7 shows that there was a rapid decrease in the ability of GTP[S] (in the presence of MgATP) to stimulate PEt accumulation. This decrease was essentially complete (90%) within 10 min. Permeabilized cells preincubated for 20 or 30 min showed only minor further decreases in activity. It should be noted that the residual (i.e. remaining after > 10 min preincubation) PLD activity was sufficient to catalyze a 4-fold increase in PEt accumulation in response to 100 μM -GTP[S], 1 mM-MgATP and 1 μM - Ca^{2+} . As previously noted, MgATP and micromolar Ca^{2+} greatly potentiate GTP[S]-dependent PLD activity when electropermeabilized cells are simultaneously exposed to these stimuli. We also designed experiments to test whether the time-dependent decrease/inactivation of regulated PLD activity noted in Fig. 7 was due primarily to a loss/inactivation of GTP[S] action or the loss/inactivation of the potentiating effects of MgATP/ Ca^{2+} (Table 3). In this experiment, a 20 min preincubation of permeabilized cells before the (simultaneous) addition of 100 μM -GTP[S], 1 mM-MgATP and 1 μM - Ca^{2+} was accompanied by a 90% decrease in PEt accumulation relative to that observed when these agents were simultaneously added immediately after permeabilization. Permeabilized cells which were exposed to 100 μM -GTP[S], but no MgATP, during this initial 20 min preincubation period, as well as during the subsequent 60 min incubation, showed a 3-fold increase in PEt accumulation (similar to that illustrated in Fig. 3). However,

Fig. 7. Time-dependent decrease in the magnitude of GTP[S]-dependent PLD activity after electropermeabilization of HL-60 granulocytes

Cells were permeabilized in basic assay medium containing 1 mM-EGTA, 2 mM- MgCl_2 and 0.5% ethanol. The cells were then preincubated at 37 °C for the indicated times before addition of 100 μM -GTP[S], 1 mM-MgATP and sufficient CaCl_2 to yield 1 μM free Ca^{2+} . The cells were then incubated for an additional 60 min at 37 °C. PEt ($\bullet$, $\circ$) and PA ($\blacksquare$, $\square$) were assayed and quantified as described in the Materials and methods section: $\bullet$, $\blacksquare$, data from cells incubated with GTP[S] and MgATP; $\circ$, $\square$, data from control permeabilized cells incubated for 60 min in the absence of these agents. Each point represents the average ($\pm$ range) of duplicate determinations; in most cases, the range bars are smaller than the symbols. In this experiment, each incubation vial contained 3×10^6 cells which had incorporated 403 300 c.p.m. of [^3H]oleic acid into the bulk phospholipid pool.

Table 3. Retention of regulated PLD activity by GTP[S] treatment of electropermeabilized HL-60 cells

Cells were permeabilized in basic assay medium containing 1 mM-EGTA and 2 mM- MgCl_2 . The cells were then incubated at 37 °C. Portions of the permeabilized cells were then supplemented at the indicated times (after permeabilization) with 100 μM -GTP[S], 1 mM-MgATP, or sufficient CaCl_2 to yield 1 μM free Ca^{2+} . Reactions were terminated at either 60 min or 80 min after permeabilization. PEt was assayed and quantified as described in the Materials and methods section. Each point represents the average ($\pm$ range) of duplicate determinations. In this experiment, each incubation vial contained 3×10^6 cells which had incorporated 403 300 c.p.m. of [^3H]oleic acid into the bulk phospholipid pool; c.p.m. in PEt have been normalized per 100 000 c.p.m. in bulk phospholipid.

Addition		Reaction terminated (time after permeabilization)	PEt accumulation (^3H c.p.m./10 ⁵ c.p.m. total phospholipid)
($t = 0$ min)	($t = 20$ min)		
–	–	60 min	209 $\pm$ 1
GTP[S]/MgATP/ Ca^{2+}	–	60 min	6658 $\pm$ 156
–	–	80 min	220 $\pm$ 10
–	GTP[S]/MgATP/ Ca^{2+}	80 min	865 $\pm$ 111
GTP[S]	–	80 min	671 $\pm$ 20
GTP[S]	MgATP/ Ca^{2+}	80 min	4440 $\pm$ 95

when these GTP[S]-treated cells were additionally exposed (after the 20 min preincubation) to 1 mM-MgATP and 1 μM - Ca^{2+} , there was a 19-fold increase in PEt accumulation; this was only 30% lower than the net PEt accumulation observed in the perme-

abilized cells which were immediately treated with GTP[S], MgATP and $1\text{ }\mu\text{M}$ - Ca^{2+} . Thus GTP[S] pre-treatment appears greatly to attenuate the loss/inactivation of regulated PLD activity which occurs during prolonged incubation of electropermeabilized HL-60 cells.

DISCUSSION

The use of electropermeabilized HL-60 granulocytes has allowed us to identify separate, but complementary, roles for guanine-nucleotide- and adenine-nucleotide-dependent processes in the regulation of PLD. Our results suggest that guanine nucleotides play an obligatory role in the regulation of PLD activity in these cells. Conversely, ATP appears to activate proteins/reactions which, in themselves, do not activate PLD, but rather act to potentiate the guanine-nucleotide-dependent regulation of the effector enzyme. In addition, Ca^{2+} ions act further to modulate positively the guanine-nucleotide- and adenine-nucleotide-dependent activation of the phospholipase.

Role of guanine nucleotides

The observed activation of PLD by guanine nucleotides is presumably mediated by a GTP-binding regulatory protein (G-protein). Several sets of data support this hypothesis. (1) The introduction of non-hydrolysable GTP analogues (GTP[S] and p[NH]ppG) into electropermeabilized HL-60 granulocytes induces a pronounced and persistent stimulation of PA formation and, in the presence of ethanol, the accumulation of PET. (2) The action of GTP[S] was highly dependent on the presence of Mg^{2+} in the millimolar range. At very low free Mg^{2+} , the magnitude of GTP[S]-induced accumulation of either PA or PET was measurable but small. There was a sharp increase in GTP[S]-regulated PLD activity as free Mg^{2+} was elevated from 0.5 to 5 mM. In the absence of guanine nucleotide, there was little change in PLD activity over this range of $[\text{Mg}^{2+}]$. This type of Mg^{2+} -dependence is consistent with the mode of G-protein action wherein millimolar Mg^{2+} is required for GTP[S]-induced G-protein subunit dissociation, with attendant formation of stable, biologically active, complexes of α -subunit-GTP[S]- Mg^{2+} [45]. It is likely that Mg^{2+} may play an additional role in the regulatory action of ATP discussed below. (3) The stimulatory effect of GTP[S] was attenuated in the presence of GDP and GDP[S]. These last two nucleoside diphosphates have been shown to attenuate or inhibit a wide variety of processes known (or presumed) to be regulated by both heterotrimeric and non-oligomeric G-proteins. It is also important to note that, in the presence of GTP[S] (or p[NH]ppG), significant increases in PLD activity could be observed in the virtual absence of either adenine nucleotides (Figs. 3 and 4) or free Ca^{2+} ions (Fig. 6). Thus the presumed G-protein involved in this phenomenon appears to be both necessary and sufficient to activate the phospholipase D effector.

Role of adenine nucleotides

Our results indicate that ATP plays an important role in potentiating the GTP[S]-dependent activation of PLD in HL-60 cells. It should be stressed that, in the absence of guanine nucleotides, ATP had no effect on the accumulation of PET and induced only a very modest accumulation of PA (Fig. 4); this latter observation may reflect production of PA via diacylglycerol kinase action. Several mechanisms may underlie the ability of ATP to potentiate GTP[S]-dependent activation of PLD. ATP might play a role in maintaining suitable levels of the major phospholipid substrate(s) for PLD. The action of ATP in maintaining adequate levels of polyphosphoinositides during incubations *in vitro* of permeabilized cells [47] or isolated membranes [48] has been extensively characterized. However,

previous studies have indicated that choline-containing (both 1,2-diacyl-PC and 1-*O*-alkyl-2-acyl-PC) [6,9-12,17] or ethanolamine-containing [49,50] phospholipids are the principal substrates for PLD in HL-60 cells and most other cells. This appears to be true regardless of whether endogenous or exogenous phospholipids are utilized as substrates. Choline-containing phospholipids constitute approx. 60% of the total phospholipid pool in HL-60 granulocytes [11]. Our previous studies (M. Xie, L. S. Jacobs & G. R. Dubyak, unpublished work) have indicated that such choline-containing lipids constitute 50% of the total phospholipid pool labelled with [^3H]oleic acid. Moreover, we find that less than 5% of the total [^3H]oleic acid-labelled phospholipid pool is metabolized during a 60 min incubation of permeabilized HL-60 granulocytes in the presence of $100\text{ }\mu\text{M}$ -GTP[S], 200 nM - Ca^{2+} and 1 mM -MgATP (results not shown). Thus it is unlikely that ATP potentiates PLD activity as a result of maintaining suitable substrate levels.

We [43,46,51] and others [52,53] have reported that HL-60 cells express P_2 -purinergic receptors which, when occupied by ATP or certain other nucleotides, can activate rapid hydrolysis of phosphoinositides by G-protein-dependent mechanisms. We have also determined that occupation of these ATP receptors in intact HL-60 cells induces a rapid activation of PLD; this effect of ATP is eliminated in pertussis-toxin-treated cells (M. Xie, L. S. Jacobs & G. R. Dubyak, unpublished work). Therefore, the ability of ATP to potentiate GTP[S]-dependent PLD activity in the permeabilized HL-60 cells might occur as a result of occupation of these G-protein-coupled P_2 -purinergic receptors. Agonist occupation of such receptors would accelerate GDP/GTP[S] exchange by the coupled G-protein(s). The ability of ATP to potentiate guanine-nucleotide-dependent PLD activity in hepatocyte membranes [6] and in saponin-permeabilized endothelial cells [18] has been ascribed to the occupation of similar P_2 -purinergic receptors. However, several lines of evidence from the present study suggest that such receptors are not responsible for the effects of ATP observed in permeabilized HL-60 cells. First, UTP is equipotent to, and as efficacious as, ATP in activating both PI-PLC [43,51] and PLD (M. Xie, L. S. Jacobs & G. R. Dubyak, unpublished work) in intact HL-60 granulocytes. Conversely, UTP (0.01 – 1 mM) induced no potentiation of GTP[S]-dependent PET/PA accumulation in the permeabilized cells. Likewise, ATP[S] is as efficacious as ATP in activating PLD in intact cells (M. Xie, L. S. Jacobs & G. R. Dubyak, unpublished work), but is considerably less effective than ATP in potentiating PLD activity in the permeabilized cells (Table 2).

It is more likely that the ability of ATP to modulate PLD activity positively may involve an ATP-driven phosphotransferase reaction (e.g. kinase or phosphohydrolase), rather than an occupation of P_2 -purinergic receptors. This hypothesis is supported by a number of observations. First, ATP-induced stimulation was highly dependent on the presence of millimolar $[\text{Mg}^{2+}]$. Such a Mg^{2+} -dependence is characteristic of most kinases and ATPases, although, as noted above, Mg^{2+} is also required for optimal G-protein activation. Second, the non-hydrolysable ATP analogue p[CH $_2$]ppA was unable to mimic the stimulatory effect of ATP. Rather, in the presence of p[CH $_2$]ppA there was substantial inhibition of ATP-potentiated GTP[S]-dependent PLD activity. In this regard, p[CH $_2$]ppA may compete with ATP for nucleotide-binding sites on the putative ATP-utilizing regulatory enzyme. Kiss & Anderson [50] have similarly demonstrated that ATP, but not non-hydrolysable ATP analogues, can stimulate a PE-specific PLD activity in membranes derived from NIH 3T3 cells; this action of ATP was potentiated in the presence of GTP[S].

A more complicated pattern of results was observed when PLD was monitored in the presence of ATP[S]. Although poorly metabolized by phosphohydrolases, this ATP analogue can serve as substrate for kinases. Even in the absence of added guanine nucleotides, ATP[S] activated PLD. Several lines of evidence suggested that the mechanism whereby ATP[S] activates PLD was distinct from that underlying GTP[S]. First, the activation of PLD by ATP[S] was decreased, rather than potentiated, in the presence of excess ATP. Second, co-incubation of the permeabilized cells with both ATP[S] and GTP[S] produced an additive effect on PLD activity. Finally, the ATP[S]-induced stimulation observed in the presence of a 10-fold molar excess of GDP[S] was decreased by only 25 %; this contrasted with the > 60 % decrease in GTP[S]-stimulated activity measured in the presence of GDP[S]. H.p.l.c. analysis of the ATP[S] used in these studies indicated no contamination by GTP[S] or other guanine nucleotides; the major contaminant was ADP (~ 10 %). These results suggest that ATP[S] may provide substrate for the thiophosphorylation of amino acid residues on proteins; such thiophosphorylated sites are generally more resistant than oxyphosphorylated sites to the action of protein phosphatases [54]. In the presence of vigorous phosphatase activity, ATP[S], but not ATP, may facilitate increased steady-state levels of key phosphorylated sites on protein(s) involved in regulation of PLD activity.

Thus, our results indicate that optimal activation of PLD in HL-60 cells appears to involve both guanine-nucleotide- and adenine-nucleotide-dependent regulatory proteins. It is noteworthy that Grinstein and colleagues [37–40] have demonstrated that the ability of GTP[S] to activate superoxide release in electroporabilized neutrophils and HL-60 cells is extremely dependent on the presence of ATP. Moreover, ATP[S], vanadate (in the presence of ATP) or GTP[S] (also in the presence of ATP) can independently induce tyrosine phosphorylation and superoxide generation. Thus the regulation of both PLD and the NADPH oxidase in permeabilized phagocytes involves a combination of G-protein-regulated- and ATP-regulated mechanisms. It remains to be determined whether the regulation of these disparate functions involves a common set of G-proteins or ATP-dependent enzymes.

Role of Ca^{2+} ions

Incubation of intact cells with Ca^{2+} ionophores results in enhanced PLD activity [9,15,24]. Receptor-induced activation of PLD in neutrophils and HL-60 cells has been shown to be highly dependent on the extracellular $[\text{Ca}^{2+}]$. In addition, Ca^{2+} (in the millimolar ranges) greatly potentiated the GTP[S]-stimulated PLD activity observed in homogenates from HL-60 granulocytes [16]. In the electroporabilized HL-60 granulocytes, we observed that Ca^{2+} itself had little effect on PLD activity in the absence of GTP[S]. Likewise, $[\text{Ca}^{2+}]$ less than 200 nM did not affect GTP[S]-dependent PLD activity. Conversely, when the $[\text{Ca}^{2+}]$ was raised to levels present in agonist-stimulated cells (1–10 μM), a 2.5-fold potentiation of the GTP[S]-dependent activity was noted (Fig. 6). Virtually identical effects of Ca^{2+} on GTP[S]-dependent PLD activity have been observed in membranes isolated from rabbit platelets [31]. Such effects of Ca^{2+} on PLD stand in sharp contrast to the Ca^{2+} -dependence characterizing guanine-nucleotide-dependent PI-PLC activity in HL-60 membranes [43,55], streptolysin-permeabilized HL-60 cells [56] or electroporabilized GH_3 cells [30]. Thus these data indicate that GTP[S] can activate PLD in permeabilized HL-60 cells under conditions where there is minimal or no activation of the PI-PLC signalling cascade. GTP[S]-induced activation of phosphatidylcholine breakdown (by either PLC- or PLD-type reactions) in hepatocyte membranes has also been reported to be

independent of $[\text{Ca}^{2+}]$ below 0.1 mM [5,6]. Such results lend further support to the hypothesis [12] that PLD can be directly regulated by G-proteins by mechanisms which are independent of either PI-PLC activation or Ca^{2+} mobilization.

Lability of guanine-nucleotide-dependent PLD activity in electroporabilized cells

Electroporabilization protocols similar to that used in this study have been used to characterize the regulation of superoxide release [37–40] and secretion [57] in a variety of phagocyte cell types. We selected this particular method of cell permeabilization in order to prevent or minimize the loss of soluble regulatory/catalytic macromolecules which might be involved in PLD-type signalling. Indeed, HL-60 cells permeabilized by our electroporation protocol retained at least 80 % of the total soluble protein that can be released by digitonin-induced permeabilization (results not shown). Thus we were surprised to observe that the absolute magnitude of the GTP[S]-dependent MgATP-potentiated PLD activity rapidly ($t_{1/2} = 5\text{--}6$ min) decreased by approx. 90 % when the electroporabilized cells were pre-incubated before addition of GTP[S] and MgATP (Fig. 7). This is not due to re-sealing of the pores, since the electroporabilized cells remain hyper-permeable to ethidium bromide (M_r 394, versus M_r 584 for ATP and M_r 563 for GTP[S]) throughout this incubation. Given the relatively small membrane lesions produced by electroporation, it is also unlikely that this decrease in activity is due to an actual loss (to the extracellular space) of PLD effector enzymes or regulatory G-proteins/kinases. Rather, this phenomenon is indicative of a functional uncoupling or desensitization of the relevant regulatory processes. It is noteworthy that Smolen & Sandborg [57] have reported that Ca^{2+} -dependent ATP-potentiated secretion from electroporated neutrophils is similarly desensitized/uncoupled upon washing the cells. The 'desensitization' processes observed in our studies may be due to reversal of covalent modifications (e.g. dephosphorylation) on key regulatory or catalytic proteins. Alternatively, optimal interaction of the various macromolecules involved in PLD regulation may involve topographical relationships (e.g. subcellular localization of the PLD effector enzymes) which are perturbed upon electroporation. Significantly, when GTP[S] was present during this preincubation period, the ability of MgATP to potentiate PLD activity was largely preserved. These observations indicate that the respective actions of GTP[S] and MgATP on PLD activity can be resolved into two distinct regulatory processes. One can hypothesize that the presence of GTP[S] induces rapid stabilization of a membrane-associated G-protein/PLD effector complex. In the absence of MgATP, there is relatively low catalytic activity by this putative G-protein/PLD effector complex. The rate of catalysis via the PLD effector is increased by MgATP, acting through either a regulatory kinase or an energy-providing phosphotransferase.

It is tempting to speculate that PLD regulation in HL-60 cells and, perhaps, other cell types may involve amphipathic PLD effector enzymes which can be 'translocated' to, or variably associated with, membranes containing the putative regulatory G-proteins and phosphotransferases. As previously noted, membranes prepared from PMA-treated platelets have been reported to contain 18-fold more GTP[S]-dependent PLD activity than is found in membranes from untreated platelets [31]. In related studies (M. Xie & G. R. Dubyak, unpublished work), we have noted that membranes prepared from HL-60 granulocytes (pre-labelled with $[\text{^3H}]$ oleic acid) contain only minor amounts of GTP[S]-dependent PLD activity even when this activity is assayed in the presence of various concentrations of MgATP, Ca^{2+} or detergents (oleate, cholate). Similar to membranes from PMA-treated platelets [31], membranes prepared from PMA-treated

HL-60 cells contain 3–4 times more GTP[S]-dependent activity. Even more intriguing is our observation that washed membranes, prepared from permeabilized GTP[S]-treated HL-60 cells, contain 10-fold higher amounts of a MgATP-dependent PLD activity. Such phenomena lend further support to the hypothesis that PLD activity in various cell types may be co-ordinately, but differentially, regulated by G-proteins, kinases and Ca^{2+} .

This work was supported in part by NIH grant GM-36387. G. R. D. is an Established Investigator of the American Heart Association.

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Received 10 December 1990/20 March 1991; accepted 5 April 1991