Phospholipase D activity in phagocytic leucocytes is synergistically regulated by G-protein- and tyrosine kinase-based mechanisms

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The regulation of phospholipase D (PLD)-type effector enzymes by G-proteins and protein kinases/phosphatases was characterized in the U937 human promonocytic leucocyte line. PLD activity was assayed by measuring (in the presence of 1% ethanol) the accumulation of phosphatidylethanol in cells permeabilized with β-escin, a saponin-like detergent. Basal PLD activity was very low when cells were permeabilized and incubated in cytosol-like medium containing micromolar [Ca^{2+}]. When this medium was supplemented with exogenous MgATP or guanosine 5′-[γ-thio]triphosphate (GTP[S]), PLD activity increased by 9- and 14-fold respectively. Cells permeabilized in the absence of exogenously added MgATP, but in the presence of 1 μM vanadate/100 μM H_2O_2, also exhibited a modest 12-fold increase in PLD activity. However, the simultaneous presence of either GTP[S] plus exogenous MgATP or GTP[S] plus vanadate/H_2O_2 (and endogenous MgATP) induced similar 60–75-fold increases in the rate and extent of phosphatidylethanol accumulation. These latter effects of vanadate/H_2O_2 were strongly correlated with the very rapid accumulation of multiple tyrosine-phosphorylated proteins. Other studies utilized cells which were permeabilized in the presence of GTP[S] and then washed before assay of PLD. These cells retained ~60% of the MgATP-regulatable PLD activity (EC_{50} ≃ 100 μM MgATP) observed in freshly permeabilized non-washed cells. In the absence of GTP[S] pre-treatment, washed cells retained minimal PLD activity. Genistein, a tyrosine kinase inhibitor, significantly attenuated the ability of MgATP to stimulate PLD activity and accumulation of tyrosine-phosphorylated proteins in the washed GTP[S]-treated cells. These data suggest that PLD activity in myeloid leucocytes involves co-ordinate regulation by both G-protein(s) and tyrosine phosphorylation.

INTRODUCTION

Phospholipase D (PLD, EC 3.1.4.4) catalyses the hydrolysis of phospholipids (preferentially phosphatidylcholine), producing phosphatidic acid and the free polar head group. In the presence of a primary alcohol, this enzyme also catalyses a transphosphatidylation reaction in which the phosphatidyl moiety of the substrate phospholipid is transferred to alcohol so as to produce the corresponding phosphatidylalcohol [1–3]. PLD activities have been characterized in both membrane and soluble fractions derived from mammalian tissues and cells [4–7]. However, no intracellular PLD enzyme of mammalian origin has been purified to homogeneity, and it remains to be determined whether mammalian phosphatidylcholine-PLD activities represent a superfamily of distinct isoenzymes analogous to the superfamily of PtdIns-phospholipase C (PLC) effector enzymes [8]. Receptor-linked regulation of PLD activity has been extensively characterized in phagocytic leucocytes [9–15] and other cell types [9–23]. In most cases, these receptors belong to the superfamily of G-protein-coupled receptors. However, the mechanisms by which diverse receptor agonists activate these PLD activities remain poorly defined. Pharmacological and biochemical studies indicate that the regulation of PLD effector enzymes in various cells/tissues may involve direct roles for protein kinase C [23–34] or G-proteins [16–18,20–22,33–41].

In addition to regulation by G-protein-coupled receptors, PLD effector enzymes can be stimulated by growth-factor agonists for receptor tyrosine kinases [42] or by pharmacological agents (e.g. vanadate) which enhance the accumulation of tyrosine-phosphorylated proteins [43,44]. Bourgoin and Grinstein [44] have reported that the ability of vanadate peroxides to stimulate PLD in electropermeabilized washed HL-60 granulocytes is strongly correlated with enhanced tyrosine phosphorylation of proteins and is repressed by some, but not all, tyrosine kinase inhibitors. These investigators also indicated that this activation of PLD by vanadate peroxides did not directly involve protein kinase C- or G-protein-based mechanisms. Thus G-proteins, tyrosine phosphorylation and protein kinase C have all been implicated in the regulation of PLD activity in phagocytic leucocytes. These signalling elements/reactions may represent independent pathways for activation of distinct PLD isoenzymes. Alternatively, input from these three regulatory elements may converge on a single PLD effector enzyme via either parallel or serial signal-transduction cascades. Significantly, the stimulation of PLD, but not PtdIns-PLC, in human neutrophils by agonists (*N*-formylmethionyl-leucyl-phenylalanine, platelet activating factor, leukotriene B_4) for G-protein-coupled receptors is significantly attenuated by inhibitors of tyrosine kinases, but not by inhibitors of protein kinase C [43]. A rapid accumulation of tyrosine-phosphorylated proteins has been noted in intact phagocytic leucocytes stimulated by agonists for such receptors [43,45–47] and in permeabilized leucocytes directly stimulated with non-hydrolysable guanine nucleotides [48].

These latter data strongly suggest that non-receptor tyrosine kinases may be involved in the regulation of phagocyte PLD activity by G-protein-coupled receptors. We have previously reported that, in electropermeabilized HL-60 granulocytes, PLD was synergistically stimulated in the presence of both guanosine 5′-[γ-thio]triphosphate and MgATP [39]. Similar effects have been subsequently described in undifferentiated HL-60 cells

Abbreviations used: PLD, phospholipase D; PLC, phospholipase C; GTP[S], guanosine 5′-[γ-thio]triphosphate; PEt, phosphatidylethanol.

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permeabilized with streptolysin O [41]. We hypothesized that the regulation of PLD activity in phagocytic leucocytes may involve co-ordinate actions of both G-proteins and protein kinases. In the present study, we employed the U937 human promonocytic leucocyte line to characterize further the mechanisms underlying the synergistic actions of GTP[S] and ATP in regulating cellular PLD activity. We present data which strongly suggest that this synergy involves regulatory input from tyrosine kinases and phosphatases.

EXPERIMENTAL

Culture and radioisotopic labelling of cells with [^{3}H]oleic acid

Culture and maintenance of U937 cells in Iscove's variant of Dulbecco's modified Eagle medium plus 10% calf serum was performed as previously described [49,50]. Lipid pools were radioisotopically labelled by incubation of cells [(3–5) $\times 10^6$/ml] in culture medium supplemented with [^{3}H]oleic acid (1–2 μCi/ml) for 90 min. Some experiments were performed with HL-60 cells differentiated into granulocytes by a 2-day exposure to 0.5 mM dibutyryl cyclic AMP. The culture, differentiation and radioisotopic labelling of HL-60 granulocytes followed our previously described methods [39,40].

Permeabilization of U937 or HL-60 cells

To study the regulation of PLD under defined conditions *in vitro*, we employed radiolabelled cells which were rapidly permeabilized by incubation (at 37 °C) with β-escin, which, like saponin or digitonin, is a cholesterol-abstracting glycoside. β-Escin was dissolved in ethanol, and stock solutions (5 mg/ml) were protected from light and stored at −20 °C. It should be stressed that the rate of permeabilization induced by a particular concentration of β-escin is significantly influenced by the effective cell concentration. Likewise, different lots of commercially available β-escin were characterized by modest differences in potency. In general, we employed cell suspensions containing 3×10^6 cells/ml and β-escin in the 25–35 μg/ml range. Studies with the fluorescent probe ethidium bromide (which fails to stain intact cells) revealed that under these conditions > 95% of the β-escin-treated cells were rendered permeable within 3 min and remained so throughout a 1 h incubation at 37 °C (results not shown).

PLD activity was studied in two different populations of β-escin-permeabilized U937 (or HL-60) cells. (1) In certain experiments, PLD activity was directly assayed in cells which were simultaneously exposed to β-escin and the various agents (e.g. GTP[S], MgATP) being tested. We refer to experiments performed under such conditions as involving 'acutely permeabilized cells'. (2) In other experiments, U937 cells were first permeabilized by incubation with β-escin for 5 min and then washed and resuspended in fresh assay medium. PLD was subsequently assayed in these permeabilized cells. We refer to these as 'permeabilized/washed cells'. In a limited number of studies, we utilized U937 or HL-60 cells which were electropermeabilized by previously described methods [39,40].

In some experiments, U937 cells were metabolically inhibited by using conditions which previously have been shown to deplete > 90% of endogenous ATP in phagocytic leucocytes [44]. Briefly, intact U937 cells were suspended in basal salt solution (BSS: 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 15 mM $CaCl_2$, 25 mM Hepes, pH 7.5) supplemented with either 5 mM D-glucose or 5 mM 2-deoxyglucose plus 1 μM antimycin A. These cells were incubated at 37 °C for 20 min before washing, permeabilization and PLD assay as described below.

Assay of PLD activity in acutely permeabilized cells

Labelled cells were washed twice with ice-cold Ca^{2+}-free BSS. Washed cells were then resuspended in the basic glutamate assay medium (120 mM potassium glutamate, 20 mM potassium acetate, 3 mM $MgCl_2$, 20 mm Na-Hepes, pH 7.4, 1 mM EGTA, 1 mg/ml BSA) to 3×10^6 cells/ml and warmed to 37 °C. Portions (0.5 ml) of cell suspension were then diluted (1:2) into pre-warmed medium containing (final concns.) ethanol (1%), β-escin (25 μg/ml), and sufficient $CaCl_2$ to yield 1 μM free Ca^{2+}. The assay medium into which the cells were diluted also contained the various agents (e.g. GTP[S], MgATP, Na_3VO_4, H_2O_2) being tested. Incubations were carried out for various times (routinely 20 min) at 37 °C. The reactions were terminated by addition of 5 ml of chloroform/methanol (2:1, v/v). The lipids were extracted, dried and analysed by t.l.c. as previously described [39,40]. The radioactivity co-migrating with the [^{3}H]phosphatidylethanol ([^{3}H]PEt), [^{3}H]phosphatidic acid and bulk [^{3}H]phospholipid standards was quantified by liquid-scintillation spectrophotometry. PLD activity was routinely assayed by monitoring the accumulation of [^{3}H]PEt content (normalized to the content of total ^{3}H-labelled phospholipid).

Assay of PLD in permeabilized/washed U937 cells

Labelled cells were first washed with Ca^{2+}-free BSS as described above. The cells were then resuspended (3×10^6/ml) in the basic potassium glutamate medium and warmed to 37 °C; β-escin (25 μg/ml) was added and the suspension was incubated a further 5 min. The cells were then gently pelleted (300 *g*, 6 min) and the supernatant was removed. The cells were resuspended in ice-cold potassium glutamate medium and re-centrifuged; this wash step was repeated. The permeabilized/washed cells were stored on ice for up to 20 min before the assay of PLD exactly as described above. In some experiments, 100 μM GTP[S] was included during the initial incubation with β-escin. These GTP[S]-pretreated cells were then processed and washed (in potassium glutamate medium lacking GTP[S]) as previously described.

Western-blot analysis of tyrosine-phosphorylated proteins

Unlabelled U937 cells were used for parallel studies of protein tyrosine phosphorylation. Acutely permeabilized cells or permeabilized/washed cells were incubated and stimulated exactly as described for the PLD assays. At selected times, 1 ml samples of the cells were rapidly pelleted (6000 *g*, 10 s) and the supernatant was removed. The cell pellet (1.5×10^6 cells/sample) was suspended in 150 μl of a previously described [51] lysis buffer containing 20 mM Tris/HCl (pH 8), 137 mM NaCl, 1 mM Na_3VO_4, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethanesulphonyl fluoride, 20 μM leupeptin and 0.15 unit/ml aprotinin (protease inhibitors were added immediately before cell lysis). The lysate was vortex-mixed, incubated on ice for 20 min, and then centrifuged (12000 *g*, 4 min) to remove insoluble cytoskeleton and nuclei. A 125 μl portion of the supernatant was supplemented with 125 μl of SDS/PAGE sample buffer containing 125 mM Tris/HCl (pH 6.8), 4% SDS, 20% glycerol and 10% β-mercaptoethanol. After heating (90 °C, 5 min), 50 μl samples of the processed lysates (2.5×10^5 cell equivalents) were applied to individual lanes of a Laemmli-type SDS/polyacrylamide gel (11.8% acrylamide). After electrophoresis, the proteins were transferred to nitrocellulose (Schleicher and Schull) by using a Bio-Rad Trans-Blot cell. The nitrocellulose was rinsed in a standard blotting solution containing 150 mM NaCl, 10 mM Tris/HCl (pH 7.4), 1 mM EDTA and 0.05% Tween-20, and then blocked by incubation (60 min,

Table 1 Regulation of PLD activity in permeabilized U937 promonocytes

U937 promonocytes were radiolabelled and prepared for assay as described in the Experimental section. Before assay, the cells (resuspended in basic potassium glutamate medium) were divided into three groups and pre-treated as follows. Group I cells were untreated and incubated for 5 min at 37 °C; group II cells were permeabilized (Perm.) (25 μg/ml β-escin) and incubated for 5 min. at 37 °C; group III cells were permeabilized (25 μg/ml β-escin) and incubated in the presence of 100 μM GTP[S] for 5 min at 37 °C. After the 5 min initial incubation, each group of cells was diluted (1:5) with ice-cold medium, pelleted by centrifugation, and resuspended in fresh ice-cold medium; this washing procedure was then repeated. After the second wash and resuspension, the cells were diluted (1:2) into pre-warmed medium containing (final concns.): 1 μM free [Ca^{2+}], ethanol (1%), and, for the group I cells only, β-escin (25 μg/ml). The assay medium was variously supplemented with GTP[S] (100 μM), MgATP (2 mM), H_2O_2 (100 μM) or Na_3VO_4 (1 μM). After a 20 min incubation, the samples were extracted and assayed for [^{3}H]PEt content [normalized to the content of total ^{3}H-labelled phospholipid (PL)] as described in the Experimental section. Data for acutely permeabilized cells represent means (±S.E.M.) from 5–15 experiments (numbers in parentheses). Data for washed cells represents the average (±range) of results from two separate experiments.

Additions to assay medium			PEt accumulation (^{3}H c.p.m./20 min per 10^5 c.p.m. of PL)			
GTP[S] (100 μM)	MgATP (2 mM)	VO_4^{3-}/H_2O_2 (1 μM/100 μM)	Pre-treatment of cells...	None	Perm. (−GTP[S]) Washed	Perm. (+GTP[S]) Washed
−	−	−		242±38 (15)	125±21	505±55
+	−	−		3418±364 (15)	506±84	722±140
−	+	−		2105±189 (11)	450±14	5878±383
+	+	−		14272±1053 (15)	2484±85	8063±61
−	−	+		2926±406 (14)	224±6	968±103
+	−	+		18556±1175 (15)	562±96	1331±215
−	+	+		6245±887 (5)	686±291	6994±776
+	+	+		18393±3109 (5)	2526±78	7729±180

25 °C) in blotting solution supplemented with 3% BSA and 1% ovalbumin. After washing, the blot was incubated (60 min, 25 °C) in blocking solution supplemented with 30 ng/ml of the anti-phosphotyrosine PY20 antibody (ICN) covalently coupled to horseradish peroxidase. All incubations (blocking, washing, and probing) were performed in glass cylinders continuously rotated on the rotisserie of a Hybaid hybridization oven. After repeated washing (4×) with standard blotting solution, the horseradish peroxidase-labelled proteins were detected by using the Amersham Enhanced Chemiluminescence ('ECL') reagents. Luminescence was assayed by exposing autoradiography film to the blot for times ranging from 5 to 60 min.

Data presentation

Individual experiments were performed at least twice. Unless otherwise indicated, results from single representative experiments are illustrated.

Materials

GTP[S] was from Boehringer Mannheim. All other nucleotides and β-escin were from Sigma. [9,10-^{3}H(n)]Oleic acid and [2-*palmitoyl*-9,10-^{3}H(n)]-L-α-dipalmitoyl phosphatidylcholine were from Amersham. Anti-phosphotyrosine antibody PY20 was from ICN. Genistein, daidzein, erbstatin analogue (methyl 2,5-dihydroxycinnamate) and lavendustin B were from LC Services; herbimycin was from Calbiochem. Whatman silica-gel 60A K6 plates were used for t.l.c.

RESULTS

Synergistic effects of GTP[S], MgATP and vanadate peroxides on PLD activity in acutely permeabilized U937 promonocytes

When U937 promonocytes were suspended in a cytosol-like permeabilization medium supplemented with 25 μg/ml β-escin and 1 μM Ca^{2+}, only minor accumulation of [^{3}H]PEt was detected during a 20 min incubation (Table 1). However, when either 100 μM GTP[S] or 2 mM MgATP was included in this permeabilization medium, PEt accumulation was increased by mean values of 14-fold or 9-fold respectively. Significantly, when cells were permeabilized in the presence of both GTP[S] and MgATP, there was a synergistic 59-fold (mean value; range 30–102-fold) increase in PEt production. These results are qualitatively similar to those previously observed in electropermeabilized HL-60 granulocytes [39] and in undifferentiated HL-60 promyelocytes permeabilized with streptolysin O [41].

Previous studies have indicated that vanadate peroxides induce a marked accumulation of tyrosine-phosphorylated proteins in intact neutrophils [43] and electropermeabilized HL-60 cells (both granulocytes and undifferentiated promyelocytes [44,48]). In electropermeabilized HL-60 granulocytes, this is accompanied by the activation of PLD [44]. The ability of vanadium compounds to inhibit tyrosine phosphatase activity is well documented [52], as is the ability of such compounds to mimic or enhance biological responses known to be stimulated by activated tyrosine kinases [53]. Accordingly, we tested whether vanadate peroxides (added as 1 μM vanadate plus 100 μM H_2O_2) might similarly modulate the GTP[S]- and MgATP-dependent regulation of PLD in U937 cells. In the absence of GTP[S] and added MgATP, vanadate/H_2O_2 induced a mean 12-fold increase (range 4–17-fold) in PEt accumulation. The presence of 2 mM MgATP facilitated a further 2-fold increase in this vanadate-dependent PLD activity (26-fold increase over control; range 14–50-fold). Most significant, however, was the mean 77-fold increase (range 40–119-fold) in PEt accumulation (over control), which was observed when cells were permeabilized in the presence of both vanadate/H_2O_2 and GTP[S]. The additional presence of MgATP did not facilitate a further increase in this vanadate- and GTP[S]-dependent activity. The absolute magnitudes of the observed increases in PLD activity are noteworthy, as they represent the respective transphosphatidylation (to ^{3}H-labelled PEt) of 14% (in the presence of GTP[S] and MgATP, but no vanadate) or > 18% (in the presence of GTP[S] and vanadate, with or without MgATP) of the initial pool of ^{3}H-labelled phospholipids (PL) during a 20 min incubation in the presence of 1% ethanol. Smaller amounts of phosphatidic acid (~ 2% of the initial PL pool) were also accumulated under these assay conditions (results not shown). The same patterns of PLD activation were observed

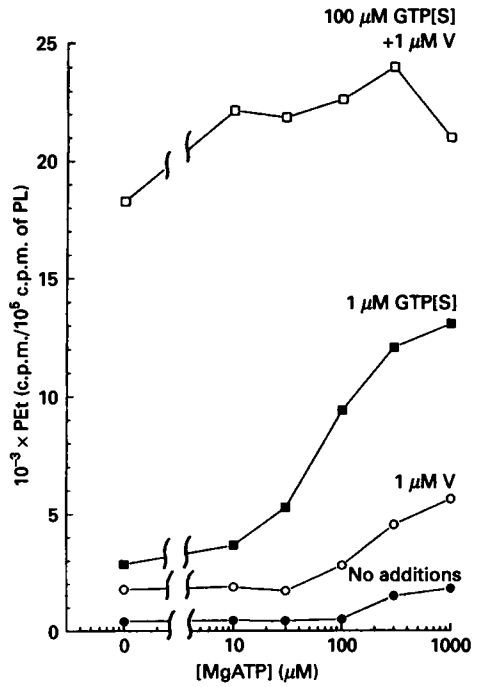

Figure 1 PEt accumulation in acutely permeabilized U937 cells stimulated with GTP[S] or vanadate/peroxide: effects of exogenously added MgATP or depletion of endogenous MgATP

(a) U937 promonocytes were radiolabelled, permeabilized, and incubated as described in Table 1 legend. The permeabilization medium, which contained the indicated concentrations of exogenously added MgATP, was also supplemented with no other agents (●), with 100 μM GTP[S] alone (■), with 1 μM Na_3VO_4/100 μM H_2O_2 alone (V; ○), or with 1 μM Na_3VO_4/100 μM H_2O_2 plus 100 μM GTP[S] (□). After a 20 min incubation, the samples were extracted and assayed for [^{3}H]PEt content [normalized to the content of total ^{3}H-labelled phospholipid (PL)] as described in the Experimental section. Data represent the values from a single experiment. Similar concentration–response relationships were observed in two other experiments.

Table 2 Effects of ATP depletion on PLD activity in acutely permeabilized U937 promonocytes

Radiolabelled U937 cells were metabolically inhibited by preincubation with 2-deoxyglucose and antimycin A as described in the Experimental section. The ATP-depleted cells and control cells (identically preincubated in the absence of 2-deoxyglucose and antimycin A) were then acutely permeabilized in the presence of various combinations of GTP[S] (30 μM), MgATP (1 mM), and vanadate/H_2O_2 (1 μM/100 μM). PEt accumulation after a 20 min incubation at 37 °C was assayed as described in Figure 1 legend. Data points represent the values from a single experiment. A similar pattern was observed in one other experiment. Abbreviation: PL, total ^{3}H-labelled phospholipid.

Additions to assay medium			PEt accumulation (^{3}H c.p.m./20 min per 10^5 c.p.m. of PL)	
GTP[S]	MgATP	Vanadate/H_2O_2	Control	ATP-depleted
−	−	−	245	654
+	−	−	3534	2602
−	+	−	2185	2409
+	+	−	24656	22151
−	−	+	1762	802
+	−	+	15407	3007
−	+	+	6204	6926
+	+	+	24529	22640

when differentiated HL-60 granulocytes were acutely permeabilized in the presence of the various combinations of GTP[S], MgATP and vanadate/H_2O_2 (results not shown). Likewise, similar synergistic interactions between GTP[S] and vanadate/H_2O_2 were noted in differentiated HL-60 granulocytes which were permeabilized by our previously described electroporation protocol [39]. It should also be noted that no significant accumulation of PEt was observed when U937 or HL-60 cells were incubated in medium containing the various combinations and concentrations of GTP[S], MgATP and vanadate/H_2O_2, but lacking β-escin. Higher concentrations of vanadate/H_2O_2 (> 10 μM) did activate significant PEt accumulation in intact U937 cells.

Given the relative metabolic stability of non-physiological phosphatidylalcohols, PEt accumulation will act as an integrator of PLD activity over time. Under all assay conditions, PEt accumulation was rapid during the initial 10 min after initiation of permeabilization and then reached a plateau between 10 and 20 min (results not shown). It is noteworthy that similar initial rates of PEt accumulation were observed in the presence of GTP[S] plus MgATP, but no vanadate/H_2O_2, or in the presence of GTP[S] plus vanadate/H_2O_2, but no added MgATP.

Figure 1 illustrates the concentration–response relationships which characterize the effects of MgATP on PEt accumulation in U937 cells acutely permeabilized under four conditions: (1) in the absence of other agents; (2) in the presence of 100 μM GTP[S]; (3) in the presence of 1 μM vanadate/100 μM H_2O_2 but no GTP[S]; or (4) in the presence of vanadate, H_2O_2 and GTP[S]. The presence of MgATP induced a several-fold potentiation of PEt accumulation in each of the first three conditions. A similar EC_{50} (in the 100–150 μM range) characterized these effects of added MgATP. In contrast, added MgATP (in the 10–2000 μM range) exerted only a small and variable potentiating effect on PEt accumulation by cells permeabilized in the presence of GTP[S] and vanadate. The ability of vanadate/H_2O_2 to potentiate GTP[S]-dependent PLD activity in the absence of exogenously added MgATP indicate that tyrosine-phosphorylated proteins rapidly accumulate during the initial minutes after permeabilization, due to tyrosine kinase activity which is supported by endogenous MgATP. When intact cells were depleted of endogenous MgATP before permeabilization and PLD assay, the ability of vanadate/H_2O_2 to potentiate GTP[S]-dependent PLD activity was absolutely dependent on inclusion of exogenous MgATP in the permeabilization/assay medium (Table 2).

We also monitored the accumulation of tyrosine-phosphorylated proteins in extracts prepared from U937 cells which were permeabilized and treated under conditions identical with those used for the PLD assays (Figure 2). A very modest accumulation of three immunoreactive bands (with approximate masses of 56, 46 and 43 kDa) was observed in the cells incubated for 5 or 10 min in the presence of GTP[S] and MgATP, but without vanadate/H_2O_2. In contrast, the inclusion of vanadate/H_2O_2 in the permeabilization medium rapidly induced the significant accumulation of multiple tyrosine-phosphorylated proteins in the absence or presence of exogenously added MgATP. The relative levels of all these phosphorylated products were already maximal at the earliest time point assayed (5 min) and then progressively declined in cells incubated for 10 or 20 min. The additional presence of GTP[S] did not substantively alter the pattern of protein tyrosine phosphorylation noted in vanadate/H_2O_2-treated cells.

Thus micromolar concentrations of vanadyl hydroperoxides can very rapidly induce a massive accumulation of tyrosine-phosphorylated proteins during the initial phases of β-escin-

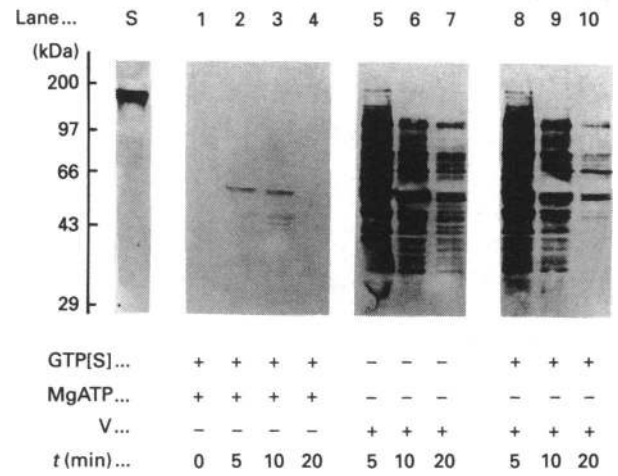

Figure 2 Time course characterizing the accumulation of tyrosine-phosphorylated proteins in acutely permeabilized U937 cells

U837 promonocytes (which were not radiolabelled) were permeabilized and incubated as described in Table 1 legend. The permeabilization medium was also supplemented as indicated with 100 μM GTP[S] plus 2 mM MgATP, with 1 μM Na_3VO_4/100 μM H_2O_2 alone (V), or with 1 μM Na_3VO_4/100 μM H_2O_2 plus 100 μM GTP[S]. At the indicated time points, cell extracts were prepared; the extracts were subsequently processed for SDS/PAGE and Western-blot analysis of tyrosine-phosphorylated proteins as described in the Experimental section. The lane labelled 'S' (for standard) contained a membrane extract prepared from epidermal-growth-factor (EGF)-stimulated A431 cells; the 170 kDa EGF receptor is the major tyrosine-phosphorylated protein in this extract. The molecular masses (in kDa) of standard proteins are indicated on the left scale. Immunoblots from a single experiment are shown. Similar time courses were observed in four other experiments.

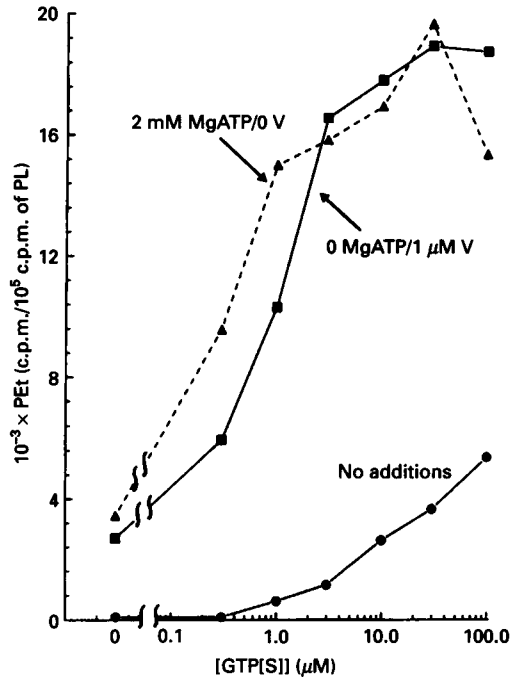

Figure 3 Concentration–response relationships characterizing the effects of GTP[S] on PEt accumulation in acutely permeabilized U937 cells: potentiation by vanadate/H_2O_2 treatment or inclusion of exogenous MgATP

U937 promonocytes were radiolabelled, permeabilized, and incubated as described in Table 1 legend. The permeabilization medium, which contained the indicated concentrations of GTP[S], was also supplemented with no other agents (●), with 1 μM Na_3VO_4 (V) plus 100 μM H_2O_2 (■), or with 2 mM MgATP (▲). After a 20 min incubation, the samples were extracted and assayed for [^{3}H]PEt content [normalized to the content of total ^{3}H-labelled phospholipid (PL)] as described in the Experimental section. Data represent the values from a single experiment. Similar concentration–response relationships were observed in two other experiments.

induced permeabilization. However, this accumulation is not sufficient to drive maximal activation of PLD in the absence of GTP[S]. Figure 3 shows the concentration-dependence which characterizes this action of GTP[S]. For comparison, the effects of GTP[S] concentration on PEt accumulation in the presence of 2 mM MgATP (but no vanadate) are also illustrated. Similar shifts in the GTP[S] concentration–response relationship were noted when protein phosphorylation was supported by endogenous MgATP in the presence of a tyrosine phosphatase inhibitor, or when phosphorylation was supported by exogenous MgATP in the absence of a tyrosine phosphatase inhibitor. Additional experiments (results not shown) examined the ability of other guanine nucleotides (guanosine 5′-[$\beta\gamma$-imido]triphosphate, GTP, GDP and guanosine 5′-[β-thio]-diphosphate) to potentiate the vanadate-activated PEt accumulation. When tested at 100 μM, only guanosine 5′-[$\beta\gamma$-imido]triphosphate mimicked the effects of GTP[S]; at 1 mM, GTP induced a modest potentiation.

The stimulatory actions of vanadate/peroxide on both PLD activity and the accumulation of tyrosine-phosphorylated proteins were observed when the vanadate concentration was progressively increased from 0.1 μM to the 0.3–1 μM range; maximal accumulation was noted with 3 μM vanadate. These effects reached a plateau as the vanadate concentration was further increased up to 30 μM (results not shown). PLD activity, but not the accumulation of tyrosine-phosphorylated proteins, was significantly decreased at vanadate concentrations $\geqslant$ 100 μM (results not shown). PEt accumulation decreased to near baseline levels in the presence of 1 mM vanadate (plus 1 mM H_2O_2). Additional studies (S. J. Schomisch and G. R. Dubyak, unpublished work) using purified cabbage PLD have indicated that vanadium compounds (at sub-millimolar concentrations) can directly inhibit PLD-type enzymes. Thus at sub-millimolar concentrations vanadium compounds may have effects on PLD-based signalling which are unrelated to inhibition of tyrosine phosphatases. The ability of vanadate to activate accumulation of both PEt and tyrosine-phosphorylated proteins was highly dependent on the additional presence of H_2O_2. This peroxide-dependence correlates with previous findings that peroxides of vanadate are much more potent than vanadate itself in inhibiting tyrosine phosphatases in electropermeabilized HL-60 granulocytes [44].

Regulation of PLD activity in permeabilized/washed U937 cells

In a previous study [39], we reported that the magnitude of the GTP[S]/MgATP-dependent PLD activity showed a time-dependent decrease after electropermeabilization of HL-60 cells. We have observed essentially the same phenomenon in β-escin-permeabilized U937 cells (Table 1) and HL-60 cells (results not shown). U937 cells which were first incubated in β-escin-supplemented medium at 37 °C for 5–10 min and then washed/resuspended in fresh medium exhibited significantly lower rates ($\sim$ 85% decrease) of PEt accumulation when subsequently challenged with GTP[S], alone or in combination with 2 mM MgATP. In contrast with its actions in acutely permeabilized cells, vanadate/H_2O_2 had no significant effect on PEt accumulation in these permeabilized/washed cells even when the assay medium was supplemented with GTP[S] or MgATP (alone or in combination). Cells were also pelleted after β-escin-induced

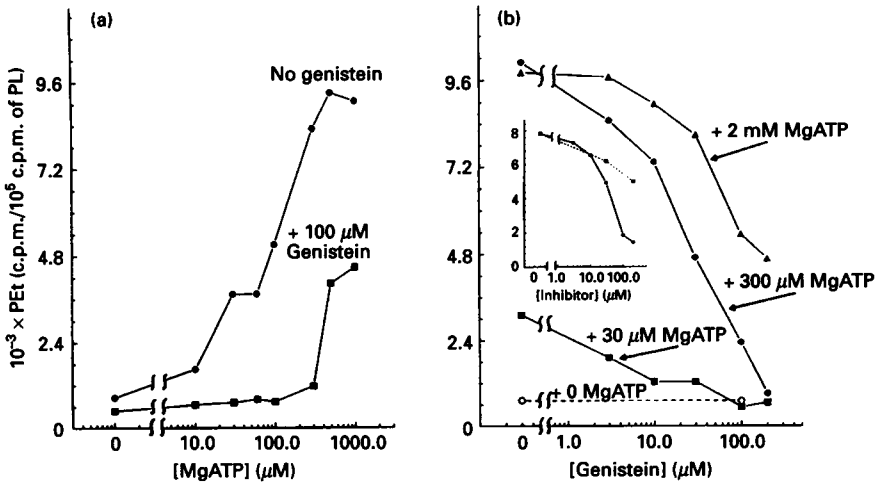

Figure 4 MgATP-dependent PEt accumulation in permeabilized/washed U937 cells: inhibition by genistein, a tyrosine kinase inhibitor

(a) U937 promonocytes were radiolabelled, permeabilized in the presence of GTP[S], and then washed (twice) as described for the 'Group III cells' in Table 1 legend. Two batches of the permeabilized/washed cells were then preincubated (25 °C) for 15 min with either 100 μM genistein (■) or an equivalent volume of dimethyl sulphoxide vehicle (●). The pre-treated cells were then stimulated with indicated concentrations of MgATP in the presence of 1% ethanol. After a 20 min incubation, the samples were extracted and assayed for [^{3}H]PEt content [normalized to the content of total ^{3}H-labelled phospholipid (PL)] as described in the Experimental section. Data represent the values from a single experiment. Similar results were observed in two other experiments. (b) U937 promonocytes were permeabilized in the presence of GTP[S] and washed (twice) as described above. Samples of the permeabilized/washed cells were preincubated (25 °C) for 15 min with the indicated concentrations of genistein or dimethyl sulphoxide vehicle. The pre-treated cells were then stimulated (in the presence of 1% ethanol) with 2 mM MgATP (▲), 300 μM MgATP (●), 30 μM MgATP (■), or no MgATP (○). After a 20 min incubation, the samples were extracted and assayed for [^{3}H]PEt content [normalized to the content of total ^{3}H-labelled phospholipid (PL)] as described in the Experimental section. Data represent the values from a single experiment. Similar results were observed in one other experiment. The inset shows results from a separate experiment in which permeabilized/washed U937 cells were pre-treated with the indicated concentrations of genistein (●——●) or daidzein (■ · · · · ■) before stimulation with 300 μM MgATP.

permeabilization, and the proteins released into supernatants were analysed by SDS/PAGE. There was minimal loss of proteins with molecular masses > 20 kDa during the first 5 min after addition of the detergent. By 10 min, however, there was a very significant loss of proteins up to ~ 70 kDa in mass.

Parallel samples of U937 cells were permeabilized with β-escin in the presence of GTP[S], followed by washing (twice) and resuspension in fresh assay medium lacking GTP[S]. These cells exhibited a significant central rate of PEt accumulation, and this rate was only modestly augmented by inclusion of additional GTP[S] in the assay medium. When the assay was supplemented with 2 mM MgATP alone, a 10-fold increase in PEt accumulation was observed. When the medium contained both MgATP and additional GTP[S], these permeabilized/washed cells exhibited a rate of PEt accumulation which was only 2-fold lower than that observed in acutely permeabilized cells assayed under identical conditions. Despite the considerable amount of GTP[S]/ATP-dependent PLD activity associated with these latter cells, PEt accumulation was not appreciably potentiated when the cells were stimulated with maximally activating concentrations of MgATP in the additional presence of vanadate/H_2O_2.

Thus β-escin-induced permeabilization and washing of U937 cells is accompanied by the loss, inactivation or reorganization of some regulatory/catalytic components which constitute the GTP[S]- and MgATP-regulated PLD activity observed in acutely permeabilized cells. Rapid occupation of G-proteins by GTP[S] during permeabilization, but before washing, attenuates this 'loss' of regulated PLD activity. Given the high affinity of the relevant G-protein(s) for GTP[S] (Figure 3), it is likely that this protein is occupied by GTP[S] even after extensive washing. These washed cells apparently retain both the G-protein (already in the GTP[S]-activated state) and the ATP-dependent regulatory factor. This may explain: (1) the substantial basal rate of PEt accumulation observed when these cells were assayed in medium lacking additional GTP[S] and MgATP; and (2) the marked activation of PLD when the assay medium included MgATP, but no additional GTP[S].

Figure 4 shows the concentration–response relationship which characterizes the activation of PLD by MgATP in these permeabilized/GTP[S]-pretreated/washed cells. The concentrations of MgATP which induced threshold, half-maximal ($EC_{50} \simeq 100$ μM) and optimal rates of PEt accumulation were similar to those noted in acutely permeabilized cells (Figure 1). As indicated in Table 1, vanadate/H_2O_2 did not increase the efficacy of MgATP. However, a modest, but reproducible, shift in this concentration/response relationship was observed in the presence of vanadate/H_2O_2 (results not shown). This suggested that these permeabilized/GTP[S]-pretreated/washed cells could be used for further characterizing the role of tyrosine phosphorylation in the regulation of the GTP[S]-dependent PLD.

Permeabilized/washed cells (which had been treated with GTP[S]) were additionally preincubated for 15 min in the presence or absence of 100 μM genistein, an inhibitor of certain tyrosine kinases [54], and then assayed for PEt accumulation in medium supplemented with various concentrations of MgATP. The PEt accumulation supported by [MgATP] ⩽ 200 μM was completely inhibited in the genistein-treated cells. Increasing [MgATP] to millimolar levels partially reversed this inhibitory action. These results are consistent with previous reports that the inhibition of epidermal-growth-factor receptor kinase activity by genistein was competitive with ATP [54]. The experiment depicted in Figure 4(b) further supports the possibility that a similar competitive interaction characterizes genistein-induced inhibition of the MgATP-dependent PLD in U937 cells. The relative IC_{50} values which characterized this effect of genistein were highly dependent on the concentration of MgATP used to stimulate PLD: ~ 100 μM with 2 mM MgATP, ~ 30 μM with 0.3 mM MgATP, and ~ 3 μM with 30 μM MgATP. Previous studies

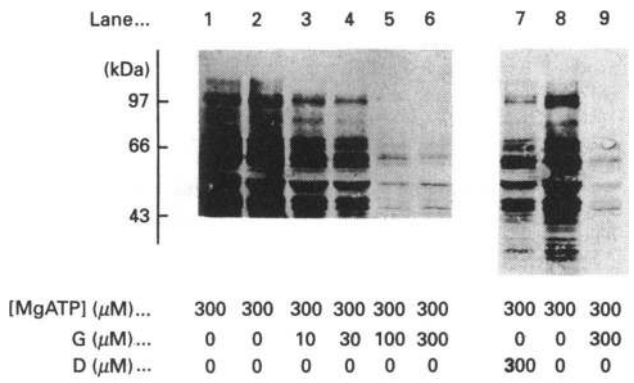

Figure 5 Accumulation of tyrosine-phosphorylated proteins in permeabilized/washed U937 cells: inhibition by genistein

The experimental protocol described for Figure 3 was used to study the accumulation of tyrosine-phosphorylated proteins in permeabilized/washed cells which were preincubated with the indicated concentrations of genistein (G) or daidzein (D) before stimulation with 300 μM MgATP. Vanadate (1 μM) and H_2O_2 (100 μM) were included in the assay medium to inhibit tyrosine phosphatases. Immunoblots from a single experiment are shown. Similar results were observed in two other experiments. The molecular masses (in kDa) of standard proteins are indicated on the left-hand scale.

have indicated that daidzein (4′,7-dihydroxyisoflavone) is less potent than genistein (4′,5,7-trihydroxyisoflavone) in inhibiting tyrosine kinases *in vitro* [54]. Daidzein was also less potent than genistein in inhibiting the MgATP-activated PLD in permeabilized/washed U937 cells (Figure 4b, inset). Other reported inhibitors of tyrosine kinases were also tested under similar assay conditions. No significant inhibition of the MgATP-activated PLD was observed after a 15 min preincubation of permeabilized/washed cells with 10 μM lavendustin B [55], 100 μM erbstatin analogue [44], or 1 μg/ml herbimycin a [56]. In contrast with this lack of herbimycin effect after short-term exposure, an overnight incubation of intact U937 cells with 1 μg/ml herbimycin produced a 50% decrease in the rate and extent of PEt accumulation in permeabilized cells under all assay conditions (results not shown).

The abilities of MgATP and genistein respectively to activate and to inhibit PEt accumulation in these permeabilized/GTP[S]-treated/washed cells were well correlated with the effects of these agents on the accumulation of tyrosine-phosphorylated proteins (Figure 5). MgATP-dependent accumulation of these phosphorylated proteins was potentiated in the presence of vanadate/H_2O_2 and inhibited in the presence of genistein. The concentration–response relationship describing this effect of genistein was similar to that characterizing its action on PEt accumulation. When 300 μM MgATP was used to support phosphorylation, $\geqslant$ 100 μM genistein was required to decrease very significantly the accumulation of tyrosine-phosphorylated proteins. When tested at equimolar concentrations, daidzein was less effective than genistein in inhibiting tyrosine phosphorylation.

DISCUSSION

These experiments confirm and significantly extend the recent report by Bourgoin and Grinstein [44] concerning the ability of vanadate/H_2O_2 to inhibit tyrosine phosphatases co-ordinately and to activate PLD in electropermeabilized HL-60 granulocytes. However, those investigators observed additive, rather than synergistic, effects on PLD when the cells were co-stimulated with GTP[S] and vanadate peroxides. By analogy with the activation of distinct PtdIns-PLC isoenzymes by G-proteins rather than by tyrosine kinases, these findings suggested that there may be multiple PLD isoenzymes in phagocytic cells which are likewise independently regulated by G-proteins or by tyrosine kinases. In contrast, our results suggest that a common PLD effector enzyme in these cells may be activated by convergent signals derived from both G-protein- and tyrosine kinase-based signalling cascades. It should be noted that Bourgoin and Grinstein [44] utilized HL-60 cells which was first electropermeabilized, washed and preincubated before the assay of PLD activity. We have previously observed that when electropermeabilized HL-60 cells are first incubated for several minutes before addition of GTP[S], there is a progressive and time-dependent decrease in the rate of PEt accumulation which can be synergistically stimulated by GTP[S] and MgATP [39]. This attenuation in GTP[S]/MgATP-dependent PLD activity is significantly decreased if the cells are exposed to GTP[S] during or immediately after permeabilization. Geny and Cockcroft have reported essentially identical findings using undifferentiated HL-60 cells permeabilized with streptolysin O [41]. Data from the present study (Table 1) indicates a similar attenuation of GTP[S]/MgATP-dependent activity after permeabilization of U937 cells with β-escin.

Thus it appears that the GTP[S]-mediated regulatory process requires a near-intact cytosolic environment for optimal activation of PLD. Permeabilization of cells by any method will necessarily be accompanied by time-dependent changes in the content or organization of cytosolic molecules. Presumably, entry of GTP[S] and activation of the putative G-protein(s) involved in PLD regulation must be sufficiently rapid so as to precede the major disruption of cytosol content/organization which accompanies sustained permeabilization of cells. Significantly, a GTP[S]-dependent PLD activity, which can be measured in homogenates prepared from HL-60 cells [36,37] or human neutrophils [38], cannot be detected when cytosolic or membrane fractions isolated from these homogenates are separately assayed. However, recombination of the cytosolic and membrane fractions is accompanied by reconstitution of the GTP[S]-dependent PLD activity [37,38]. Cytosol was found to provide an essential heat-inactivatable factor(s). Guanine-nucleotide-dependent PLD cascades in phagocytic leucocytes may thus involve multiple regulatory and/or catalytic elements derived from both soluble and particulate compartments of the cell. An activated membrane-associated G-protein may constitute an anchoring element for the assembly of a supramolecular signalling complex.

Our data support the findings of Uings et al. [43] that treatment of intact neutrophils with tyrosine kinase inhibitors greatly attenuates the activation of PLD, but not PtdIns-PLC, by the G-protein-coupled receptors for *N*-formylmethionyl-leucyl-phenylalanine, leukotriene B_4 and platelet-activating factor. However, the identity of the tyrosine kinase activity involved in PLD regulation in phagocytic leucocytes remains undefined. Bourgoin and Grinstein [44] noted that only certain tyrosine kinase inhibitors attenuated the accumulation of PEt and tyrosine-phosphorylated proteins in electropermeabilized HL-60 cells. Our studies indicated that genistein was also an effective inhibitor of the GTP[S]/MgATP-activated PLD in U937 cells. In contrast, we observed no significant inhibition when permeabilized cells were similarly treated with 10 μM lavendustin B or 100 μM erbstatin analogue. However, other studies demonstrated a marked decrease in GTP[S]/MgATP/vanadate-activated PLD when U937 cells are exposed to 1 μg/ml herbimycin for prolonged

(15 h), but not short (15 min) periods. Recent studies by Rhee and colleagues have indicated that prolonged herbimycin treatment of U937 cells attenuates Fc-receptor-induced tyrosine phosphorylation of PtdIns-PLC-γl by inhibiting a non-receptor tyrosine kinase associated with cross-linked Fc receptors [57]. Prolonged herbimycin treatment of lymphocytes induces an apparent down-regulation of *fyn* and *lck*, two non-receptor tyrosine kinases [56]. Such observations suggest that a non-receptor tyrosine kinase may also be involved in the regulation of PLD in phagocytic cells. The abilities of phorbol esters, exogenous diacylglycerols and Ca^{2+} ionophores to stimulate PLD activity in phagocytic leucocytes and most other cell types suggest that serine/threonine kinases, such as PKC, are also involved in the regulation of PLD [23–34,41]. Additional studies are needed to evaluate whether a common PLD effector enzyme can be co-ordinately regulated by signalling cascades involving both tyrosine kinase and serine/threonine kinases.

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