Phospholipase D activation by P_{2Z} -purinoceptor agonists in human lymphocytes is dependent on bivalent cation influx

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The role of bivalent cations in ATP-stimulated phospholipase D (PLD) activity was investigated in human leukaemic lymphocytes. Cells were labelled with [3H]oleic acid and incubated with extracellular ATP or benzoylbenzoic ATP in the presence of 1 mM Ca^{2+} and butanol, and PLD activity was assayed by the accumulation of [3H]phosphatidylbutanol ([3H]PBut). ATP stimulated PLD activity in a dose-dependent manner, and the inhibitory effects of suramin, oxidized ATP and extracellular Mg^{2+} suggested that the effect of ATP was mediated by P_{2Z} purinoceptors known to be present on lymphocytes. Thapsigargin increased cytosolic [Ca^{2+}] but did not stimulate PLD activity, whereas preloading cells with a Ca^{2+} chelator reduced cytosolic

[Ca^{2+}] and, paradoxically, potentiated ATP-stimulated [3H]PBut accumulation. ATP-stimulated [3H]PBut formation was supported by both Ba^{2+} and Sr^{2+} when they were substituted for extracellular Ca^{2+} . Addition of EGTA to block bivalent cation influx inhibited the majority of ATP-stimulated PLD activity. Furthermore ATP-stimulated PLD activity showed a linear relationship to extracellular [Ba^{2+}], and ATP-induced $^{133}Ba^{2+}$ influx also had a linear dependence on extracellular [Ba^{2+}]. These results suggest that ATP stimulates PLD activity in direct proportion to the influx of bivalent cations through the P_{2Z} -purinoceptor ion channel and that this PLD activity is insensitive to changes in bulk cytosolic [Ca^{2+}].

INTRODUCTION

Phospholipase D (PLD) is activated by a diverse range of receptor agonists in a wide variety of cell types and is thought to have an important role in cell signal transduction. It hydrolyses plasma-membrane phosphatidylcholine producing phosphatidic acid and choline. Agonist activation of PLD occurs either directly or, more commonly, by an indirect pathway through the initial stimulation of phosphoinositide-specific phospholipase C (PI-PLC), leading to the simultaneous production of diacylglycerol and inositol trisphosphate (IP_3)-mediated release of Ca^{2+} from internal stores. Phorbol esters and calcium ionophores both activate PLD in many cell types, supporting the concept that PLD activation is controlled by protein kinase C (PKC) and/or cytosolic Ca^{2+} (reviewed by Shukla and Halenda [1], Billah [2] and Exton [3]). In most studies of intact cells, the use of extracellular and/or intracellular Ca^{2+} chelators indicate that a rise in cytosolic [Ca^{2+}] is necessary for both direct and indirect activation of PLD [4–6]. However, in subcellular fractions, a similar increase in [Ca^{2+}] does not stimulate PLD activity [3]. The reported discrepancy between intact and broken cells in their Ca^{2+} requirement for PLD activation is unexplained.

Extracellular ATP and other agonists for P_2 purinoceptors raise cytosolic [Ca^{2+}], and, in some cell types, these agonists also stimulate PLD activity [7–12]. P_2 purinoceptors have been classified into four broad groups (P_{2X} , P_{2Y} , P_{2U} and P_{2Z}) based on pharmacological profiles of agonists and antagonists and their mechanism of transmembrane signalling (reviewed by Gordon [13] and Dubyak and El-Moatassim [14]). Stimulation of P_{2Y}

and P_{2U} purinoceptors, which are coupled to a G-protein and PI-PLC effector enzyme, causes a release of internal Ca^{2+} stores which is often associated with phosphoinositide hydrolysis. The P_{2X} and P_{2Z} purinoceptor subtypes are cation-selective ion channels which allow a Ca^{2+} influx when ATP binds to the receptor. However, the P_{2Z} purinoceptor, found on mast cells, macrophages and lymphocytes, has a weaker affinity for ATP (EC_{50} approx. 90 μM) than the P_{2X} purinoceptor of excitable cells (EC_{50} approx. 1–10 μM). Recently El-Moatassim and Dubyak [7,15] reported that benzoylbenzoic ATP (BzATP), a selective P_{2Z} agonist, activated PLD in murine macrophages. However, murine macrophages express both P_{2U} and P_{2Z} receptors [7,16,17], which complicates any study of ATP-stimulated PLD activity in this cell type. In contrast, human leukaemic lymphocytes express the P_{2Z} type of purinoceptor in isolation, which makes them an ideal cell for studying the features of ATP-stimulated PLD activation. Both the ionic selectivity and agonist/antagonist profile of the lymphocyte P_{2Z} purinoceptor and its associated ion channel have been well characterized [18–23]. Thus extracellular ATP increases the permeability of lymphocytes to a variety of cations such as Ca^{2+} , Na^+ , K^+ , Ba^{2+} (137 Da) and ethidium $^+$ (314 Da), although this channel shows considerable selectivity for Ca^{2+} over univalent cations [24]. The ATP^{4-} species is the agonist for the P_{2Z} receptor, as addition of Mg^{2+} , which forms the inactive species $MgATP^{2-}$ and thereby reduces the concentration of ATP^{4-} , rapidly closes the cation channel [21]. The most potent ligand for the P_{2Z} purinoceptor is BzATP [7,22]. Cation fluxes through the P_{2Z} -purinoceptor ion channel are inhibited by extracellular Na^+ ,

Abbreviations used: PLD, phospholipase D; PI-PLC, phosphoinositide-specific phospholipase C; PKC, protein kinase C; [3H]PBut, [3H]phosphatidylbutanol; IP_3 , inositol trisphosphate; FSBA, fluoroethylsulphonylbenzoyladenosine; BzATP, benzoylbenzoic ATP; HMA, 5-(NN -hexamethylene) amiloride; BAPTA, 1,2-bis-(o -aminophenoxy)ethane- NNN' -tetra-acetic acid; AM, acetoxymethyl ester; pp[CH $_2$]pA, adenosine 5'-[α -methylene]triphosphate; ATP[S], adenosine 5'-[γ -thio]triphosphate.

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amiloride analogues, suramin and various irreversible inhibitors such as fluorosulphonyl benzoyl adenosine (FSBA), oxidized ATP and the stilbene derivative 4,4'-di-isothiocyanostilbene-disulphonic acid [20–23, 25, 26]. In the present study, extracellular ATP has been shown to activate PLD in intact human leukaemic lymphocytes by stimulation of P_{2u} purinoceptors. The dependence of ATP-stimulated PLD activity on extracellular cations has been studied, and the special properties of Ba^{2+} as a permeant have been exploited to demonstrate a link between bivalent cation influx through the P_{2u} -operated ion channel and activation of PLD.

MATERIALS AND METHODS

Materials

Ficoll-Paque (*d* 1.077) was obtained from Pharmacia (Uppsala, Sweden). ATP, BzATP, ADP, oxidized ATP, adenosine, UTP, FSBA, phosphatidylcholine, phosphatidylethanolamine and phosphatidic acid standards were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [9,10- 3H]Oleic acid and Hyperfilm MP were purchased from Amersham International (Amersham, Bucks., U.K.). TLC plates (LK6D) came from Whatman (Maidstone, Kent, U.K.). En^3Hance and $^{133}Ba^{2+}$ were from Dupont (Boston, MA, U.S.A.). Fura 2-acetoxymethyl ester (AM) and 1,2-bis-(*o*-aminophenoxy)ethane-*NNN'*-tetra-acetic acid (BAPTA)-AM were obtained from Molecular Probes (Eugene, OR, U.S.A.). Thapsigargin, adenosine 5'-[$\alpha\beta$ -methylene]triphosphate (pp[CH₂]pA), 2-methylthio-ATP and the amiloride analogue 5-(*NN*-hexamethylene) amiloride (HMA) were from Research Biochemicals (Natick, MA, U.S.A.). Adenosine 5'-[γ -thio]triphosphate (ATP[S]) was from Boehringer-Mannheim (Sydney, Australia). Suramin was a gift from Bayer (Sydney, Australia). Anti-IgD antibody (sheep anti-human, IgG fraction) was from Silenus Laboratories (Hawthorn, Vic, Australia).

Lymphocyte preparation

Lymphocytes were isolated from heparinized venous blood (20 ml) taken from patients with B-cell chronic lymphocytic leukaemia and diluted in 2 vol. of RPMI 1640 medium. Mononuclear cells were separated by density-gradient centrifugation over Ficoll-Paque. After being washed, the monocytes were removed by adherence to plastic tissue culture flasks by incubation for 40 min at 37 °C. Cyto-centrifuge specimens showed that more than 99 % of the remaining mononuclear cells were small mature lymphocytes.

PLD assay

Lymphocytes (1×10^7 cells/ml) were cultured at 37 °C for 18–24 h in RPMI 1640 medium supplemented with gentamicin (40 μ g/ml), 10 % heat-inactivated fetal calf serum and [3H]oleic acid (2–5 μ Ci/ml; specific radioactivity 10 Ci/mmol) to label membrane phospholipids. Under these conditions, 61 ± 2.5 and 22 ± 4.2 % (mean $\pm$ S.D., $n = 9$) of the phospholipids labelled were phosphatidylcholine and phosphatidylethanolamine respectively. In some experiments 10 or 30 μ M BAPTA-AM was added to the flask for the final 30 min of culture. Labelled cells were washed twice in chilled medium (145 mM NaCl, 5 mM KCl, 10 mM Hepes, 5 mM glucose, 1 mM Ca^{2+} and 0.1 % BSA, pH 7.4), followed by a final wash in Hepes-buffered 150 mM KCl or 150 mM NaCl plus 5 mM glucose, 1 mM Ca^{2+} and 0.1 % BSA. Aliquots (1 ml) containing 1×10^7 cells were warmed to 37 °C in the presence of 30 mM butanol (0.27 %, v/v) with or without 1 mM $CaCl_2$ in glass culture tubes for 5 min before addition of agonist, and then incubated for 15 min with gentle

mixing. The PLD reaction was terminated by addition of 1 ml of 20 mM $MgCl_2$ and the sample placed on ice, centrifuged at 1000 *g* for 5 min and 1.0 ml of ice-cold methanol added to the pellet. Cell viability was 95 % or higher as assessed by Trypan Blue exclusion.

Lipid extraction and analysis

Membrane lipids were extracted by the method of Bligh and Dyer [27] as modified by Metz and Dunlop [28]. Briefly, 2 ml of acidified chloroform (chloroform/HCl, 200:1, v/v) was added to cells in methanol, the sample vigorously vortexed and lipids were extracted overnight at 4 °C under N_2 . Phase separation was achieved by the addition of 0.75 ml of 0.5 M KCl containing 50 mM EDTA. After vortexing and centrifugation at 1000 *g* for 5 min, the lower organic phase containing lipids was removed and the chloroform evaporated under a stream of N_2 . The lipid film was reconstituted in 20 μ l of chloroform/methanol (2:1, v/v), vortexed and 10 μ l samples were applied to heat-activated (110 °C; 15 min) TLC plates. Phospholipids were separated using the solvent system, ethyl acetate/iso-octane/acetic acid/water (13:2:3:10, by vol.) under saturating conditions. Phosphatidylcholine, phosphatidylethanolamine and phosphatidic acid standards (10 mg/ml in chloroform) and [^{14}C]PBut standard prepared by the method of Bonser and Thompson [29] (10 μ l of each) were spotted on adjacent lanes. Standard and sample spots were located by I_2 vapour and autoradiography of En^3Hance -sprayed TLC plates. The [3H]PBut and [3H]phospholipid spots were scraped into scintillant fluid (2,5-diphenyloxazole in toluene, 4 g/l), vortexed and counted in a Packard TriCarb 500 liquid-scintillation counter. The R_f value obtained for sample spots of [3H]PBut was 0.43 ± 0.03 (mean $\pm$ S.D., $n = 16$).

Calculations of concentration of ATP^{4-} species

The ATP concentration required to achieve a constant ATP^{4-} concentration (230 μ M) in all experiments was calculated using an updated version (3.0) of a previously published program, Bound and Determined [30].

Cytosolic Ca^{2+} , Ba^{2+} and Sr^{2+} measurements by fluorimetry

Washed lymphocytes suspended at 1×10^7 /ml in Hepes-buffered medium (10 mM Hepes, 145 mM NaCl, 5 mM KCl, 5 mM glucose, 0.1 % BSA and 1 mM $CaCl_2$, pH 7.4) were loaded with 2 μ M fura 2-AM by incubation at 37 °C for 20 min in the dark. In some experiments, cells were subsequently loaded with 10 or 30 μ M BAPTA-AM for 20 min, washed and incubated for a further 20 min at 37 °C to allow for AM hydrolysis. Cells were washed twice in Hepes-buffered medium and resuspended at 2×10^6 /ml in KCl medium (20 mM Hepes, 150 mM KCl, 5 mM glucose and 0.1 % BSA, pH 7.4) and kept in the dark. Ca^{2+} , Ba^{2+} , Sr^{2+} (1 mM) or 0.2 mM EGTA was added just before ATP or other agonists. Bivalent cations, Ca^{2+} , Ba^{2+} and Sr^{2+} bind with fura 2 to give a fluorescent signal [31]. Changes in cytosolic bivalent cation concentrations in stirred cell suspensions at 37 °C were monitored by a Johnson Foundation fluorimeter [32] with excitation at 340 nm and emission at 500 nm. Calibration of maximum and minimum signals was performed after each run by adding digitonin (25 μ g/ml, final concentration) followed by EGTA (final concentration 6 mM in 50 mM Tris/HCl, pH 8.5) [33].

Uptake of $^{133}Ba^{2+}$

$^{133}Ba^{2+}$ (0.5 mCi/ml, specific radioactivity 3 mCi/mg) was used to measure Ba^{2+} influx into lymphocytes stimulated with ATP.

BaCl₂ solutions (0.4–4.0 mM, 1 or 2 μ Ci/ml) in Ca²⁺-free Hepes KCl medium were equilibrated at 37 °C and then added to equal volumes of washed lymphocytes suspended in KCl medium at 37 °C (final concentration 1.25×10^7 cells/ml) just before the addition of 230 μ M ATP⁴⁻ at time zero. ¹³³Ba²⁺ influx was determined by removing 800 μ l aliquots of reaction mixture after 0, 15, 30 and 45 s and mixing with 200 μ l of ice-cold 50 mM MgCl₂ in Hepes-buffered medium layered over 250 μ l of phthalate oil mixture (di-*n*-butylphthalate/di-*n*-octylphthalate 7:3, v/v) [34] in Eppendorf tubes. The tubes were immediately centrifuged at 8000 *g* for 30 s to separate the cells from the medium. Subsequently, the medium and oil were aspirated and cell pellets were counted in a Wallac Wizard 3 Automatic Gammacounter. The specific radioactivity of ¹³³Ba²⁺ was also measured to allow the calculation of ¹³³Ba²⁺ uptake in nmol per 10⁷ cells. Isotope uptake at the zero time point for each Ba²⁺ concentration was subtracted from subsequent time points. Rate of ¹³³Ba²⁺ uptake (i.e. influx) at each Ba²⁺ concentration was calculated by least-squares analysis.

Data presentation and analysis

The quantity of [³H]PBut is reported as a percentage of total ³H-labelled cellular phospholipids on each lane of the TLC plates. PLD assays were performed in triplicate and data are expressed as means $\pm$ S.E.M. Statistical analysis of data was by Student's *t* test.

RESULTS

ATP-stimulated PLD activity in lymphocytes from patients with chronic lymphocytic leukaemia

PLD activity was measured in the presence of butanol, as PLD catalyses the transphosphatidyl reaction which yields the stable end product [³H]PBut. Extracellular ATP stimulated PLD activity in human lymphocytes, and [³H]PBut reached a maximum by 15 min (Figure 1a). PLD activity, defined as [³H]PBut accumulation at 15 min, increased as the concentration of ATP was raised, reaching a maximum at 1 mM (Figure 1b) with an EC₅₀ of 513 ± 48 μ M ATP (113 μ M ATP⁴⁻). Mean basal [³H]PBut accumulation was 0.13 ± 0.01 % of total [³H]phospholipids per 15 min (*n* = 12). BzATP, another P_{2z} agonist, was more potent than ATP in stimulating PLD activity with an EC₅₀ of 379 ± 36 μ M (Figure 1b). Other known agonists for the P_{2z} receptor, 2-methylthio-ATP and ATP[S], were less effective and produced maximal responses that were 40 and 20 % respectively of the response obtained with ATP. pp[CH₂]pA, ADP, UTP and adenosine all failed to stimulate PLD activity (results not shown). ATP-induced [³H]PBut accumulation varied considerably between different patients [1.04 ± 1.02 % (mean $\pm$ 1 S.D.) per 15 min, 12 patients]. However, there was consistency in lymphocyte responsiveness for any individual patient (e.g. 1.47, 1.56, 1.58 % [³H]PBut per 15 min for three assays several months apart).

Inhibitors of ATP-induced PLD activity

Both suramin and oxidized ATP, which are inhibitors of the P_{2z} purinoceptor in murine macrophages and human lymphocytes [22,25], prevented PLD activation by extracellular ATP (Table 1). Our previous work has shown that bivalent cation influx stimulated by extracellular ATP is attenuated by high concentrations of extracellular Na⁺ [20]. When K⁺ was replaced with the same concentration of Na⁺ (150 mM), ATP-stimulated PLD activity was reduced by 77 ± 6.5 % (*n* = 14) (Table 1). Permeant fluxes through the P_{2z}-ligand-gated ion channel are blocked

Figure 1 ATP-stimulated [³H]PBut accumulation in human lymphocytes

[³H]Oleic acid-labelled lymphocytes resuspended at 1×10^7 /ml in buffer containing 150 mM KCl, 10 mM Hepes, 5 mM glucose, 0.1 % BSA and 1 mM Ca²⁺ were equilibrated to 37 °C for 5 min. In (a) cells were incubated with (■, ▲) or without (□) 910 μ M ATP (230 μ M ATP⁴⁻) in the presence (■, □) or absence (▲) of 30 mM butanol. In (b) cells were incubated with ATP (□) or BzATP (■) for 15 min in the presence of 30 mM butanol and 1 mM Ca²⁺. [³H]PBut was expressed as (a) percentage of total [³H]phospholipids (means $\pm$ S.E.M., *n* = 3) from one of four experiments, and (b) percentage of the maximal response to 1.0 mM ATP which was taken as 100% (means $\pm$ S.E.M. from three to nine determinations).

Table 1 Inhibition of ATP-stimulated PLD activation in human lymphocytes

[³H]Oleic acid-labelled lymphocytes (1.0×10^7 /ml) in Hepes-buffered KCl medium supplemented with 30 mM butanol and 1 mM Ca^{2+} were incubated with or without 910 μM ATP (230 μM ATP⁴⁻) for 15 min, and [³H]PBut was measured. Some cells were incubated with or without 300 μM oxidized ATP for 60 min at 37 °C in buffer containing 145 mM NaCl, 5 mM KCl, 10 mM Hepes, 5 mM glucose, 1 mM Ca^{2+} and 0.1% BSA, washed and then incubated with ATP in KCl medium. Suramin (300 μM) or Mg^{2+} (10 mM) was added to the cell suspensions in KCl medium just before ATP. In some experiments, cells were incubated with ATP when suspended in Hepes-buffered NaCl medium. Results are expressed as percentages of the response by the same cells suspended in KCl medium. Results are means $\pm$ S.E.M. for the number of experiments shown, in parentheses.

Conditions	[³ H]PBut (% of control)
KCl medium (control)	100 (14)
Oxidized ATP	1.6 ± 1.22 (3)
Suramin	15.4 ± 11.8 (3)
Mg^{2+}	1.1 ± 1.10 (5)
NaCl medium	23.0 ± 6.5 (14)

when Mg^{2+} is added in 2–10-fold molar excess over extracellular ATP [16,21]. Similarly, in the present experiments, Mg^{2+} added to ATP-stimulated cells reduced [³H]PBut accumulation to $1.1 \pm 1.1\%$ of maximal activity (Table 1). Paradoxically, other inhibitors, such as FSBA and HMA, both of which permeate cells, significantly stimulated basal PLD activity ($P < 0.002$) and thus were not studied further (results not shown).

Role of Ca^{2+} in ATP-induced PLD activation

The relationship between ATP-stimulated PLD and changes in cytosolic [Ca^{2+}] was then studied. Addition of ATP to cells suspended in Ca^{2+} -containing medium caused a rapid increase in cytosolic [Ca^{2+}] to values above 1.0 μM , but no change in cytosolic [Ca^{2+}] was observed in Ca^{2+} -free medium containing 0.2 mM EGTA (Figure, 2 top). Likewise, ATP increased [³H]PBut accumulation 10-fold in Ca^{2+} -containing medium but only produced a 2-fold increase in Ca^{2+} -free medium containing EGTA (Figure, 2 top). These results suggest that either a rise in cytosolic [Ca^{2+}] or an influx of this cation stimulated PLD activity. These two possibilities were distinguished by PLD measurement in cells treated with thapsigargin, a specific inhibitor of the endoplasmic reticulum ATPase that sequesters Ca^{2+} [35,36]. Thapsigargin raised cytosolic [Ca^{2+}] to levels above 1.0 μM in the presence of extracellular Ca^{2+} and to about 400 nM in its absence, but PLD was not activated under either condition (Figure 2, bottom). When surface IgD present on these B-lineage leukaemic lymphocytes [37] was cross-linked with anti-IgD antibody, a rise in intracellular [Ca^{2+}] was observed as the result of release of Ca^{2+} from internal stores, but this rise in cytosolic [Ca^{2+}] did not stimulate PLD (results not shown). PLD activity was also measured in cells loaded with BAPTA-AM in order to buffer the rise in intracellular [Ca^{2+}] produced by ATP. In these cells, ATP-stimulated PLD activity was not inhibited but rather enhanced as the cytosolic BAPTA concentration was increased (Figure 3). Taken together, these results show that ATP-stimulated PLD activity shows no clear relationship to variations in cytosolic [Ca^{2+}].

Ba^{2+} and Sr^{2+} support ATP-stimulated PLD activity

In the presence of extracellular Ca^{2+} , ATP stimulated a rapid rise in cytosolic [Ca^{2+}], but the levels then decline over 10 min because of removal of Ca^{2+} by homeostatic mechanisms (Figure

Figure 2 Effect of ATP and thapsigargin on cytosolic [Ca^{2+}] and PLD activity in lymphocytes

Fura-2-loaded cells suspended in Hepes-buffered KCl medium were stimulated with (top) 230 μM ATP⁴⁻ or (bottom) 5 μM thapsigargin in the presence of 1 mM Ca^{2+} or 0.2 mM EGTA as indicated. In a parallel experiment, [³H]oleic acid-labelled lymphocytes suspended in Hepes-buffered KCl medium with 30 mM butanol were stimulated with (top) 230 μM ATP⁴⁻ or (bottom) 5 μM thapsigargin for 15 min, and [³H]PBut was measured. Mean values $\pm$ S.E.M. ($n = 3$) are shown. Basal [³H]PBut production in the absence of agonists was 0.14 ± 0.02 and $0.11 \pm 0.004\%$ in Ca^{2+} - and EGTA-containing media respectively. Results are from a single experiment representative of three. *Significant increase in PLD activity ($P < 0.001$).

4). In contrast, when Ba^{2+} and Sr^{2+} , which are also permeants of the P_{zy} -operated ion channel [21], were substituted for Ca^{2+} , the cytosolic levels of these two cations remained elevated for at least 15 min (Figure 4). These results indicate that, on entry into the cell, Ba^{2+} and Sr^{2+} are neither sequestered into the stores nor pumped from the cell by transport Ca^{2+} -ATPases [31]. All three bivalent cations supported ATP-stimulated PLD activity (Figure 4).

Bivalent cation influx determines PLD activity

In further experiments the relation between PLD activity and bivalent cation influx through the P_{zy} -operated ion channel was studied. ATP-stimulated PLD activity showed a linear relationship to extracellular [Ba^{2+}] over the range 0–2 mM (Figure 5). Furthermore, ATP-induced $^{133}\text{Ba}^{2+}$ uptake also showed a linear relationship to extracellular [Ba^{2+}] (Figure 5). The interdependence of PLD activation and bivalent cation influx was further supported by the observation that, when Mg^{2+} was added 5 min after lymphocytes had been stimulated with ATP in Ca^{2+} -containing medium, no further [³H]PBut accumulated (Figure 6). Similarly, when excess EGTA was added to ATP-stimulated cells, PLD activity abruptly ceased (Figure 6).

Figure 3 Effect of BAPTA on cytosolic $[Ca^{2+}]$ and PLD activity in ATP-stimulated lymphocytes

Cells sequentially loaded with fura 2 and BAPTA and suspended in Hepes-buffered KCl medium were incubated with $230 \mu M$ ATP $^{4-}$ in the presence of 1.0 mM Ca^{2+} . In a parallel experiment, $[^3H]$ PBut accumulation over 15 min was determined in $[^3H]$ oleic acid-labelled lymphocytes loaded with BAPTA and incubated with 30 mM butanol plus $230 \mu M$ ATP $^{4-}$. Mean values $\pm$ S.E.M. ($n = 3$) are shown. Basal $[^3H]$ PBut values (0.12 ± 0.04 , 0.07 ± 0.01 and $0.18 \pm 0.01\%$ for 0 , 10 and $30 \mu M$ BAPTA respectively) were subtracted. Results are from a single experiment representative of two. *Significant increase in PLD activity ($P < 0.001$).

Figure 4 Bivalent cations permeate the P_{2z} channel and stimulate PLD activity

Fura-2-loaded cells suspended in Hepes-buffered KCl medium were incubated with $230 \mu M$ ATP $^{4-}$ in the presence of 1.0 mM Ba^{2+} , Sr^{2+} or Ca^{2+} . In a parallel experiment, $[^3H]$ PBut accumulation over 15 min was determined in $[^3H]$ oleic acid-labelled lymphocytes incubated with 30 mM butanol plus $230 \mu M$ ATP $^{4-}$. Mean values $\pm$ S.E.M. ($n = 3$) are shown. Basal $[^3H]$ PBut production in the absence of ATP was 0.11 ± 0.002 , 0.12 ± 0.01 and $0.12 \pm 0.004\%$ when Ba^{2+} , Sr^{2+} or Ca^{2+} respectively were present in the medium. Each bivalent cation significantly increased ATP-stimulated PLD activity; * $P < 0.001$. Results are from a single experiment representative of three.

DISCUSSION

ATP-stimulated PLD activity is mediated by P_{2z} purinoceptors

This study shows that extracellular ATP, acting through the P_{2z} purinoceptor, stimulated PLD activity in human lymphocytes. This conclusion supports that of El-Moatassim and Dubyak [7,15] who showed that BzATP, an agonist for the P_{2z} purinoceptor, activated PLD of murine macrophages. However, murine macrophages express several types of P_2 purinoceptors, in contrast with human lymphocytes, which have only the P_{2z} purinoceptor subclass. This together with the features of the ATP-stimulated PLD activity make it very likely that the effect is a P_{2z} -purinoceptor-mediated response. The rank order of

Figure 5 Extracellular ATP produces a parallel increase in $^{133}Ba^{2+}$ influx and PLD activity

PLD activity (■) was measured in $[^3H]$ oleic acid-labelled lymphocytes suspended in Hepes-buffered KCl medium with 30 mM butanol and Ba^{2+} (0 – 2 mM) plus $230 \mu M$ ATP $^{4-}$ for 15 min at $37^\circ C$. Basal $[^3H]$ PBut accumulation was $0.13 \pm 0.01\%$ for several concentrations of extracellular Ba^{2+} . Mean values $\pm$ S.E.M. ($n = 3$) are shown. In a parallel experiment with cells from the same patient, $^{133}Ba^{2+}$ influx (□) at $37^\circ C$ was measured at extracellular Ba^{2+} concentrations of 0.2 – 2.0 mM plus $230 \mu M$ ATP $^{4-}$. Results are from a single experiment, representative of two.

Figure 6 Mg^{2+} and EGTA inhibit ATP-stimulated PLD activation in human lymphocytes

$[^3H]$ oleic acid labelled-lymphocytes suspended in Hepes-buffered KCl medium with 1 mM Ca^{2+} and 30 mM butanol were incubated with (■, ◆, ▲) or without (□) $230 \mu M$ ATP $^{4-}$ for 5 min after which Mg^{2+} (◆) or EGTA (▲) both final concentrations 2 mM, or an equal volume of buffer (■) was added. $[^3H]$ PBut was measured. Values are means $\pm$ S.E.M. ($n = 3$) from a single experiment, representative of four.

potency for purinoceptor agonists in producing maximal stimulation of PLD activity was BzATP $>$ ATP $>$ 2-methylthio-ATP (P_{2y} agonist) $\gg$ ATP[S]. Other potential agonists, pp[CH $_2$]pA (P_{2x} -specific), UTP (P_{2U} -specific), ADP (P_{2T} -specific) and adenosine (P_1 -purinoceptor agonist), did not stimulate PLD activity. This rank order of potency of nucleotide agonists is similar to that reported for producing Ba^{2+} influx through the P_{2z} -operated ion channel [22]. Mg^{2+} inhibited both ATP-stimulated PLD activation and opening of the P_{2z} -operated ion channel indicating that ATP $^{4-}$ was the preferred agonist species. Table 1 shows that suramin, a non-specific P_2 -purinoceptor antagonist, and oxidized ATP, a potent and irreversible inhibitor of the lymphocyte P_{2z} -operated ion channel, both prevented ATP-stimulated $[^3H]$ PBut

accumulation. High concentrations of extracellular Na^+ , which inhibits ATP-induced cation fluxes, also reduced ATP-stimulated PLD activity. Thus occupancy of P_{2z} purinoceptors or influx of Ca^{2+} through the P_{2z} -operated ion channel leads to stimulation of PLD activity.

Role of Ca^{2+} in ATP-stimulated PLD activity

It is well known that Ca^{2+} has a role in the activation of PLD, since Ca^{2+} ionophores and many Ca^{2+} -mobilizing agonists stimulate this effector enzyme. However, the exact role of Ca^{2+} in agonist-mediated PLD activation is not well defined [3,38]. In broken or permeabilized cell preparations, Ca^{2+} does not appear to be responsible for PLD activation. For example, concentrations of Ca^{2+} that approximated intracellular values (0.1–1 μM) did not stimulate PLD activity in rat liver plasma membranes, broken human neutrophils or permeabilized HL-60 cells [39–42]. Similarly, Ca^{2+} was not required for bradykinin-stimulated PLD activity in subcellular fractions of MDCK cells, but was necessary for the effect of bradykinin in intact cells [43]. Other studies on intact cells have confirmed the requirement for extracellular Ca^{2+} in receptor-stimulated PLD activation. For example, P_{2y} receptor agonists stimulated PLD activity in bovine aortic endothelial cells and MDCK cells [4,44] in the presence of extracellular Ca^{2+} , but not when EGTA was present. In these and other studies, Ca^{2+} -dependent stimulation of PLD by Ca^{2+} ionophore or receptor agonists (many of which activate PI-PLC enzymes) was attributed to a rise in cytosolic $[\text{Ca}^{2+}]$ [5,6]. In contrast, in the present study changes in cytosolic $[\text{Ca}^{2+}]$ in human lymphocytes did correlate with PLD activity. Thus thapsigargin, which raises cytosolic $[\text{Ca}^{2+}]$ by both release from cellular organelles and Ca^{2+} influx through the refilling channel, was unable to generate significant $[\text{Ca}^{2+}]$ accumulation, despite a large increase in cytosolic $[\text{Ca}^{2+}]$ and a vigorous Ca^{2+} influx (Figure 2, bottom). Similarly, Pfeilschifter and Merriweather [11] showed that thapsigargin did not stimulate significant PLD activity in mesangial cells. B-lymphocytes from patients with chronic lymphocytic leukaemia possess surface immunoglobulin (IgD), and cross-linking the surface IgD activates the PI-PLC cascade and raises cytosolic $[\text{Ca}^{2+}]$ [45]. Again, this rise failed to activate lymphocyte PLD. Similarly, El-Moatassim and Dubyak [7] reported that Ca^{2+} -mobilizing agonists such as platelet-activating factor and UTP, which are also coupled to the PI-PLC pathway, did not stimulate PLD activity in murine macrophages. Taken together, these results suggest that a release of Ca^{2+} from internal stores does not stimulate PLD activity in murine macrophages, mesangial cells or human lymphocytes. Agonist-stimulated changes in cytosolic $[\text{Ca}^{2+}]$ can be buffered with the Ca^{2+} chelator BAPTA, which is introduced into the cell as the membrane-permeant precursor BAPTA-AM [46]. Loading lymphocytes with BAPTA prevented the ATP-stimulated rise in cytosolic $[\text{Ca}^{2+}]$, but potentiated rather than inhibited PLD activity (Figure 3). These results contrast with other studies on human neutrophils and erythroleukaemia cells, and also rat mast cells and vascular smooth-muscle cells, which showed that agonist-stimulated PLD activity was reduced in BAPTA-loaded cells [5,6,47,48]. Unlike lymphocytes, these cell types either do not possess the P_{2z} purinoceptor or, in the case of mast cells, were not stimulated with P_2 agonists. Although in some cell types PLD activation appears to be dependent on IP_3 -mediated release of Ca^{2+} from internal stores, in human lymphocytes at least, PLD activity is not responsive to changes in cytosolic $[\text{Ca}^{2+}]$, but rather is dependent on Ca^{2+} influx through the P_{2z} -operated ion channel.

Ba^{2+} influx through the P_{2z} -operated ion channel activates PLD

The major finding of our study is that PLD activity was directly related to influx of Ba^{2+} through the P_{2z} -operated ion channel. Ba^{2+} is a convenient surrogate for Ca^{2+} and has some advantages over Ca^{2+} in studying the functional effects of bivalent cations in lymphocytes. For example, Ba^{2+} -fura 2 has similar spectral properties to Ca^{2+} -fura 2, so that Ba^{2+} uptake can be measured fluorimetrically [31] (Figure 4). Moreover, the unidirectional Ba^{2+} influx can be readily quantified using $^{133}\text{Ba}^{2+}$, since once inside the cell, this cation is neither pumped nor sequestered by transport ATPases. Ba^{2+} is a good permeant for the P_{2z} -operated ion channel with values for ATP-stimulated Ba^{2+} influx that approximate those for Ca^{2+} [21]. Furthermore, both Ba^{2+} and Sr^{2+} share with the Ca^{2+} the ability to support ATP-dependent PLD activity in intact cells (Figure 4). Similarly, Blache and Ciavatti [49,50] showed that thrombin-stimulated phospholipase A_2 activity in intact rat platelets, which requires extracellular Ca^{2+} , can also be stimulated by Sr^{2+} and Ba^{2+} . Our studies using $^{133}\text{Ba}^{2+}$ suggest that PLD activation can be related to ATP-induced influx of this bivalent cation. A good correlation between ATP-stimulated $^{133}\text{Ba}^{2+}$ influx and ATP-stimulated PLD activity over a range of extracellular Ba^{2+} concentrations was observed (Figure 5). In addition, PLD activity ceased immediately the permeating bivalent cation was removed by addition of excess EGTA. With the observed relationship between ATP-stimulated PLD activity and bivalent cation influx through the P_{2z} -operated ion channel, it is possible that PLD was activated by high local concentrations of these cations which accumulate in the subplasma-membrane region. Indeed, several investigators [51,52] have shown that agonist-stimulated Ca^{2+} influx raises Ca^{2+} concentrations to several micromolar in the subplasma-membrane region. Such $[\text{Ca}^{2+}]$ changes cannot be accurately measured by fura 2, which largely measures concentrations of bulk cytosolic Ca^{2+} . Furthermore, although BAPTA has high affinity for Ca^{2+} and rapid Ca^{2+} -binding kinetics, these undetected increases in $[\text{Ca}^{2+}]$ in the microenvironment near the P_{2z} -operated ion channels may occur so rapidly [53] that PLD activity is potentiated even while total cytosolic $[\text{Ca}^{2+}]$ is adequately buffered (Figure 3). It is also possible that, in ATP-stimulated lymphocytes, these local increases in Ba^{2+} or Ca^{2+} near the plasma membrane may mediate translocation of PLD or a putative regulatory G-protein and/or PKC to the membrane.

Although our data show that bivalent cation influx through the P_{2z} -operated ion channel activates PLD in intact cells, Figure 5 shows that a small fraction of Ca^{2+} (or Ba^{2+})-independent PLD activity was usually observed. In lymphocytes, that ATP-stimulated but Ca^{2+} -independent PLD activity is small in comparison with that in murine macrophages, in which most of the PLD activity is Ca^{2+} -insensitive [7]. Thus both the Ca^{2+} -dependent and -independent PLD of human lymphocytes appears to be closely associated with, or is part of, the P_{2z} purinoceptor, and furthermore PLD activation appears to be tightly coupled to bivalent cation influx associated with stimulation of this receptor.

Although the physiological significance of receptor-mediated PLD activation is not yet apparent, it may have a role in membrane-remodelling events. For example, recent reports that ADP ribosylation factor, a ubiquitous small GTP-binding protein that regulates vesicle-mediated protein trafficking and membrane-fusion events, stimulated PLD activity in HL-60 granulocytes suggests that PLD may be associated with secretion [54,55]. Furthermore earlier studies have linked PLD activity to secretion in neutrophils, mast cells and pancreatic β -cells [6,28,56]. In human leukaemic lymphocytes, ATP-stimulated PLD activity may also have a role in membrane-remodelling

events associated with lymphocyte transmigration and recirculation by modification of the plasma membrane at points of cell-to-cell contact, where local concentrations of ATP are significant.

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