Regulation of phospholipase D and primary granule secretion by P2-purinergic- and chemotactic peptide-receptor agonists is induced during granulocytic differentiation of HL-60 cells.

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Research Article

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Regulation of Phospholipase D and Primary Granule Secretion by P_2-Purinergic- and Chemotactic Peptide-Receptor Agonists Is Induced during Granulocytic Differentiation of HL-60 Cells

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Abstract

We have compared the abilities of extracellular ATP (acting via P_2-purinergic receptors) and formylated peptides (FMLP) to stimulate both phospholipase D (PLD)-based signal transduction and primary granule (azurophilic) secretion in HL-60 cells induced to differentiate along the granulocytic pathway. In undifferentiated HL-60 cells, neither ATP nor FMLP elicited significant PLD activation or increased secretion despite the previously documented ability of ATP to stimulate large increases in polyphosphoinositide hydrolysis and Ca^{2+} mobilization. Conversely, within 1 d after induction of granulocytic differentiation by dibutyryl cAMP, both ATP and FMLP induced large increases in azurophilic secretion and corresponding increases in PLD activity. ATP-activated PLD activity was near-maximal within 1 d after dibutyryl cAMP treatment, while the FMLP-induced activity increased continuously over 4 d, with a maximal level twice that stimulated by ATP. Additional experiments characterized the activation of PLD by receptor-independent pathways at different stages of differentiation; these included studies of phorbol ester action in intact cells and GTPγS action in electropermeabilized cells. An apparent role for guanine nucleotide-binding regulatory proteins in PLD regulation was also indicated by the significant reduction in FMLP- and ATP-stimulated PLD activity observed in cells pretreated with pertussis toxin. At all stages of differentiation, there was good correlation between the relative efficacies of ATP versus FMLP in stimulating both secretion and PLD activity. These data indicate: (*a*) that the receptor-regulated phospholipase D signaling pathway is induced during differentiation of myeloid progenitor cells; and (*b*) that differential activation of this signaling system by various Ca^{2+}-mobilizing receptor agonists may underlie the differential regulation of secretion and other phagocyte functions by such agents. (*J. Clin. Invest.* 1991. 88:45–54.) Key words: phagocytes • signaling • phospholipases • secretion

Introduction

The HL-60 human promyelocytic leukemia cell line can be terminally differentiated into neutrophil-like cells by a variety of inducing agents, such as dibutyryl cyclic AMP, DMSO, and retinoic acid (reviewed in reference 1). Such cells exhibit the majority of cell-specific functions characteristic of normal neutrophils. These include chemotaxis, lysosomal enzyme secretion, phagocytosis, agonist-stimulated superoxide release, and bacterial killing (2). Differentiation is also accompanied by expression of cell surface receptors for formylated chemotactic peptides (e.g., FMLP) (2), leukotriene B_4 (LTB_4) (3), and platelet-activating factor (PAF)[1] (4). All of these latter agonists can activate (albeit with varying efficacies) the various neutrophil-specific functions. Moreover, the receptors for these three agonists are functionally coupled to the phosphatidylinositol-specific phospholipase C (PI-PLC) effector enzyme(s) (4, 5).

We and others have previously demonstrated that human neutrophils and other phagocytic cells express a subtype from the family of so-called P_2-purinergic receptors for extracellular ATP (6–8). Occupation of these receptors induces inositol phospholipid hydrolysis and Ca^{2+} mobilization in differentiated HL-60 cells and neutrophils (6–10). Significantly, these receptors for ATP, in contrast with the receptors for FMLP, LTB_4, and PAF, are expressed in undifferentiated HL-60 cells, as well as other myeloid progenitor cells (6). The kinetics and magnitudes characterizing ATP-induced inositol triphosphate (IP_3) accumulation and Ca^{2+} mobilization are similar in undifferentiated and differentiated (by dibutyryl cAMP) HL-60 cells (6, 9–11). In differentiated HL-60 cells, ATP and FMLP are equally efficacious in stimulating PI-specific phospholipase C activity and Ca^{2+} mobilization (6, 7, 9). However, ATP, LTB_4, and PAF are considerably less efficacious than FMLP in stimulating superoxide generation or primary granule secretion in human neutrophils and differentiated HL-60 granulocytes (3–5, 7, 10, 12–13). These and other observations suggest that the activation of PI-PLC signaling cascades may play a necessary, but not sufficient, role in the differential stimulation of neutrophil responses by various Ca^{2+}-mobilizing receptor agonists.

Recent attention (reviewed in references 14 and 15) has been directed towards defining the role of phosphatidylcholine and other choline-containing lipids in receptor-mediated signal transduction. In certain cells, diglycerides can be derived from choline phospholipids either by a direct phospholipase C-type reaction or by an indirect pathway involving a primary production of phosphatidic acids by phospholipase D (PLD)-type catalysis (16–17). Studies using phagocytic leukocytes (neutrophils and differentiated HL-60 cells) have provided some of the most compelling evidence for the existence of physiologically significant PLD-based signaling pathways (17–23). Recent

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1. *Abbreviations used in this paper:* BSS, basal salt solution; G-proteins, guanine nucleotide binding regulatory proteins; PA, phosphatidic acid; PAF, platelet-activating factor; PC, phosphatidylcholine; PEt, phosphatidylethanol; PI-PLC, phosphatidylinositol-phospholipase C; PLD, phospholipase D; PMA, phorbol myristate acetate.

studies have indicated that receptor-stimulated PLD activity may play an essential role in the functional activation of superoxide generation in human neutrophils (23). Our study had two aims. The first was to determine whether differentiation of HL-60 cells along the granulocytic pathway is accompanied by altered expression of receptor-dependent and receptor-independent pathways for phospholipase D regulation. The second aim was to establish whether differentiation-related changes in the magnitude of receptor-dependent phospholipase D activity can be correlated with differentiation-related changes in receptor-dependent release of azurophilic secretory granules.

Methods

Cells. HL-60 cells (obtained from the American Type Culture Collection, ATCC) were routinely cultured in Iscove's medium (Gibco Laboratories, Grand Island, NY) supplemented with 25 mM Hepes and 10% calf bovine serum (HyClone Laboratories, Logan, UT). The cells were maintained in a humidified atmosphere of 92.5% air, 7.5% CO_2. It should be noted that these experiments were performed over an 18-mo period using HL-60 cells derived from four different frozen stocks. Each stock was passaged and used for no more than 4 mo.

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/ml transferrin, 5 μg/ml insulin, and 5 ng/ml sodium selenite. 500 μM dibutyryl cAMP was included in this medium as a differentiating agent (2). Functional responses in such cells were assayed at various times (1–4 d) after induction of differentiation.

Isotopic labeling of cellular lipids. 15–18 h before experiments, [^{3}H]glycerol (5 μCi/ml) or [^{3}H]oleic acid (0.3–0.5 μCi/ml) was added directly to cell cultures. Under these conditions, > 90% of the added [^{3}H]oleic acid was incorporated into chloroform-soluble cellular lipids; ~ 100,000–150,000 cpm/10^6 cells was associated with the total phospholipid pool. [^{3}H]glycerol labeling was less efficient with ~ 10,000 cpm/10^6 cells being incorporated into total phospholipids. Phosphatidylcholine (50%) and phosphatidylethanolamine (30%) were the predominant species labeled with either [^{3}H]glycerol or [^{3}H]oleic acid.

Preparation of standard phosphatidylethanol. Phosphatidylethanol (PEt) standard was prepared using cabbage PLD and synthetic dipalmitoyl phosphatidylcholine (PC) by modification of a previously described method (24). In brief, 1.3 mM dipalmitoyl PC, 0.1 M Na acetate buffer, pH 5.6, 37.5 mM $CaCl_2$, 0.5 mM SDS, 3 M ethanol, and 1.5 mg of cabbage PLD were incubated in a total vol of 5 ml. After a 3-h incubation at 36°C, the reaction was terminated with 25 ml chloroform/methanol (2:1). The tube was vigorously mixed and then centrifuged at 1,500 rpm for 10 min. The upper and middle phases were reextracted with an equal volume of chloroform/methanol (2:1). The two lower phases were combined and dried by vacuum centrifugation (Speed Vac 200 Concentrator; Savant Instruments, Inc., Hicksville, NY). The dried lipids were resuspended in 300 μl chloroform/methanol (2:1) and stored at −70°C. Greater than 90% of the product was PEt with phosphatidic acid (PA) constituting the remaining 10% of product mass.

Separation of PLD reaction products. The products of PLD-catalyzed reactions (PEt and PA) were resolved by thin-layer chromatography on 20 × 20-cm silica gel 60 glass plates (Universal Scientific, Inc., Atlanta, GA). Plates were developed with a solvent system consisting of ethyl acetate/iso-octane/acetic acid (9:5:2) (25). This system provided adequate resolution of PA (R_f = 0.24) and PEt (R_f = 0.36) from each other, from neutral lipids/fatty acids (R_f = 0.75–1), and from the major cellular phospholipids (R_f = 0).

Electropermeabilization of HL-60 cells. Labeled cells were washed twice with ice-cold Ca^{2+}-free basal salt solution (BSS) (125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 25 mM Hepes, pH 7.5). The cells were resuspended in ice-cold K glutamate buffer (120 mM K glutamate, 20 mM K acetate, 20 mM NaHepes, pH 7.4, plus 1 mM EGTA, 5 mM glucose, 1 mg/ml BSA) to a concentration of 2 × 10^7 cells/ml. The cells were permeabilized by subjecting the suspension to nine successive discharges of 2.5 kV/cm from a 4-μf capacitor (under these conditions the capacitative discharge was characterized by a 100-μs time constant (26). Cells were manually resuspended after groups of three discharges. Aliquots of the permeabilized cell suspensions were immediately distributed into reaction tubes.

Phospholipase D assay. All PLD-type enzymes (or putative enzymes) studied thus far can also catalyze unique transphosphatidylation reactions whereby short-chain alcohols (e.g., ethanol) can serve as phosphatidyl acceptors to yield metabolically stable, but physiologically "inert," phosphatidyl alcohols (15, 24). Consistent with this "signature" reaction are previous findings that, upon activation by Ca^{2+}-mobilizing agonists in the presence of short-chain alcohols, differentiated HL-60 cells and neutrophils show large increases in phosphatidyl alcohol content at the expense of reduced phosphatidic acid accumulation. Thus, the accumulation of either [^{3}H]oleoyl-labeled PEt or [^{3}H]-glycerol-labeled PEt was measured as the definitive index of PLD activation in this study. Labeled cells were washed twice with Ca^{2+}-free BSS (125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 25 mM Na-Hepes, pH 7.5) and then resuspended in BSS supplemented with 1 mM $CaCl_2$, 5 mM glucose, 1 mg/ml BSA to the desired cell density. The reaction vol was 1 ml which contained either 10^7 [^{3}H]glycerol-labeled cells or 2.5 × 10^6 [^{3}H]oleic acid-labeled cells. The cells were preincubated with cytochalasin B (5 μM, unless otherwise indicated) in the presence or absence of ethanol (0.5% or 85 mM, unless otherwise indicated) for 5 min at 37°C. The reaction was initiated by the addition of 2–10 μl of appropriately concentrated agonist. For studies using electropermeabilized cells, the reaction protocol was as follows: 0.1 ml permeabilized cells was added to 0.9 ml K-glutamate buffer previously supplemented with the various agents to be tested and prewarmed to 37°C. For both intact cell and permeabilized cell studies, reactions were stopped, after appropriate incubation times, by the addition of 5 ml chloroform/methanol (2:1). Phosphatidic acid and phosphatidylethanol standards (0.5–2 μg) were also added to the extracts. The lipids were extracted, dried by vacuum centrifugation, and then redissolved in 50 μl chloroform/methanol (2/1). These samples were spotted as 10-mm lines on the silica gel plates. After development as described previously, the lipids were located by staining with Coomassie blue dye (0.03 g Coomassie blue per 100 ml of 100 mM NaCl in 30% methanol) followed by destaining with 100 mM NaCl in 30% methanol (27). The silica gel areas containing PEt, phosphatidic acid, and bulk cell phospholipid (at origin) were removed, resuspended in 5 ml scintillation fluid (Bio-Safe II; Research Products International Corp., Mount Prospect, IL), and thoroughly mixed. After extraction for 24 h, the radioactivity in ^{3}H-PEt and ^{3}H-PA was quantitated by liquid scintillation spectrophotometry.

Secretion assay. Release of myeloperoxidase from azurophilic granules was determined as the index of secretion (28). HL-60 cells were washed twice with ice-cold Ca^{2+}-free BSS and then resuspended at 4 × 10^6 cells/ml in BSS supplemented with 1 mM $CaCl_2$, 5 mM glucose, 1 mg/ml BSA. The cell suspension (250 μl aliquots) was distributed into 1.5-ml centrifuge tubes and then preincubated in the presence of 5–10 μM cytochalasin B for 5 min at 37°C. Secretion was initiated by the addition of 5–10 μl aliquots of appropriately concentrated secretagogues and was terminated by rapid cooling of the reaction tubes in ice-water, followed by centrifugation at 1,500 rpm for 10 min at 4°C. Immediate rapid centrifugation (12,000 *g;* 15 s) was used to stop secretion after very short (< 1 min) incubations. A 100-μl aliquot of each cell-free supernatant was transferred into a 13 × 100-mm glass test tube for assay of myeloperoxidase activity. The total cellular content of myeloperoxidase was determined by treating the cells with 0.2% Triton X-100 before centrifugation. Myeloperoxidase activity was assayed by adding 1 ml substrate solution (0.1 M Na citrate buffer, pH 5.5/0.1% *o*-dianisidine in ethanol/1 mM H_2O_2, 10:1:1, plus 0.05% Triton X-100) to the 100-μl sample of cell supernatent or whole cell extract. The samples were incubated for 1 min at room temperature and the reaction was stopped by addition of 1 ml 35% perchloric acid. After brief centrifugation to remove any precipitated protein, the absorbance

at 560 nm was measured. Secretion data are expressed as the percentage of total cell myeloperoxidase released to the extracellular medium.

Data presentation. All assays in individual experiments were performed in duplicate or triplicate. Each experiment was repeated two to three times. Data points in most figures and tables represent the mean±standard error or the average±range of results from two to three similar experiments.

Results

Phospholipase D regulation during differentiation of HL-60 cells. Fig. 1 *A* illustrates the profiles of phospholipase D activation by two Ca^{2+}-mobilizing receptor agonists, ATP and FMLP, that are observed in [^{3}H]-oleic acid–labeled HL-60 cells at various stages of dibutyryl cAMP-induced differentiation. 100 μM ATP and 1 μM FMLP were used to ensure near-maximal occupancy of their respective receptors. These reactions were also performed in the presence of 0.5% ethanol to facilitate generation of the ^{3}H-phosphatidylethanol product (Fig. 1 *A*) via PLD-catalyzed transphosphatidylation. The basal ^{3}H-cpm associated with the PEt band in unstimulated cells largely represented nonspecific background radioactivity since it was also observed in cells incubated in the absence of ethanol (data not shown). In undifferentiated HL-60 cells, neither 100 μM ATP nor 1 μM FMLP induced significant PEt formation. Conversely, within 1 d after induction of differentiation, both ATP and FMLP stimulated significant PEt accumulation. In these "1-d" granulocytes, the magnitudes of PEt formation elicited by ATP and FMLP were approximately equal (fivefold stimulation over basal). The relative magnitude of ATP-stimulated PEt accumulation was not significantly increased in cells treated with dibutyryl cAMP for longer (2–4 d) times. This contrasted with the progressive increase in FMLP-stimulated PEt formation observed in the same preparations of differentiated cells. Thus, there was a significant difference between the relative efficacies characterizing ATP- versus FMLP-stimulated PEt production in cells differentiated in vitro for progressively longer times. By day two, the magnitude of PEt formation triggered by FMLP was only slightly higher than that elicited by ATP. On day 3, FMLP induced 1.7- to 2-fold more PEt accumulation than ATP. After 4 d, FMLP stimulated approximately twofold more PEt production than that observed in the ATP-treated cells. Corresponding changes in ATP- and FMLP-stimulated PA accumulation were observed at similar stages of dibutyryl cAMP-induced differentiation (data not shown). Unstimulated cells (both undifferentiated and differentiated) contained significant amounts of [^{3}H]-oleic acid–labeled PA and the absolute amount of radioactivity associated with these basal PA levels varied by a factor of 3 between experiments. In undifferentiated HL-60 cells, ATP induced a 1.5-fold increase in PA production while no change was triggered by FMLP. This is not surprising since undifferentiated HL-60 cells express negligible numbers of FMLP receptors (29). We have previously (9) demonstrated that these undifferentiated cells respond to micromolar ATP with a large increase in PI-PLC–mediated inositol polyphosphate accumulation. Therefore, the PA production elicited by ATP in such cells may be due to phosphorylation of diacylglycerol generated by PI-PLC. After dibutyryl cAMP-induced differentiation, FMLP also elicited substantial PA formation while the accumulation triggered by ATP was further increased. Table I summarizes data on the differentiation-related changes in receptor-dependent PLD activity as observed in cells prelabeled with [^{3}H]-glycerol versus [^{3}H]-oleic acid. Similar profiles of ATP- and FMLP-stimulated PEt accumulation were observed regardless of which radiolabeled metabolite was used.

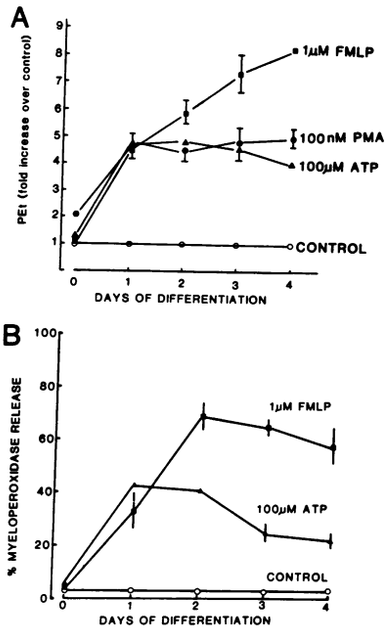

Figure 1. Changes in receptor-mediated PLD activation and primary granule secretion in dibutryl cAMP-induced differentiation of HL-60 cells. (*A*) HL-60 cells were cultured in the absence or presence of 500 μM dibutyryl cAMP. At various times after induction of differentiation, aliquots of the cells were removed and cellular phospholipids were labeled with ^{3}H-oleic acid as described in Methods. Labeled cells were washed and prepared for the assay of receptor- and phorbol ester–stimulated PLD activity. In each assay, cells were pretreated with 5 μM cytochalasin B for 5 min. 2.5 × 10^6 cells were aliquoted into individual tubes containing 0.5% ethanol and either no agonist (○), 100 μM ATP (▲), 1 μM FMLP (■), or 100 nM PMA, (●). ATP- and FMLP-stimulated cells were then incubated at 37°C for 2 min while PMA-stimulated cells were incubated for 15 min. Lipids were extracted, dried, and analyzed as described in Methods. For each experiment, the ^{3}H-PEt was expressed as fold-stimulation over control. Values represent the average±range of data from two separate cell preparations. The respective amounts of radioactivity associated with PEt in the different control cell extracts were as follows (in ^{3}H-cpm/10^5 cpm of total phospholipid): Day 0: 208 (±30); day 1: 152 (±34); day 2: 306 (±97); day 3: 213 (±10); day 4: 171 (±79). (*B*) HL-60 cells were cultured in the absence or presence of 500 μM dibutyryl cAMP. At various times after induction of differentiation, aliquots of the cells were removed, washed, and prepared for the assay of receptor-stimulated myeloperoxidase release as described in Methods. Cells were pretreated with 5 μM cytochalasin B for 5 min. 1 × 10^6 cells were aliquoted into each tube containing either no agonist (○), 100 μM ATP (▲), or 1 μM FMLP (■). After 10 min incubation at 37°C, tubes were transferred on ice and centrifuged. Supernatants were analyzed for myeloperoxidase release. Data points represent the average±range of results from two separate cell preparations.

The above data indicate that receptor-regulated phospholipase D activity increases during differentiation. Billah et al. (19) have demonstrated that phorbol esters (e.g., phorbol myristate acetate, PMA) can also activate phospholipase D in differentiated (by DMSO) HL-60 cells by a mechanism that is only partially attenuated by protein kinase C inhibitors. They also reported that undifferentiated HL-60 cells exhibited much lower levels of this PMA-stimulated PLD activity. We tested whether a similar increase in receptor-independent, PMA-stimulated PLD activity can be observed during dibutyryl cAMP-induced differentiation. Since the rate of PMA-induced PLD activation is considerably slower than receptor-induced activation (18), the labeled cells were incubated with PMA for

Table I. Comparison of ATP- versus FMLP-Stimulated PLD Activity in HL-60 Cells Labeled with ^{3}H-Glycerol or ^{3}H-Oleic Acid

Cell type	Label	N	PLD activity			Fold-increase over basal	
			Basal	ATP	FMLP	ATP	FMLP
			cpm in PEt/10^5 cpm total phospholipid				
Undifferentiated	Glycerol	3	120±13	147±43	151±72	1.2	1.2
Differentiated (3 d)	Glycerol	6	88±34	530±130	1024±184	6.0	11.6
Undifferentiated	Oleic Acid	3	116±37	156±52	107±34	1.3	0.9
Differentiated (2 d)	Oleic Acid	4	77±9	332±13	428±42	4.3	5.6
Differentiated (3 d)	Oleic Acid	8	91±4	359±12	618±46	3.9	6.8

HL-60 cells were cultured for 2 or 3 d in the absence or presence of 500 μM dibutyryl cAMP. 15–18 h before experiments, the cultures were supplemented with either (^{3}H)-glycerol or (^{3}H)-oleic acid. Cells were isolated, washed, and assayed for PEt accumulation as described in methods. All incubations were performed at 37°C in the presence of 5 μM cytochalasin B and 0.5% ethanol. Cells were stimulated for 2 min with either FMLP (1 μM) or ATP (100 μM). For individual experiments, the radioactivity associated with PEt was normalized to the total radioactivity associated with the bulk phospholipid pool. Data show the mean (±standard error) of results from the indicated number (*N*) experiments.

15 min rather the 2-min exposure to ATP or FMLP. Fig. 1 *A* shows that the rate of phorbol ester (PMA)-activated PEt accumulation, like receptor-activated PEt accumulation, is very low in undifferentiated HL-60 cells. However, within 1 d after induction of differentiation, there was a fivefold increase in the rate of PMA-activated PEt release. This rate did not further increase in cells differentiated for longer periods of time.

Earlier studies (9–11) have indicated that pretreatment of HL-60 cells with pertussis toxin can very substantially attenuate the activation of PI-PLC by P_2-purinergic- and FMLP receptors. We have previously demonstrated (11) that incubation of these cells with toxin (0.1–1 μg/ml) for prolonged periods (15–18 h) is required to produce near-complete ADP-ribosylation of the predominant 40–41-kD toxin substrate (the alpha subunits of G_{i-2} and G_{i-3}). The ability of FMLP to stimulate PEt accumulation was completely inhibited in such toxin-treated cells (Table II). ATP-induced PEt accumulation was also significantly, but incompletely (70% inhibition) reduced. This inhibition of ATP-induced signal transduction is consistent with our previous observations that P_2-purinergic agonists can activate a twofold increase in $InsP_3$ accumulation and substantial Ca^{2+} mobilization in pertussis-intoxicated HL-60 cells containing < 1% of the normal content unmodified $G_{i-2/3}$ (9, 11). FMLP- and ATP-induced PEt accumulation were also reduced by 88% and 65%, respectively, in cells treated for 2 h with 300 ng/ml pertussis toxin. These data suggest an obligatory role for guanine nucleotide-binding regulatory proteins (G-proteins) in the receptor-mediated activation of PLD in these cells. Additional studies with hepatocyte membranes (30), HL-60 cell lysates (31, 32), and saponin-permeabilized endothelial cells (33) have also indicated suggested that G-proteins can activate phospholipase D-catalyzed reactions in nonintact cell preparations. In related studies (Xie, M., and G. R. Dubyak, unpublished observations), we have characterized a similar role for putative G-proteins in PLD regulation by using HL-60 cells permeabilized by electroporation. Table III shows PLD activation by calcium and GTPγS (a nonhydrolyzable GTP analogue) in electropermeabilized HL-60 cells. In permeabilized, undifferentiated cells, 200 nM Ca^{2+} alone had no effect on PEt formation while either 1 mM Ca^{2+} alone, or 200 nM Ca^{2+} plus 100 μM GTPγS, induced formation of low, but detectable, amounts of PEt. 1 mM Ca^{2+} plus 100 μM GTPγS acted synergistically to stimulate the accumulation of fivefold more PEt than was observed with either agent alone. In fully differentiated (3 d post dibutyryl cAMP) cells, the absolute magnitude of PEt formed in response to any of the above stimuli was increased 9- to 10-fold. PA levels also increased proportionately in response to these stimuli (data not shown). It is noteworthy that free [Ca^{2+}] as high as 1 mM has relatively low efficacy in stimulating PLD activity in these cells, given that 1 mM Ca^{2+} maximally acti-

Table II. Effects of Pertussis Toxin Pretreatment on FMLP- and ATP-Stimulated PLD Activities in Differentiated HL-60 Cells

Preincubation	Assay conditions	PEt accumulation
		cpm/10^5 cpm total PL/2 min (N)
Control	Control	126±13 (3)
	FMLP (1 μM)	535±96 (3)
	ATP (100 μM)	274±19 (3)
Pertussis toxin	Control	98±28 (2)
300 ng/ml	FMLP (1 μM)	118±22 (2)
2 h	ATP (100 μM)	149±4 (2)
Pertussis toxin	Control	100±29 (3)
100 ng/ml	FMLP (1 μM)	97±20 (3)
18 h	ATP (100 μM)	148±15 (3)

HL-60 cells were cultured for 3 d in the presence of 500 μM dibutyryl cAMP. 18 h before experiments, the cultures were supplemented with (^{3}H)-oleic acid and divided into 2–3 equal aliquots. 1 aliquot was immediately supplemented with 100 ng/ml pertussis toxin. In two experiments, a second aliquot was supplemented with 300 ng/ml pertussis toxin 2 h before assay of PLD activity. Cells were isolated, washed, and assayed for PEt accumulation as described in Methods. All incubations were performed at 37°C in the presence of 5 μM cytochalasin B and 0.5% ethanol. Cells were stimulated for 2 min with either FMLP (1 μM) or ATP (100 μM). For individual experiments, the radioactivity associated with PEt was normalized to the total radioactivity associated with the bulk phospholipid pool. Data show the mean (±standard error) or average (±range) of results from (*N*) experiments.

Table III. Calcium- and Guanine Nucleotide-dependent PLD Activities in Electroporated HL-60 Cells

Incubation conditions	Cell type	PEt accumulation
		cpm/10⁵ cpm total PL/60 min
Control	Undifferentiated	97±34
	Differentiated	213±61
200 nM Ca^{2+}	Undifferentiated	85±16
	Differentiated	244±50
200 nM Ca^{2+} plus 100 μM GTPγs	Undifferentiated	208±37
	Differentiated	2,053±74
1 mM Ca^{2+}	Undifferentiated	196±41
	Differentiated	941±42
1 mM Ca^{2+} plus 100 μM GTPγs	Undifferentiated	627±83
	Differentiated	5,883±328

HL-60 cells were cultured for 3 d in the absence or presence of 500 μM dibutyryl cAMP. 15–18 h before experiments, the cultures were supplemented with (^{3}H)-oleic acid. Cells were isolated, electroporated, and assayed for PEt and phosphatidic acid accumulation as described in Methods. All incubations were performed at 37°C in the presence of 0.5% ethanol and 1 mM MgATP. Cells were incubated for 60 min in the presence of the indicated concentrations of free Ca^{2+} and/or GTPγs. For individual experiments, the radioactivity associated with PEt was normalized to the total radioactivity associated with the bulk phospholipid pool. Data shown represent the mean (±standard error) of three separate experiments.

vates PI-PLC in similarly permeabilized and treated HL-60 cells (Xie, M., and G. R. Dubyak, unpublished observation).

Activation of primary granule secretion during differentiation of HL-60 cells. Fig. 1 *B* illustrates the profiles of ATP- and FMLP-induced primary granule secretion during differentiation of HL-60 cells. The percentage release of total myeloperoxidase under various conditions was used as an index of such secretion, given that both undifferentiated and differentiated HL-60 cells contain similar amounts of this primary granule enzyme. In undifferentiated cells, very little (< 4%) release of myeloperoxidase was elicited by either 100 μM ATP or 1 μM FMLP (Fig. 1 *B* and Table IV). Conversely, within 1 d after induction of differentiation, ATP could elicit the rapid release of ~ 40% of total myeloperoxidase content while FMLP stimulated the secretion of ~ 30% of this enzyme. By day two, occupation of FMLP receptors induced secretion of 70–75% of the enzyme while the response to ATP was unchanged from that observed on day 1. After 3–4 d of differentiation, the maximal secretory responses to either FMLP or ATP were modestly decreased relative to the values observed on day 2.

Table IV. Comparison of ATP- versus FMLP-stimulated Primary Granule Release

Cell type	Percentage release of total myeloperoxidase		
	Control	ATP(UTP)	FMLP
Undifferentiated	1.4±0.1 (5)	4.3±0.6 (5)	2.3±0.3 (3)
Differentiated	3.4±0.6 (16)	26.1±2.4 (16)	60.8±4.0 (16)

HL-60 cells were cultured for 3 d in the absence or presence of 500 μM dibutyryl cAMP. Cells were isolated, washed, and assayed for myeloperoxidase release as described in Methods. All incubations were performed at 37°C in the presence of 5 μM cytochalasin B. Cells were stimulated for 10 min with either FMLP (1–3 μM), ATP (30–100 μM), or UTP (30–100 μM). Data show the mean (±standard error) or results from the indicated number (in parentheses) experiments.

Comparison of PLD activation and primary granule secretion elicted by ATP and FMLP in differentiated HL-60 granulocytes. Stimulation of HL-60 granulocytes with either ATP or FMLP (in the presence of 85 mM ethanol) resulted in the rapid (maximal within 60 s) accumulation of PEt after a brief lagtime (Fig. 2 *A*). When assays were performed in the absence of ethanol, both agonists induced a rapid (75% maximal at 30 s)

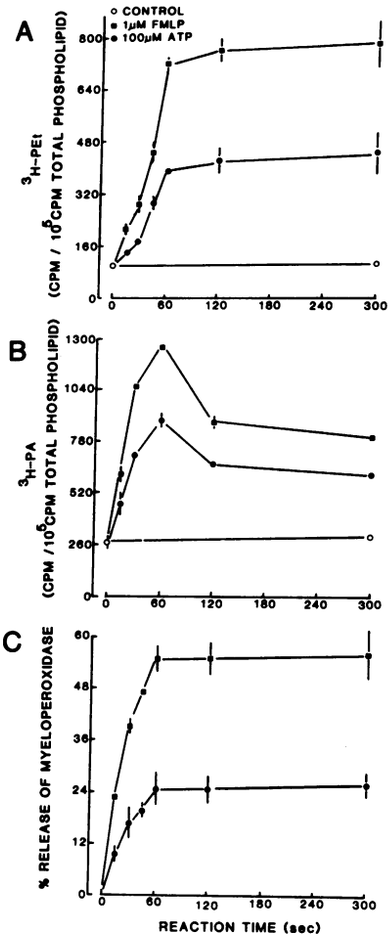

Figure 2. Kinetics of PLD activation and primary granule secretion by ATP and FMLP in differentiated HL-60 granulocytes. (*A*) PEt accumulation. 3-d differentiated HL-60 cells were labeled with ^{3}H-oleic acid as described in Methods. Cells were washed and pretreated with 5 μM cytochalasin B. 2.5 × 10^6 cells were aliquoted into tubes containing 0.5% ethanol and either 100 μM ATP (●), 1 μM FMLP (■), or no agonist (○). After incubation for various times at 37°C, reaction was stopped; lipids were extracted, dried, and analyzed by TLC. PEt accumulation was normalized relative to the total amount of ^{3}H in the bulk phospholipids. Data points represent the average±range of results from two separate preparations. (*B*) PA accumulation. Assays were performed as above but in the absence of added ethanol. PA accumulation was normalized to the total amount ^{3}H in the bulk phospholipids. Data points represent the average±range of results from two separate preparations. (*C*) Primary granule secretion. 3-d differentiated HL-60 cells were pretreated with 5 μM cytochalasin B for 5 min. 1 × 10^6 cells were aliquoted into tubes containing either 100 μM ATP (●) or 1 μM FMLP (■). After incubation for various times at 37°C, tubes were transferred on ice and rapidly centrifuged. Supernatants were analyzed for myeloperoxidase content as described in Methods. Results were presented as percentage release of total (i.e., released by Triton X-100) content of cellular myeloperoxidase. Data points represent the average±range of results from two separate preparations.

production of ^{3}H-labeled PA (Fig. 2 *B*). The maximal levels of PEt formed in response to either agonist remained constant for at least 5 min. However, after peaking at 60 s, PA levels decreased by ~ 50% during the next 5 min. Although the time courses characterizing ATP- and FMLP-induced PLD activation were similar, the maximal levels of PEt or PA formed in response to ATP was 40–50% less than that elicited by FMLP. Fig. 2 *C* illustrates the time course of primary granule secretion as stimulated by 100 μM ATP or 1 μM FMLP. As for PLD activation, these agonists induced a similarly rapid release of myeloperoxidase attaining a maximum at 60 s. Longer incubations did not result in further enzyme release. Similar rates and extents of ATP- and FMLP-induced secretion were observed when β-glucouronidase release, rather than myeloperoxidase release, was monitored (not shown).

The concentration-response relationships characterizing ATP- and FMLP-stimulated phosphatidylethanol formation are illustrated in Fig. 3 *A*. The threshold concentrations for ATP- and FMLP-stimulated PEt formation were ~ 0.3 μM and 1 nM, respectively. ATP and FMLP produced near-maximal stimulation at 30 μM and 1 μM, respectively. These profiles suggest that both ATP and FMLP activate HL-60 cell PLD by receptor-mediated mechanisms. Moreover, these concentration-response relationships are similar to those characterizing PI-PLC activation by ATP and FMLP (11). Additional studies (not shown) revealed that the nucleotide selectivity characterizing P_2-purinergic receptor-stimulated PLD activity was similar to that characterizing nucleotide-induced activation of PI-PLC and Ca^{2+} mobilization (6, 7, 34) in these cells. Significantly, UTP and ATP were equally potent and efficacious in activating PLD.

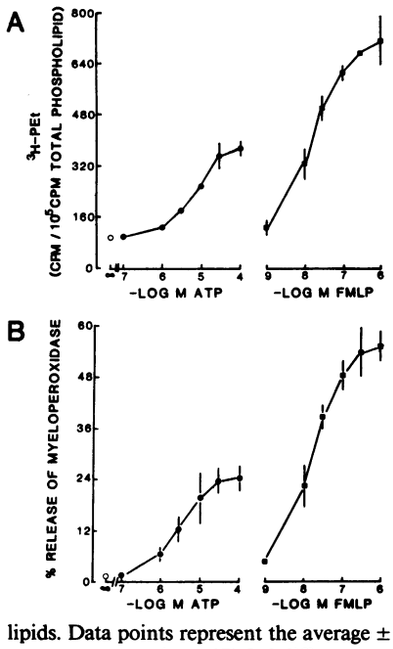

Figure 3. Concentration-response relationships characterizing the activation of PLD or primary granule secretion by ATP and FMLP in differentiated HL-60 granulocytes. (*A*) 3-d differentiated HL-60 cells (labeled with ^{3}H-oleic acid) were assayed for receptor-stimulated PLD activity as described in the Fig. 2 legend. Cells were stimulated for 2 min (at 37°C) with either no agonist (○) or the indicated concentrations of ATP (●) or FMLP (■). PEt accumulation was normalized relative to the total amount of ^{3}H in the bulk phospholipids. Data points represent the average ± range of results from two separate preparations. (*B*) 3-d differentiated HL-60 cells were assayed for receptor-stimulated primary granule secretion as described in the Fig. 2 legend. Cells were stimulated for 5 min (at 37°C) with the indicated concentrations of either ATP (●) or FMLP (■). Results were presented as percentage release of total (i.e., released by Triton X-100) content of cellular myeloperoxidase. Data points represent the average±range of results from two separate preparations.

Similar concentration-response relationships characterized ATP- and FMLP-stimulated myeloperoxidase release in 3-d differentiated cells (Fig. 3 *B*). In such cells, maximally activating concentrations of FMLP induced release of ~ 55–65% of the total cellular myeloperoxidase content (Table IV). Conversely, maximally activating concentrations of ATP stimulated only a 24–28% release. As for PLD activation, UTP and ATP were equally efficacious in stimulating secretion (Table IV and refs. 7 and 10). Thus, the relative differences between ATP- and FMLP-induced PLD activation and secretion were similar, with FMLP being twice as efficacious as ATP in HL-60 granulocytes.

Role of cytochalasin B on receptor-induced activation of PLD and secretion. Previous studies (21–23) have established that maximal FMLP-induced activation of PLD in neutrophils and HL-60 granulocytes requires that these cell types be pretreated with cytochalasin B. The data presented in Fig. 4 *A* indicate that the ability of ATP to maximally stimulate PLD in HL-60 granulocytes was likewise dependent on the presence of cytochalasin B. Maximal PEt formation was observed in the presence of 5 μM cytochalasin B. The EC_{50} for this cytochalasin B effect was 2 μM; cytochalasin B by itself was without effect on PEt accumulation. FMLP-induced PEt production in these same cells was characterized by an identical dependence on [cytochalasin B] (not shown).

Fig. 4 *B* illustrates the effect of cytochalasin B on the ability of 100 μM ATP and 1 μM FMLP to stimulate primary granule secretion in these cells. In the absence of cytochalasin B treatment, ATP elicted no significant release of myeloperoxidase. In contrast, FMLP induced the release of 6% of total cellular enzyme content. Pretreatment of the cells with cytochalasin B greatly augmented both ATP- and FMLP-triggered secretion. Induction of maximal secretion by either receptor agonist required the presence of 5 μM cytochalasin B. However, the presence of 1 μM cytochalasin B was sufficient to support near-maximal (70–80%) myeloperoxidase release in response to FMLP or ATP; this contrasted with the 2 μM EC_{50} characterizing the effects of this agent on receptor-mediated PEt accumulation. Cytochalasin B by itself did not alter basal granule release.

Effects of ethanol on receptor-stimulated PA production and primary granule secretion. The extent of FMLP- and ATP-stimulated PEt accumulation continuously increased as the ethanol concentration in assay was increased 0–140 mM (0–0.8% vol/vol) (data not shown). As shown in Fig. 5 *A*, the extent of agonist-stimulated PA accumulation reciprocally decreased with increasing ethanol concentration. This decrease in PA formation presumably reflects the increased use of ethanol versus water as the nucleophilic acceptor in PLD-catalyzed phosphatidyltransferase reaction. In this concentration range, ethanol had no effect on ATP- and FMLP-induced Ca^{2+} mobilization in these cells (data not shown) nor did it appreciably alter basal levels of PA (Fig. 5 *A*). At the highest ethanol concentration tested in these experiments (0.8% or 136 mM), the extent of ATP-stimulated PA accumulation was reduced to near-control levels. FMLP-induced PA accumulation was reduced by ~ 30% compared to that observed in control cells.

The presence of ethanol also resulted in a concentration-dependent inhibition of both FMLP- and ATP-stimulated myeloperoxidase release (Fig. 5 *B*). The concentration-response curve characterizing the inhibitory effect of ethanol on ATP-elicited secretion paralleled that describing inhibition of FMLP

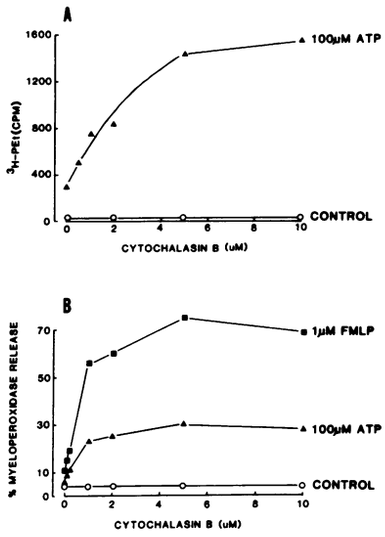

Figure 4. Effects of cytochalasin B on ATP-induced PLD activation and ATP- and FMLP-induced primary granule secretion in HL-60 granulocytes. (*A*) 2-d differentiated HL-60 cells (labeled with ^{3}H-oleic acid) were assayed for receptor-stimulated PLD activity as described in the Fig. 2 legend. Cells were preincubated with the indicated concentration of cytochalasin B for 5 min. Cells were then aliquoted into tubes containing 0.5% ethanol and either no agonist (○) or 100 μM ATP (▲). After a 2-min incubation at 37°C, the reaction was stopped. Data show the ^{3}H-PEt accumulation measured in a single preparation of cells. In this experiment, each assay tube contained 2.5×10^6 cells which had incorporated 356,000 cpm of ^{3}H-oleic acid into the total phospholipid pool. These data are representative of two similar experiments. (*B*) 3-day differentiated HL-60 cells were assayed for receptor-stimulated myeloperoxidase secretion as described in the Fig. 2 legend. Cells were pretreated with the indicated concentrations of cytochalasin B for 5 min before stimulation (10 min, 37°C) with no agonist (○), 100 μM ATP (▲), or 1 μM FMLP (■). Data shown result from a single preparation of cells; these data are representative of two similar experiments.

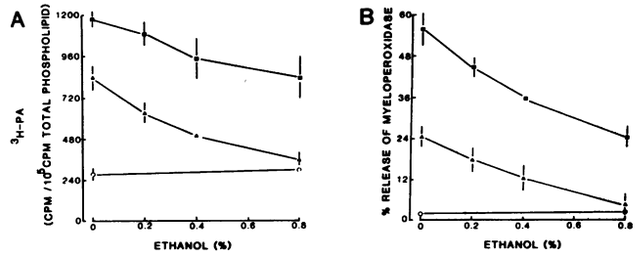

Figure 5. Concentration-response relationships characterizing the inhibitory effects of ethanol on receptor-stimulated ^{3}H-PA accumulation and on receptor-stimulated primary granule secretion in differentiated HL-60 granulocytes. (*A*) 3-d differentiated HL-60 cells (labeled with ^{3}H-oleic acid) were assayed for receptor-stimulated PLD activity as described in the Fig. 2 legend. Cells were pretreated with 5 μM cytochalasin B for 5 min and then transferred to tubes containing the indicated concentration of ethanol. Cells were stimulated for 60 s (at 37°C) with no agonist (○), 100 μM ATP (▲), or 1 μM FMLP (■). PA accumulation was normalized relative to the total amount of ^{3}H in the bulk phospholipids. Data points represent the average±range of results from two separate preparations. (*B*) 3-d differentiated HL-60 cells were assayed for receptor-stimulated primary granule secretion as described in the Fig. 2 legend. Cells were pretreated with 5 μM cytochalasin B for 5 min and then transferred to tubes containing the indicated concentrations of ethanol. Cells were then stimulated for 5 min (at 37°C) with no agonist (○), 100 μM ATP (▲), or 1 μM FMLP (■). Results were presented as percentage release of total (i.e., released by Triton X-100) content of cellular myeloperoxidase. Data points represent the average±range of results from two separate preparations.

action. Significantly, the extent of FMLP-stimulated secretion observed in the presence of 0.8% ethanol was equivalent to that stimulated by ATP in the absence of the alcohol. Similarly, the amount of PA accumulation in cells stimulated with FMLP in the presence of 0.8% ethanol was approximately equal to that observed in ATP-treated cells assayed in the absence of ethanol. These latter observations suggested that the extent of agonist-stimulated myeloperoxidase release, as observed in the presence of various ethanol concentrations, could be correlated with the extent of net PA accumulation. This relationship between myeloperoxidase release and ^{3}H-PA levels in FMLP- and ATP-stimulated cells is plotted in Fig. 6.

Discussion

Induction of phospholipase D activity during granulocytic differentiation. The results from this study and from previous studies (19, 21) indicate that differentiated HL-60 granulocytes express a phospholipase D-type effector enzyme(s) that can be activated via several signal transduction elements or pharmacological agents. These include: FMLP receptors (21); P_2-purinergic receptors (this study); phorbol esters (19, 35); increased cytosolic [Ca^{2+}] (19); and GTP-binding regulatory proteins (Table III and refs. 31 and 32). Our results suggest that the putative PLD effector enzyme(s) that is activated by such regulatory pathways is expressed at a low level in undifferentiated HL-60 cells. This contrasts with the expression of high levels of receptor- and G-protein–regulated PI-PLC effector enzyme activities in such undifferentiated cells. While the inability of FMLP to stimulate PLD in these cells is due to the low expression of formyl peptide receptors (29), our previous studies (6, 9, 11, 34) have indicated that the efficacy and potency of ATP (and other P_2-purinergic agonists) in stimulating GTP-dependent PI-PLC activity and Ca^{2+} mobilization is similar in both undifferentiated and dibutyryl cAMP-differentiated HL-60 cells. Thus, the failure of ATP to activate PLD-type activity in the undifferentiated cells must be due to factors distal to the P_2-purinergic receptor per se. In this regard, there are at least two possibilities: (*a*) no or low expression of a suitable G-protein that might couple ATP receptors to the PLD effector enzyme; and/or (*b*)

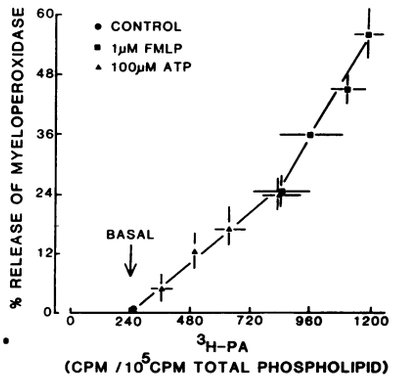

Figure 6. Correlation between the extent of primary granule secretion and the accumulation of ^{3}H-PA in differentiated HL-60 granulocytes stimulated with ATP or FMLP in the presence of various concentrations of ethanol. Data from the experiments illustrated in Fig. 5, *A* and *B*, have been replotted to show the correlation between myeloperoxidase release (*y* axis) and PA accumulation (*x* axis) observed in cells treated with no agonist (○), 100 μM ATP (▲), or 1 μM FMLP (■) in the presence of 0, 0.2, 0.4, or 0.8% (vol/vol) ethanol. Data points represent the average±range of results from two separate preparations.

no or low expression of functionally competent PLD effector enzyme(s).

Either of these possibilities could explain our observation that the magnitude of receptor-independent, GTPγS-induced PLD activity in electroporated HL-60 cells was much lower in undifferentiated cells (Table III). However, the first possibility appears less likely given: (*a*) the apparent role of a pertussis toxin sensitive G-protein(s) in mediating PLD activation by receptors in phagocytic leukocytes (Table II and ref. 21); and (*b*) previous demonstrations (36) that both undifferentiated and differentiated HL-60 cells express (albeit at somewhat different levels) G_{i-2} and G_{i-3}, but not G_o or G_{i-1}. The activation of PI-PLC by ATP is likewise substantially, but incompletely, attenuated by pertussis intoxification of both undifferentiated and differentiated HL-60 cells. Thus, undifferentiated cells express pertussis toxin–sensitive G-protein isoforms that can be activated by ATP receptors and that are likely to be involved in PLD stimulation. Moreover, phorbol ester–stimulated PLD activity was also much lower in undifferentiated HL-60 cells (Fig. 1 *A*). This suggests that the low level of receptor- and G-protein–stimulated PLD activity in such cells is most likely to due to low expression of the PLD effector enzyme in a form suitable for regulation by G-proteins or other activators. It remains to be determined whether the low level of PLD activity observed in undifferentiated HL-60 cells is due to reduced expression of the PLD enzyme per se, or the lack of some posttranslational modification(s) essential for regulation by G-proteins, phorbol esters, etc.

Upon differentiation (with dibutyryl cAMP) of HL-60 cells into neutrophil-like cells (granulocytes), the expression of receptor-regulated PLD enzyme activity is increased at least fivefold. That PMA-induced PLD activation is maximal after 1 d of dibutyryl cAMP treatment suggests that expression of functional PLD enzyme is maximal within 24 h after induction of differentiation. This suggestion is reinforced by the observation that the ability of P_2-purinergic receptors (which are already present in the undifferentiated cells) to stimulate PLD is also maximal after a 1-d differentiation period (Fig. 1). Conversely, FMLP-elicited PLD activity increased continuously through fourth day of differentiation (Fig. 1). This most likely reflects the steadily increasing numbers of chemotactic peptide receptors that are expressed during dibutyryl cAMP-induced differentiation (29).

In fully differentiated HL-60 cells, FMLP was consistently more efficacious (1.7-fold on average) than ATP in stimulating PLD activity (Figs. 1–3, 5; Table I). This was true regardless of whether ^{3}H-oleic acid or ^{3}H-glycerol was used to label cellular lipids (Table I). This contrasts with equivalent efficacies of these two agonists in stimulating PI-PLC (e.g., IP_3 production) and Ca^{2+}-mobilization (7, 9, 11). Several mechanisms may underlie the differential efficacies of ATP and FMLP as activators of PLD in these HL-60 granulocytes. First, P_2-purinergic receptors and chemotactic peptide receptors might activate the PLD via a common G-protein, but activation by the chemotactic peptide receptors may be more efficient or may induce different conformational changes in the activated alpha subunit. Recent studies by MacLeish et al. (3) have indicated that FMLP receptors and leukotriene B_4 receptors both activate $G_{i-2/3}$ (as assessed by increased GTPase and GTPγS binding) in HL-60 granulocyte membranes. However, only the FMLP receptors induced enhanced cholera toxin-catalyzed ADP-ribosylation of the alpha subunits of these G-proteins. Second, FMLP receptors and P_2-purinergic receptors may use distinct G-proteins that exhibit differential efficacies for the functional activation of PLD. That P_2-purinergic induced activation of both PI-PLC (9, 11) and PLD (Table II) is only partially inhibited in pertussis toxin–treated cells supports the notion that these receptors may interact with different G-proteins. Third, in the case of PLD activation, but not PI-PLC activation, efficacy may be related to the absolute number of receptors for FMLP versus ATP. Stimulation of PLD by several types of Ca-mobilizing receptor agonists has now been documented in multiple cell types (18, 25, 30, 33, 37, 38). It remains to be determined whether a single enzyme species is responsible for the phospholipase D activity observed under these various conditions, or whether there are multiple phospholipase D-type isozymes.

Relationship between phospholipase D-based signaling and the activation of primary granule release. Our study also provides evidence to support a role for a PLD-type signaling system in the regulation of primary granule secretion by phagocytic leukocytes. The induction of receptor-regulated PLD activation could be correlated with induction of receptor-regulated secretion (Fig. 1). It should be noted that both undifferentiated and differentiated HL-60 cells contain similar amounts of azurophilic granules (39). Moreover, as previously noted, P_2-purinergic agonists are very efficacious activators of PI-PLC in the undifferentiated cells. These findings strongly suggest that receptor-induced activation of the PI-PLC/Ca^{2+} mobilization signaling cascades is not sufficient to elicit exocytosis of the primary granules. Conversely, after induction of PLD activity in the differentiated cells the occupation of P_2-purinergic receptors can generate the necessary signals for stimulation of secretion. A possible role of PLD in agonist-induced secretion is further supported by the correlation between the differential efficacies of ATP and FMLP in stimulating secretion and PA/PEt accumulation. (Figs. 1–3, 5). We have previously noted that the two agonists produce equivalent activation of PI-PLC, but differentially activate PLD. Release of primary granules is likewise differentially stimulated by these two receptor ligands. The concentration-response relationships characterizing ATP- and FMLP-stimulated secretion closely parallel those for PLD activation as assayed by maximal PEt accumulation (Fig. 3). Likewise, the time courses describing FMLP- and ATP-stimulated myeloperoxidase release (Fig. 2 *C*) are similar to the initial rates characterizing the effects of these agonists on PA accumulation (as observed in the absence of ethanol, Fig. 3 *B*). The ability of either agonist to activate both PLD catalysis and granule release appears to be abruptly curtailed within 60 s after occupation of the receptors. It should be noted that our inability to observe ATP-induced activation of primary granule release in these undifferentiated cells contrasts with a recent study by Stutchfield and Cockcroft (40). These workers reported that both undifferentiated and differentiated HL-60 cells showed similar amounts of primary granule release in response to 100 μM ATP. We cannot account for the differences between this previous report and our observations. It may be due to the use of different sublines or passage numbers of HL-60 cells. Our studies were performed over an 18-mo time period using four separate HL-60 cell sublines that were raised from ATCC-derived stocks frozen at passage 18. Each individual subline was passaged in vitro for no more than 4 mo. In agreement with our findings, Stutchfield and Cockcroft (40) also observed that FMLP was at least twice as efficacious as ATP in stimulating granule release in the differentiated cells.

Receptor-regulated secretion and PLD activation, but not PI-PLC activation, also share a requirement for cytochalasin B pretreatment of the cells (7, 18, 21–23). The mechanism by which cytochalasin B, a potent inhibitor of actin assembly, potentiates multiple receptor-activated functions in phagocytic leukocytes remains elusive. Treatment of neutrophils with this agent is known to greatly potentiate receptor-induced stimulation of both diacylglycerol accumulation and superoxide release (13, 41–43). It is worth noting that PLD can be activated in phagocytes in the absence of cytochalasin B by agents (PMA, Ca^{2+}-ionophores, and GTPγS) which are not receptor agonists (reference 19 and Table III).

Further evidence supporting a role for PLD in the regulation of primary granule secretion is provided by the observation that both ATP- and FMLP-stimulated secretion can be inhibited, in a dose-dependent manner, by ethanol (Fig. 5 *B*). Indeed, in earlier studies (44) it was noted that long-chain primary alcohols (e.g., butanol and pentanol) can have differential effects on FMLP-activated neutrophil functions, i.e., they inhibit superoxide generation and secretion but enhance FMLP receptor affinity and increase chemotaxis. Recently, Bonser et al. (23) have demonstrated that ethanol and butanol can inhibit FMLP-stimulated superoxide generation by blocking formation of diacylglycerol without impairing inositol trisphosphate formation. Billah and colleagues have reported that most of diradylglycerol mass formed in FMLP-stimulated human neutrophils is derived from the dephosphorylation of PA formed by PLD-type catalysis (18, 21). Therefore, it is reasonable to conclude that alcohols may inhibit secretion and superoxide generation, at least in part, by diverting PLD-mediated catalysis from production of the physiological end product, phosphatidic acid, to production of the nonphysiological phosphatidylalcohols. We have observed a strong correlation between the extent of granule release and the net amount of PA, formed in response to FMLP or ATP, in cells exposed to various concentrations of ethanol (Figs. 5 and 6). These results are very similar to those of Gruchalla et al. (45) who noted that ethanol inhibits IgE-receptor–mediated mast cell degranulation in parallel with an inhibition of IgE-induced accumulation of PA. On the basis of in vitro studies with purified lipid vesicles, several investigators have proposed that PA may act as a possible "fusogenic" lipid (46, 47). Studies of phospholipase D activation (48) and exocytotic granule release (49) in platelets have led Haslam and colleagues to suggest that PLD-derived PA may play a critical role in mediating the so-called Ca^{2+}-independent, but GTPγS-dependent activation of secretion that has been observed in both platelets (48) and neutrophils (50).

While PLD-derived signals may prove necessary for maximal activation of primary granule release, it is likely that additional signaling pathways are involved. Phorbol ester treatment of intact neutrophils (51) elicits only minor release of azurophilic granules even though this agent induces the accumulation of considerable PLD-derived PA in differentiated HL-60 cells (19). Previous studies (7) have demonstrated an additional correlation between receptor-stimulated primary granule secretion and phospholipase A_2-catalyzed arachidonic acid release in HL-60 cells and human neutrophils. Thus, receptor-mediated regulation of secretion and other phagocyte functions may involve the integration of signals derived from multiple phospholipase-based transduction cascades. In this regard, our understanding of phagocyte function should be increased by studies aimed at defining the role of G-proteins and other regulatory proteins in the modulation of phospholipase D.

Acknowledgments

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References

1. Collins, S. J. 1987. The HL-60 promyelocytic leukemia cell line: proliferation, differentiation and cellular oncogene expression. *Blood.* 70:1233–1244.

2. Chaplinski, T. J., and J. E. Niedel. 1982. Cyclic nucleotide-induced maturation of human promyelocytic leukemia cells. *J. Clin. Invest.* 70:953–964.

3. McLeish, K. R., P. Gierschik, T. Schepers, D. Sidiropoulos, and K. H. Jakobs. 1989. Evidence that activation of a common G-protein by receptors for leukotriene B_4 and FMLP in HL-60 cells occurs by different mechanisms. *Biochem. J.* 260:427–434.

4. Vallavi, D. S., R. Austinhirst, and F. Snyder. 1990. Development of specific functionally active receptors for platelet-activating factor in HL-60 cells following granulocytic differentiation. *J. Biol. Chem.* 265:4261–4265.

5. Verghese, M. W., L. Charles, L. Jakoi, S. B. Dillon, and R. Snyderman. 1987. Role of a guanine nucleotide regulatory protein in the activation of phospholipase C by different chemoattractants. *J. Immunol.* 138:4374–4380.

6. Cowen, D. S., H. M. Lazarus, S. B. Shurin, S. E. Stoll, and G. R. Dubyak. 1989. Extracellular adenosine triphosphate activates calcium mobilization in human phagocytic leukocytes and neutrophil/monocyte progenitor cells. *J. Clin. Invest.* 83:1651–1660.

7. Cockcroft, S., and J. Stutchfield. 1989. ATP stimulates secretion in human neutrophils and HL-60 cells via a pertussis toxin-sensitive G-protein coupled to phospholipase C. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 245:25–29.

8. Ward, P. A., B. A. M. Walker, and B. E. Hagenlocker. 1991. Functional consequences of interactions between human neutrophils and ATP, ATPγS, and adenosine. *Ann. NY Acad. Sci.* 603:108–119.

9. Dubyak, G. R., D. S. Cowen, and L. M. Mueller. 1988. Activation of inositol phospholipid breakdown in HL60 cells by P_2-purinergic receptors for extracellular ATP: evidence for mediation by both pertussis toxin-sensitive and pertussis toxin-insensitive mechanisms. *J. Biol. Chem.* 263:18108–18117.

10. Cockcroft, S., and J. Stutchfield. 1989. The receptors for ATP and FMLP are independently coupled to phospholipases C and A_2 via G-protein(s): relationship between phospholipase C and A_2 activation and exocytosis in HL-60 cells and human neutrophils. *Biochem. J.* 263:715–723.

11. Cowen, D. S., B. Baker, and G. R. Dubyak. 1990. Pertussis toxin produces differential inhibitory effects on basal, P_2-purinergic, and chemotactic peptide-stimulated inositol phospholipid breakdown in HL-60 cells and HL-60 cell membranes. *J. Biol. Chem.* 265:16181–16189.

12. Siefert, R., R. Burde, and G. Schultz. 1989. Activation of NADPH oxidase by purine and pyrimidine nucleotides involves G-proteins and is potentiated by chemotactic peptides. *Biochem. J.* 259:813–819.

13. Truett, A. P., III, M. W. Verghese, S. B. Dillon, and R. Snyderman. 1988. Calcium influx stimulates a second pathway for sustained diacylglycerol production in leukocytes activated by chemoattractants. *Proc. Natl. Acad. Sci. USA.* 85:1549–1553.

14. Exton, J. H. 1990. Signaling through phosphatidylcholine breakdown. *J. Biol. Chem.* 265:1–4.

15. Billah, M. M., and J. C. Anthes. 1990. The regulation and cellular functions of phosphatidylcholine hydrolysis. *Biochem. J.* 269:281–291.

16. Loffelholz, K. 1989. Receptor regulation of choline phospholipid hydrolysis: a novel source of diacylglycerol and phosphatidic acid. *Biochem. Pharmacol.* 38:1543–1549.

17. Huang, C. and M. C. Cabot. 1990. Phorbol esters stimulate the accumulation of phosphatidate, phosphatidylethanol, and diacylglycerol in three cell types. Evidence for the indirect formation of phosphatidylcholine-derived diacylglycerol by a phospholipase D pathway and direct formation of diacylglycerol by a phospholipase C pathway. *J. Biol. Chem.* 265:14858–14863.

18. Billah, M. M., S. Eckel, T. J. Mullman, R. W. Egan, and M. I. Siegel. 1989. Phosphatidylcholine hydrolysis by phospholipase D determines phosphatidate and diglyceride levels in chemotactic peptide-stimulated human neutrophils: involvement of phophatidate phosphohydrolase in signal transduction. *J. Biol. Chem.* 264:17069–17077.

19. Billah, M. M., J.-K. Pal, T. J. Mullman, R. W. Egan, and M. I. Siegel. 1989. Regulation of phospholipase D in HL60 granulocytes: activation by phorbol esters, diglycerides, and calcium ionophores via protein kinase C-independent mechanisms. *J. Biol. Chem.* 264:9069–9076.

20. Mullman, T. J., M. I. Siegel, R. T. Egan, and M. M. Billah. 1990. Complement C5a activation of phospholipase D in human neutrophils: a major route to the production of phosphatidates and diglycerides. *J. Immunol.* 144:1901–1908.

21. Pai, J.-K., M. I. Siegel, R. W. Egan, and M. M. Billah. 1988. Phospholipase D catalyzes phospholipid metabolism in chemotactic peptide-stimulated HL60 granulocytes. *J. Biol. Chem.* 263:12472–12477.

22. Reinhold, S. L., S. M. Prescott, G. A. Zimmerman, and T. M. McIntyre. 1990. Activation of human neutrophil phospholipase D by three separable mechanisms. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 4:208–214.

23. Bonser, R. W., N. T. Thompson, R. W. Randall, and L. G. Garland. 1989. Phospholipase D activation is functionally linked to superoxide generation in the human neutrophil. *Biochem. J.* 264:617–620.

24. Kabayashi, M., and J. N. Kanfer. 1987. Phosphatidylethanol formation via transphosphatidylation by rat brain synaptosomal phospholipase D. *J. Neurochem.* 48:1597–1603.

25. Bocckino, S. B., P. B. Wilson, and J. H. Exton. 1987. Ca^{2+}-mobilizing hormones elicit phosphatidylethanol accumulation via phospholipase D activation. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 225:201–204.

26. Martin, T. F. J. 1987. Hormone-regulated phosphoinositide turnover in permeabilized cells and membranes. *Methods Enzymol.* 141:111–122.

27. Nakamura, K., and S. Handa. 1984. Coomassie brilliant blue staining of lipids on thin-layer plates. *Anal. Biochem.* 142:406–410.

28. Dewald, B., and M. Baggiolini. 1986. Methods for assessing exocytosis by neutrophil leukocytes. *Methods Enzymol.* 132:267–277.

29. Heiman, D. F., J. P. Gardner, W. J. Apfeldorf, and H. L. Malech. 1986. Effects of tunicamycin on the expression and function of formyl peptide chemotactic receptors of differentiated HL-60 cells. *J. Immunol.* 136:4623–4630.

30. Bocckino, S. B., P. F. Blackmore, P. B. Wilson, and J. H. Exton. 1987. Phosphatidate accumulation in hormone-treated hepaocytes via a phospholipase D mechanism. *J. Biol. Chem.* 262:15309–15315.

31. Tettenborn, C. S., and G. C. Mueller. 1988. 12-*O*-tetradecanoylphorbol-13-acetate activates phosphatidylethanol and phosphatidylglycerol synthesis by phospholipase D in cell lysates. *Biochem. Biophys. Res. Commun.* 155:249–255.

32. Anthes, J. C., S. Eckel, M. I. Siegel, R. W. Egan, and M. M. Billah. 1989. Phospholipase D in homogenates from HL-60 granulocytes: implications of calcium and G-protein control. *Biochem. Biophys. Res. Commun.* 163:657–664.

33. Martin, T. W., and K. Michaelis. 1989. P_2-purinergic agonists stimulate phosphodiesteratic cleavage of phosphatidylcholine in endothelial cells. *J. Biol. Chem.* 264:8847–8856.

34. Cowen, D. S., M. Sanders, and G. Dubyak. 1990. P_2-purinergic receptors activate a guanine nucleotide-dependent phospholipase C in membranes from HL-60 cells. *Biochim. Biophys. Acta.* 1053:195–203.

35. Liscovitch, M. 1989. Phosphatidylethanol biosynthesis in ethanol-exposed NG108-15 neuroblastoma x glioma hybrid cells: evidence for activation of a phospholipase D phosphatidyl transferase activity by protein kinase C. *J. Biol. Chem.* 264:1450–1456.

36. Murphy, P. M., B. Eide, P. Goldsmith, M. Brann, P. Gierschick, A. Spiegel, and H. L. Malech. 1987. Detection of multiple forms of G_{ia} in HL-60 cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 221:81–86.

37. Rubin, R. 1988. Phosphatidylethanol formation in human platelets: evidence for thrombin-induced activation of phospholipase D. *Biochem. Biophys. Res. Commun.* 156:1090–1096.

38. Qian, Z., and L. R. Drewes. 1989. Muscarinic acetylcholine receptor regulates phosphatidylcholine phospholipase D in canine brain. *J. Biol. Chem.* 264:21720–21724.

39. Stutchfield, J., and S. Cockcroft. 1988. Guanine nucleotides stimulate polyphosphoinositide phosphodiesterase and exocytotic secretion from HL-60 cells permeabilized with streptolysin O. *Biochem. J.* 250:375–382.

40. Stutchfield, J., and S. Cockcroft. 1990. Undifferentiated HL-60 cells respond to extracellular ATP and UTP by stimulating phospholipase C and exocytosis. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 262:256–258.

41. Honeycutt, P. J., and J. E. Niedel. 1986. Cytochalasin B enhancement of the diacylglycerol response in formyl peptide-stimulated neutrophils. *J. Biol. Chem.* 261:15900–15905.

42. Hensen, P. M., B. Zanolari, N. A. Schwartzman, and S. R. Hong. 1978. Intracellular control of human neutrophil secretion. I. C5a-induced stimulus-specific desensitization and the effects of cytochalasin B. *J. Immunol.* 121:851–855.

43. Rider, L. G., and J. E. Niedel. 1987. Diacylglycerol accumulation and superoxide anion production in stimulated human neutrophils. *J. Biol. Chem.* 262:5603–5608.

44. Yuli, I., A. Tomonaga, and R. Snyderman. 1982. Chemoattractant receptor functions in human polymorphonuclear leukocytes are divergently altered by membrane fluidizers. *Proc. Natl. Acad. Sci. USA.* 79:5906–5910.

45. Gruchalla, R. S., T. T. Dinh, and D. A. Kennerly. 1990. An indirect pathway of receptor-mediated diacylglycerol formation in mast cells. I. IgE receptor-mediated activation of phospholipase D. *J. Immunol.* 144:2334–2342.

46. Sundler, R., and D. Papahadjopoulos. 1981. Control of membrane fusion by phospholipid head groups. I. Phosphatidate/phosphatidylinositol specificity. *Biochim. Biophys. Acta.* 649:743–750.

47. Simmonds, A. C., and M. J. Halsey. 1985. General and local anaesthetics perturb the fusion of phospholipid vesicles. *Biochim. Biophys. Acta.* 813:331–337.

48. Van der Meulen, J., and R. J. Haslam. 1990. Phorbol ester treatment of intact rabbit platelets greatly enhances both the basal and guanosine 5'[-thio]-triphosphate-stimulated phospholipase D activities of isolated platelet membranes. Physiological activation of phospholipase D may be secondary to activation of phospholipase C. *Biochem. J.* 271:693–700.

49. Coorssen, J. R., M. M. L. Davidson, and R. J. Haslam. 1990. Factors affecting dense and alpha-granule secretion from electropermeabilized human platelets: Ca^{2+}-independent actions of phorbol ester and GTP S. *Cell Regul.* 1:1027–1041.

50. Barrowman, M. M., S. Cockcroft, and B. D. Gomperts. 1986. Two roles for guanine nucleotides in the stimulus-secretion sequence of neutrophils. *Nature (Lond.).* 319:504–507.

51. Wright, D. G., D. A. Bralove, and J. I. Gallin. 1977. The differential mobilization of human neutrophil granules. Effects of phorbol myristate acetate and ionophore A23187. *Am. J. Pathol.* 87:273–284.