Characterization and purification of neutrophil *ecto*-phosphatidic acid phosphohydrolase

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Phosphatidic acid and its derivatives play potentially important roles as extracellular messengers in biological systems. An *ecto*-phosphatidic acid phosphohydrolase (*ecto*-PAPase) has been identified which effectively regulates neutrophil responses to exogenous phosphatidic acid by converting the substrate to diacylglycerol. The present study was undertaken to characterize this *ecto*-enzyme on intact cells and to isolate the enzyme from solubilized neutrophil extracts. In the absence of detergent, short chain phosphatidic acids were hydrolysed most effectively by neutrophil plasma membrane *ecto*-PAPase; both saturated and unsaturated long chain phosphatidic acids were relatively resistant to hydrolysis. Both long ($C_{18:1}$) and short (C_8) chain lysophosphatidic acids were hydrolysed at rates comparable with those observed for short chain (diC_8) phosphatidic acid. Activity of the *ecto*-enzyme accounted for essentially all of the *N*-ethylmaleimide-insensitive, Mg^{2+} -independent PAPase activity recovered from disrupted neutrophils. At 37 °C and pH 7.2, the apparent K_m for dioctanoyl phosphatidic acid (diC_8PA) was 1.4×10^{-3} M. Other phosphatidic acids and lysophosphatidic acids inhibited hydrolysis of [^{32}P] diC_8PA in a rank order that correlated with competitor solubility, lysophosphatidic acids and unsaturated phosphatidic acids being much more effective inhibitors than long chain saturated phosphatidic acids. Dioleoyl

($C_{18:1}$) phosphatidic acid was an unexpectedly strong inhibitor of activity, in comparison with its ability to act as a direct substrate in the absence of detergent. Other inhibitors of neutrophil *ecto*-PAPase included sphingosine, dimethyl- and dihydro-sphingosine, propranolol, NaF and $MgCl_2$. Of several leucocyte populations isolated from human blood by FACS, including T cells, B cells, NK lymphocytes and monocytes, *ecto*-PAPase was most prevalent on neutrophils; erythrocytes were essentially devoid of activity. A non-hydrolysable, phosphonate analogue of phosphatidic acid, phosphonate 1, efficiently solubilized catalytic activity from intact neutrophils without causing cell disruption or increasing permeability. Enzyme activity in solubilized extracts was purified in the absence of detergent by successive heparin–Sephacrose, gel filtration and anion exchange chromatography. By assaying activity in renatured SDS/polyacrylamide gel slices, the molecular mass of neutrophil *ecto*-PAPase was estimated to be between 45 and 52 kDa, similar to the molecular mass of previously purified plasma membrane PAPases. Since a large portion of neutrophil plasma membrane PAPase is available for hydrolysis of exogenous substrates, *ecto*-PAPase may play an important role in regulating inflammatory cell responses to extracellular phosphatidic acid in biological systems.

INTRODUCTION

Phosphatidic acid is a biologically active phospholipid that mediates cellular responses resulting from activation of phospholipase D [1]. In addition to its well-documented involvement in intracellular signal communication, phosphatidic acid may play an important role as an extracellular messenger. Deployed on the outer surface of the plasma membrane, phosphatidic acid provides a novel method of communication between cells in direct contact [2]. Exogenous phosphatidic acid induces a number of cellular functions, including Ca^{2+} mobilization and chemotaxis of monocytes [3], Ca^{2+} mobilization and tyrosine kinase activation in neutrophilic leucocytes [4], adenylate cyclase inhibition and actin polymerization in fibroblasts [5,6] and phosphoinositide turnover in keratinocytes and cortical astrocytes [7,8]. While each of these responses has been attributed to ligation of membrane receptors for the phospholipid, the role of

phosphatidic acid receptors in cellular activation remains to be defined.

Perry et al. recently identified a novel *ecto*-phosphatidic acid phosphohydrolase (*ecto*-PAPase) on neutrophilic leucocytes [9]. Localized on the extracellular side of the plasma membrane, this enzyme rapidly hydrolysed phosphate from short chain phosphatidic acids. Diacylglycerol, continuously generated as a result of the catalytic activity of the enzyme, mediated activation of the neutrophil superoxide-generating system, probably by activating protein kinase C. Xie and Low reported similar activity on keratinocytes and demonstrated that the *ecto*-enzyme effectively hydrolysed lyso-phosphatidate in addition to short-chain phosphatidic acids [10]. Therefore *ecto*-PAPase provides a mechanism for limiting the biological effects of extracellular lysophosphatidic acid, which is an important extracellular messenger that conveys its effects by ligating a G-protein-linked plasma membrane receptor [11,12]. Cells which do not respond

Abbreviations used: CF, carrier free; diC_8PA , dioctanoyl phosphatidic acid; EGTA, ethylene glycol-bis tetra-acetic acid; HBSS, Hanks balanced salt solution; PAPase, phosphatidic acid phosphohydrolase; PE, phycoerythrin; phosphonate 1, non-hydrolysable, phosphonate analogue of phosphatidic acid; PNPP, *p*-nitrophenyl phosphate.

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to lyso-phosphatidic acid, such as neutrophilic leucocytes [4,11], may not possess lyso-phosphatidic acid receptors [11]. However, these cells display remarkable responsiveness to phosphatidic acids [4,13], indicating that a different component of receptors may be involved.

Therefore *ecto*-PAPase exerts important regulatory influences on cellular responses to both phosphatidic and lyso-phosphatidic acids. In addition, by hydrolysing available substrates, *ecto*-PAPase may effectively regulate binding of phosphatidic acid to membrane receptors. While plasma membrane PAPases have been isolated and characterized by several groups [14–17], *ecto*-PAPase has not been characterized or isolated and its distribution on various cell populations has not been established. In order to determine conditions under which the enzyme may hydrolyse substrates in biological systems, the present study was undertaken to characterize the *ecto*-PAPase on intact neutrophilic leucocytes and to assess the distribution of the *ecto*-enzyme among diverse populations of blood cells. The results demonstrate that a large portion of the PAPase activity confined to neutrophil plasma membranes can hydrolyse extracellular substrates, implying a physiologically important function for the *ecto*-enzyme in modulating the responses of cells at sites of infection and inflammation.

EXPERIMENTAL

Materials

Lipids, including the sodium salts of unlabelled phosphatidic acids and the 1,2-*sn*-diacylglycerols used to generate labelled phosphatidic acids, were obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.). A non-hydrolysable, phosphonate analogue of dioctanoyl phosphatidic acid (phosphonate 1) was synthesized from diacetone-D-mannitol as described in Perry et al. [9]. Diacylglycerol kinase was from Calbiochem (LaJolla, CA, U.S.A.) and [32 P]ATP (3000 Ci/mM) was from New England Nuclear (Boston, MA, U.S.A.). Antibodies to leucocyte cell-surface antigens conjugated to the fluorochromes, FITC or phycoerythrin (PE) were from Becton Dickinson Immunocytometry Systems (San Jose, CA, U.S.A.). Sphingosine, *N,N*-dimethylsphingosine and dihydrosphingosine were from Calbiochem. Precast SDS/polyacrylamide gels, unstained molecular-mass markers, silver staining reagents and other materials for gel electrophoresis were from Bio-Rad (Richmond, CA, U.S.A.). Gel-filtration and heparin-Sepharose resins were from Pharmacia (Uppsala, Sweden). Other chemicals and reagents, including D,L-propranolol, *N*-ethylmaleimide, *p*-nitrophenyl phosphate (PNPP), NADH, DEAE-Sepharcel, twice-crystallized papain and prestained molecular-mass markers were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Synthesis of 32 P-labelled phosphatidic acids

32 P-Labelled phosphatidic acids were synthesized by phosphorylation of diacylglycerols in the presence of [32 P]ATP and diacylglycerol kinase. Diglycerides were solubilized in 1 mM phosphatidylethanolamine/1 mM cardiolipin and incubated with 0.02 unit of diacylglycerol kinase in 350 μ l of a reaction buffer that consisted of 100 mM NaCl, 10 mM MgCl₂, 1 mM 2-mercaptoethanol, 0.7% (v/v) Triton X-100 and 50 mM Tris/HCl (pH 6.6). The diacylglycerols used included 1,2-dioctanoylglycerol (C₈), 1,2-didecanoylglycerol (C₁₀), 1,2-dimyristoylglycerol (C₁₄), 1,2-dipalmitoylglycerol (C₁₆), 1,2-dioleoylglycerol (18:1) and 1,2-dihexanoylglycerol (C₆). Each was used at a final concentration of 0.1 mM. Phosphorylation was initiated by the addition of 1–2 mCi of [32 P]ATP, without

additional unlabelled carrier in order to generate products of high specific radioactivity. After incubation for 2 h at 37 °C, the reaction was terminated with 2 ml of chloroform/methanol/HCl (1:2:0.03; by vol.). The phases were separated and the organic layers were dried and resolved by TLC developed with chloroform/methanol/20% (v/v) methylamine (60:35:10; by vol.). Various amounts of 32 P-labelled lysophosphatidic acids were generated during the kinase reactions, presumably because of contamination of the substrates with monoacylglycerols or by deacylation of the products. [32 P]Phosphatidic acid was localized by autoradiography and eluted from the silica gel with methanol. In some experiments, [32 P]lysophosphatidic acid was also localized and recovered from the silica gel. The eluates were re-extracted into chloroform, dried and sonicated in buffer containing 150 mM NaCl/1 mM EGTA/5.0 mM Hepes, pH 7.2 (NaCl/EGTA/Hepes) with the respective non-radioactive phosphatidic acid or lyso-phosphatidic acid at the final concentration used in assays of PAPase activity.

Quantification of *ecto*-PAPase

Ecto-PAPase was assessed by the release of 32 P from labelled phosphatidic acid effected by intact cells in the absence of detergent [9,10]. Phosphatidic acids were used at a final concentration of 0.2 mM, unless otherwise noted. Reactions were carried out in a final volume of 100 μ l, containing 10 μ l of labelled substrate, the indicated volumes of cells or cell extracts and NaCl/EGTA/Hepes buffer at 37 °C, unless otherwise stated. Reactions with intact cells were initiated by addition of the indicated number of cells in a volume of 10 μ l. Neutrophil extracts or fractions obtained during purification of PAPase from these extracts were used in a volume of 10–90 μ l; for these assays, the reaction was initiated by addition of 10 μ l of labelled substrate. After incubation, the reaction was terminated by the addition of 500 μ l of chloroform/methanol/HCl (1:2:0.03, by vol.). After phase partitioning, 32 P in aqueous extracts was estimated by Čerenkov radiation. Unless noted otherwise, the results represent the means $\pm$ S.D. of three determinations from a representative experiment. Each experiment was repeated at least twice for confirmation. While *N*-ethylmaleimide was not routinely included in assays of PAPase activity, activity of intact neutrophils and the extracts prepared from them was markedly resistant to the effects of the reagent at concentrations as high as 25 mM (see below). The addition of Mg²⁺ ions (5 mM MgCl₂) markedly attenuated the activity of intact neutrophils and of the neutrophil extracts prepared as described below. In these respects, activity of intact neutrophils and neutrophil extracts was similar to the activity of previously purified membrane PAPases [14–17]. Confirmation that the radioactivity measured was due to [32 P]_iphosphate and not some other water-soluble compound was given by ion-exchange HPLC analysis of the extracted material, using the method of Taylor et al. [18]. In addition, we employed a molybdenum-based extraction technique to separate [32 P]P_i from other labelled compounds in these extracts, using the procedure described by Xie and Low in studies of keratinocyte *ecto*-PAPase [10]. In accord with their results, over 95% of the water soluble radioactivity resulting from [32 P]phosphatidic acid degradation by either intact neutrophils or neutrophil extracts prepared as described below was recovered with P_i (results not shown). When added to assays before [32 P]phosphatidic acid, excess (5 mM) non-radioactive glycerol 3-phosphate did not alter this distribution of radioactivity, eliminating the possibility that the labelled P_i formed was due to catabolism of glycerol 3-[32 P]phosphate by acid or alkali hydrolases.

Cell preparations

Neutrophils were prepared from heparinized human blood after Ficoll–Hypaque density-gradient centrifugation, to remove mononuclear leucocytes, and lysis of erythrocytes in isotonic NH_4Cl [19]. Final preparations contained $< 1.0\%$ contamination with mononuclear leucocytes and no detectable erythrocytes. Erythrocytes were prepared from heparinized blood which was depleted of platelets and leucocytes by repetitive differential centrifugation and removal of the resultant buffy coats. Mononuclear leucocytes were isolated from blood by Ficoll–Hypaque density-gradient centrifugation [20]. These preparations contained few neutrophils ($< 5.0\%$) and no detectable erythrocytes. Cells were washed and resuspended in $\text{NaCl}/\text{EGTA}/\text{Hepes}$ buffer to a density of 2.5×10^7 cells/ml. For assays of PAPase activity in whole cell sonicates (Table 1), cells were resuspended in $150 \text{ mM NaCl}/1.0 \text{ mM EDTA}/5.0 \text{ mM Hepes}$, pH 7.2. EDTA was used to ensure efficient removal of Mg^{2+} ions liberated by cell disruption. Cells were disrupted by sonication using $3 \times 5 \text{ s}$ bursts delivered from the microtip of a 300 Sonic Dismembrator (Fisher), operated at 35% power. Sonicates were used, without further treatment, for determination of Mg^{2+} -independent PAPase activity, as described below.

FACS

We used FACS to isolate subpopulations of blood leucocytes for assessment of the distribution of *ecto*-PAPase. Mononuclear leucocytes were washed and resuspended in PBS at a concentration of 2.5×10^7 cells/ml. Cell preparations were incubated with one of the following antibody combinations: anti-CD4-FITC+anti-CD8-PE; anti-CD19-FITC+anti-CD56-PE; or anti-CD15-FITC+anti-CD11b-PE. For labelling, each antibody ($50 \mu\text{l}$) was incubated with cells ($150 \mu\text{l}$) for 20 min at room temperature. For labelling of neutrophils and monocytes with anti-CD15-FITC+anti-CD11b-PE, each antibody ($50 \mu\text{l}$) was added to a cell preparation which consisted of $100 \mu\text{l}$ of mononuclear leucocytes and $50 \mu\text{l}$ of washed neutrophils, also resuspended in PBS at a concentration of 2.5×10^7 cells/ml. Neutrophils were identified on the basis of their characteristic light-scattering properties and labelling with both anti-CD11b and anti-CD15 monoclonal antibodies. Monocytes were identified and segregated from neutrophils on the basis of light scattering characteristics and labelling with anti-CD11b, but not anti-CD15 antibody.

Cells were sorted with a Becton Dickinson FACS-Star^{PLUS} flow cytometer, equipped with a water-cooled Ar laser emitting at 488 nm. Lymphocytes, monocytes and neutrophils were discriminated by forward and right-angle light scatter and the fluorescence of gated cells was determined and used as the basis of cell selection. PE fluorescence (means $\pm$ S.D) was measured through a $575 \pm 26 \text{ nm}$ band pass filter and FITC fluorescence was measured through a $530 \pm 30 \text{ nm}$ band pass filter. Cells were analysed at a rate of 1000–1500 events/s and brightly fluorescent cells within respective light-scatter gates were selected into either the left (FITC) or right (PE) channel using the 'normal R' sort mode. Analysis of cells after sorting revealed that over 97% of the cells within each population met the threshold light scatter and fluorescent requirements for selection.

For determination of *ecto*-PAPase, sorted cells were pelleted by centrifugation (1200 g for 5 min), enumerated microscopically and resuspended in $\text{NaCl}/\text{EGTA}/\text{Hepes}$ buffer at a concentration of 5.0×10^5 cells/ml. The enzyme activity was quantified using 2.5×10^4 sorted cells/determination. Assay sensitivity was increased by extending the incubation period to 1 h and by decreasing by one order of magnitude the specific radioactivity of

the substrate, which was therefore used at a final concentration of $20 \mu\text{M}$ for these determinations.

Solubilization and isolation of *ecto*-PAPase

In preliminary experiments, *ecto*-PAPase was solubilized by incubation of intact cells with phosphonate 1, at 37°C . During a 15 min incubation with phosphonate 1 at a concentration of $100 \mu\text{g}/\text{ml}$, cellular *ecto*-PAPase activity was released with no microscopic indication of cellular lysis (see below). Solubilized extracts were concentrated 10–50-fold in an Amicon B15 macro-solute concentrator (Amicon Corp., Beverly, MA, U.S.A.) and, in some experiments, passed through a $1.5 \text{ cm} \times 80 \text{ cm}$ Sephadex G-100 gel-filtration column equilibrated and eluted with $\text{NaCl}/\text{EGTA}/\text{Hepes}$ buffer, before analysis. Phosphatase activity in supernatants and in gel-filtration fractions against phosphatidic acid and PNPP was assessed under the conditions described below. PNPP phosphatase activity was assessed spectrophotometrically at 405 nm, as described previously [21,22].

For enzyme purification, plasma membranes were isolated from neutrophils prepared by Ficoll–Hypaque density-gradient centrifugation of cells in pooled buffy-coat preparations obtained from 5 units of donated blood. The resultant preparations, containing $(2.5\text{--}5.0) \times 10^8$ neutrophils, were washed and finally resuspended in buffer A (1 mM PMSF , $1 \mu\text{g}/\text{ml}$ leupeptin, $1 \text{ mM di-isopropyl fluorophosphate}$, 1 mM EGTA and 150 mM NaCl , pH 7.2) at a concentration of $5 \times 10^7\text{--}1.0 \times 10^8$ cells/ml. Plasma membranes were prepared from sonicated cells as described previously [21]. Briefly, cell preparations were chilled on melting ice and disrupted by sonication. After removal of nuclei by low speed (500 g) centrifugation, sonicates (1.0 ml) were layered upon 1.5 ml cushions of 40% sucrose in $11 \text{ mm} \times 34 \text{ mm}$ thin-wall plastic ultracentrifuge tubes. After centrifugation at 43000 g in a swing-out rotor for 60 min, plasma membranes at the cytosol/sucrose interface were collected, diluted in buffer A, washed by centrifugation and resuspended in buffer A at a concentration of 3–4 mg of protein/ml. To liberate PAPase, membranes were warmed and incubated with phosphonate 1, as described above, effecting release of almost all of the membrane-associated activity. Extracted membranes were subsequently removed by ultracentrifugation at 95000 g for 30 min.

Extracts, solubilized as described above, were dialysed against 5 mM Hepes buffer (pH 5.5) containing protease inhibitors. Considerable precipitation occurred during dialysis, but PAPase activity remained soluble. As indicated below, the addition of detergent did not enhance recovery of activity during dialysis or the subsequent purification steps, but, instead, resulted in decreased specific activity. Dialysed extracts were cleared by centrifugation and applied to a $1.5 \text{ cm} \times 8 \text{ cm}$ column of heparin–Sephacryl CL-6B equilibrated with 5 mM Hepes (pH 5.5). After washing to equilibrium with 92 mM NaCl in 10 mM Hepes (pH 6.0), PAPase was eluted in approximately 80 ml of $200 \text{ mM NaCl}/10 \text{ mM Hepes}$ (pH 7.0). The eluate was dialysed extensively against 5 mM Hepes (pH 8.0) containing protease inhibitors (1 mM PMSF , $1 \mu\text{g}/\text{ml}$ leupeptin, $1 \text{ mM di-isopropyl fluorophosphate}$ and 1 mM EGTA), and concentrated in dialysis membranes to a volume of 15 ml . The concentrated extract was applied to a $1.5 \text{ cm} \times 90 \text{ cm}$ column of Sephacryl S-300 equilibrated and eluted with 5 mM Hepes (pH 8.0); fractions containing the PAPase activity which eluted after the excluded proteins were pooled (30 ml), diluted with 2 vol. of buffer containing $25 \text{ mM NaCl}/5 \text{ mM Hepes}$ (pH 7.4) and further resolved by anion-exchange chromatography, using a $1.5 \text{ cm} \times 6 \text{ cm}$ column of DEAE–Sephacel equilibrated with 5 mM Hepes (pH 7.4) and eluted with increasing concentrations of NaCl ($25 \text{ mM--}200 \text{ mM}$).

Renaturation of PAPase activity in polyacrylamide gels

Solubilized extracts were analysed by SDS/PAGE, using either precast 12% minigels (6 cm × 8 cm; BioRad Laboratories) or 10 cm × 10 cm 12% gels prepared as described by Siddiqui and Yang [23]. Resolved proteins were defined by silver staining. In addition, PAPase activity was assessed in renatured gel slices, using a modification of the procedure developed by Kameshita and Fujisawa for analysis of protein kinases [24]. In these experiments, instead of boiling the samples, they were incubated with 2 × SDS sample buffer at 37 °C for 2 h prior to application. Localization of PAPase activity was unaffected by the presence of 2-mercaptoethanol in the sample buffer; therefore, the reducing agent was not used in the experiments described. The distribution of proteins in silver-stained gels of crude, solubilized extracts boiled or not boiled in the presence or absence of 2-mercaptoethanol was strikingly similar. In some experiments, prestained molecular-mass markers were added directly to samples before electrophoresis in order to improve localization and characterization of activity within unstained gels. Preliminary experiments revealed that the prestained markers used, including triosephosphate isomerase (calibrated molecular mass, 35200 Da), lactate dehydrogenase (calibrated molecular mass, 42700 Da) and fumarase (calibrated molecular mass, 55300 Da) did not alter PAPase activity in solubilized extracts.

After electrophoresis at 25 mA for 1 h at room temperature, SDS was removed with propan-2-ol, proteins were further denatured with 6 M guanidine, and were renatured by washing the gels in buffer containing 2-mercaptoethanol and Tween-40, exactly as described by Kameshita and Fujisawa [24]. After renaturation, gels were washed in several changes of distilled water and allowed to equilibrate to pH 6.2 for 30 min. The gels were then cut into sections and assayed for PAPase activity as described below. Parallel gels were silver stained. Gel sections (approx. 3 mm × 5 mm) were each crushed in the bottom of a 12 mm × 75 mm glass tube in 250 µl of either PAPase assay buffer (pH 7.0) or, for assays at decreased pH, in 75 mM citrate buffer (pH 6.2) in order to increase sensitivity. The assay tubes were immersed in a sonication bath, at full setting, for 1.5 min at room temperature to pulverize the gel matrix. After addition of labelled substrate (2 µM), the assay tubes were incubated at 37 °C with intermittent, gentle agitation for the time intervals shown below, and the release of ³²P was determined as described above.

RESULTS

Characteristics of phosphatidic acid hydrolysis by intact neutrophils

Using a substrate concentration of 200 µM, hydrolysis of diC₈PA by intact neutrophils incubated for 30 min at pH 7.2 increased in a linear manner as the cell number was increased over the range 1 × 10⁵–5 × 10⁵ cells/assay (Figure 1a). Disruption of cells by sonication did not result in a marked increase in Mg²⁺-independent, *N*-ethylmaleimide-resistant PAPase activity (Table 1). Thus essentially all of the PAPase activity of neutrophil plasma membranes hydrolysed exogenous substrate. In subsequent experiments, enzyme activity was determined using 2.5 × 10⁵ intact cells, unless shown otherwise. Figure 1(b) illustrates the dependence of phosphatidic acid hydrolysis on the duration of incubation. The rate of hydrolysis increased linearly as the duration of incubation was increased over a 1 h time period at 37 °C. Subsequent analyses were made using a 30 min incubation period, unless otherwise noted. Figure 1(c) demonstrates the dependence of diC₈PA hydrolysis on temperature.

Figure 1 Characteristics of neutrophil ecto-PAPase

Assays were carried out using a substrate concentration of 200 µM diC₈PA. Unless indicated otherwise, hydrolysis was determined with 2.5 × 10⁵ cells/assay for 30 min at 37 °C. In (d), experiments with pH values of less than 6.5 were in 10 mM citrate buffer and those between pH 6.5 and 9.5 were in 10 mM bistris/propane buffer. Other experiments were carried out at pH 7.2 in 10 mM Hepes-buffered saline. Each point is the mean ± S.D. of three determinations.

Table 1 *N*-Ethylmaleimide-insensitive PAPase activity of intact and disrupted neutrophils

PAPase was assayed using [³²P]diC₈PA as substrate. The results are the means ± S.D. of triplicate determinations from one leucocyte preparation. This experiment was repeated twice with qualitatively similar results. *Cells were resuspended in 150 mM NaCl/1.0 mM EDTA/5.0 mM Hepes buffer (pH 7.2) before sonication. Examination by microscopy confirmed that sonication disrupted > 99% of the cells.

<i>N</i> -Ethylmaleimide (mM)	PAPase activity (nmol/30 min per 2.5 × 10 ⁵ cells)	
	Intact cells	Disrupted cells*
0	2.76 ± 0.14	2.92 ± 0.09
5	2.41 ± 0.11	2.55 ± 0.02
10	2.16 ± 0.12	2.26 ± 0.08
25	2.12 ± 0.06	2.22 ± 0.02

The rate of hydrolysis diminished rapidly at temperatures below 37 °C; at 29 °C activity was reduced by more than 60%, and little activity was observed at temperatures of ≤ 12 °C. Finally, Figure 1(d) depicts the pH-dependence of diC₈PA hydrolysis effected by intact neutrophils incubated for 30 min at 37 °C. Hydrolysis was optimal under acidic conditions and decreased rapidly with increasing alkalinity.

Experiments were undertaken to determine the influence of substrate concentration on the rate of phosphatidic acid hydrolysis effected by intact neutrophils. Typical results are illustrated in Figure 2. In 5 separate experiments using neutrophils from blood of different individuals, the apparent *K_m* was (1.41 ± 0.31) × 10⁻³ M diC₈PA and the apparent *V_{max}* was 32.6 ± 6.1 nmol of diC₈PA hydrolysed/30 min per 2.5 × 10⁵ cells (means ± S.D.).

Figure 2 Kinetics of neutrophil *ecto*-PAPase

Assays were carried out with the indicated concentrations of diC₈PA for 30 min at pH 7.2 using 2.5×10^5 cells. The results are the means $\pm$ S.D. of three determinations.

Substrate specificity of *ecto*-PAPase

As described in the Experimental section, several ³²P-labelled phosphatidic acids were synthesized using the diacylglycerol kinase reaction and the hydrolysis of each of these by intact cells was evaluated. Of the six phosphatidic acid species examined, dioctanoyl (C₈) phosphatidic acid was hydrolysed most efficiently by intact neutrophils (Table 2). Dihexanoyl (diC₆) and didecanoyl (diC₁₀) phosphatidic acids were also suitable substrates, each being hydrolysed at 33–44 % of the rate of diC₈PA. Longer chain phosphatidic acids were poor substrates of *ecto*-PAPase. Saturated myristic (diC₁₄) and palmitic (diC₁₆) phosphatidic acids were hydrolysed at approx. 15 % of the rate of hydrolysis of diC₈PA. Unsaturated dioleoyl (C_{18:1}) phosphatidic acid was hydrolysed even more slowly, with rates of less than 10 % of that observed with dioctanoyl phosphatidic acid. Lysophosphatidic acid was also efficiently hydrolysed by intact neutrophils. Both short (C₈) and long chain (C_{18:1}) lysophosphatidic acid were hydrolysed at rates comparable with those observed with the short chain parent compound.

As shown in Table 3, in the absence of exogenous non-radioactive carrier lipid, about 18 % of the available [³²P]diC₈PA was hydrolysed by 1.25×10^5 neutrophils in 15 min at 37 °C. As expected, the amount of ³²P-labelled phosphatidic acid hydrolysed was substantially reduced when unlabelled diC₈PA was added at a final concentration of 0.2 mM. We assessed the ability of other non-radioactive phosphatidic acids and lysophosphatidic acids to interfere with *ecto*-PAPase-mediated hydrolysis of carrier-free [³²P]diC₈PA. Certain lyso-phosphatidic acids, including lysomyristoyl (C₁₄) and lysopalmitoyl (C₁₆) phosphatidic acids, were potent inhibitors of enzyme activity (Table 3). The ability of other phosphatidic acids to interfere

Table 2 Substrate specificity of neutrophil *ecto*-PAPase

The ³²P-labelled phosphatidic acids and lysophosphatidic acids were used at a final concentration of 200 μ M. *The results are the means $\pm$ S.D. of 3–6 determinations. †Percentage response compared with diC₈PA as substrate.

Substrate	Chain length	Enzyme activity* (nmol/30 min per 2.5×10^5 cells)	Relative response†
Phosphatidic acid			
Dihexanoyl	C ₆	1.52 ± 0.19	44.2
Dioctanoyl	C ₈	3.44 ± 0.23	100
Didecanoyl	C ₁₀	1.15 ± 0.13	33.4
Dimyristoyl	C ₁₄	0.59 ± 0.18	17.2
Dipalmitoyl	C ₁₆	0.46 ± 0.04	13.3
Dioleoyl	C _{18:1}	0.29 ± 0.06	8.4
Lysophosphatidic acid			
Octanoyl	C ₈	2.94 ± 0.41	85.4
Oleoyl	C _{18:1}	2.29 ± 0.38	66.6

Table 3 Competitive inhibition of diC₈PA hydrolysis

*The carrier-free system measured the hydrolysis of [³²P]diC₈PA by 1.25×10^5 cells in the absence of additional non-radioactive substrate. Under these conditions, 19% of the available substrate was hydrolysed in 15 min at 37 °C. The non-radioactive phosphatidic acids listed below were added at a final concentration of 200 μ M. The results are the means $\pm$ S.D. of 3 determinations. †Percentage inhibition of hydrolysis observed in the absence of non-radioactive phosphatidic acid.

Phosphatidic acid added to 'carrier-free system	Hydrolysis of [³² P]diC ₈ PA (%)	Inhibition (%)†
None	19.0 ± 3.1	0
Diheptanoyl (C ₇)	17.0 ± 1.3	10.5
Distearoyl (C ₁₈)	15.5 ± 1.5	18.4
Lyso-distearoyl (C ₁₈)	15.5 ± 0.8	18.4
Dimyristoyl (C ₁₄)	14.7 ± 1.2	22.6
Dipalmitoyl (C ₁₆)	13.3 ± 0.2	30.0
Ditetranoyl (C ₄)	13.0 ± 1.1	31.5
Dilauroyl (C ₁₂)	9.4 ± 0.3	50.5
Dihexanoyl (C ₆)	9.3 ± 0.5	51.0
Lyso-oleoyl (C _{18:1})	8.3 ± 0.6	56.3
Didecanoyl (C ₁₀)	8.2 ± 0.5	56.8
Dioleoyl (C _{18:1})	8.1 ± 0.8	57.3
Lyso-palmitoyl (C ₁₆)	7.4 ± 1.2	61.1
Lyso-myristoyl (C ₁₄)	6.6 ± 0.2	65.3
Dilinoleoyl (C _{18:2})	6.5 ± 0.9	65.8
Dioctanoyl (C₈)	5.9 ± 0.2	68.9

with hydrolysis correlated with the degree of disparity of the chain length of each in relation to that of diC₈PA. For example, diC₆ and diC₁₀ phosphatidic acids were more effective than either diC₄ or diC₁₂ phosphatidic acids in inhibiting diC₈PA hydrolysis. Longer chain, saturated phosphatidic acids were typically non-competitive, presumably as a consequence of their solubility characteristics. For unknown reasons, dioleoyl phosphatidic acid was an unexpectedly strong inhibitor, based on its relatively weak performance as a direct substrate for *ecto*-PAPase.

Pharmacological modulators of *ecto*-PAPase

We assessed the influence of several agents on the activity of neutrophil *ecto*-PAPase. Sphingosine at a concentration of

Figure 3 Inhibition of *ecto*-PAPase

Cells were incubated with 200 μ M diC₈PA in the absence or presence of the indicated concentrations of (a) D,L-sphingosine, (b) N,N-dimethylsphingosine, (c) dihydrosphingosine, (d) D,L-propranolol, (e) NaF or (f) MgCl₂ for 30 min at 37 °C. The values represent the % of diC₈PA hydrolysis observed in the same experiment in the absence of inhibitor (means $\pm$ S.D., $n = 3$).

approx 1.25 mM caused 50% inhibition of phosphatidic acid hydrolysis (Figure 3). *N,N*-Dimethylsphingosine and dihydrosphingosine were more effective, with half maximal inhibition obtained with concentrations of less than 0.3 mM. Propranolol, a reported inhibitor of PAPase in several other systems, was an effective inhibitor of the *ecto*-enzyme, but only at relatively high concentrations (> 2.5 mM). NaF caused half maximal inhibition of *ecto*-PAPase activity at 5 mM and complete inhibition at a concentration of 50 mM, although cell disruption was not observed at this concentration. *Ecto*-PAPase activity was also markedly attenuated by MgCl₂; 5 mM MgCl₂ caused a 90% reduction in enzyme activity.

Distribution of *ecto*-PAPase

Among blood cells, erythrocytes were found to possess no detectable *ecto*-PAPase activity assessed using as many as 5×10^6 cells/assay (results not shown). We selected various leucocyte subpopulations from preparations of mononuclear leucocytes using FACS in order to determine the distribution of *ecto*-PAPase among leucocytes. For comparison, neutrophils were isolated and assayed under similar conditions. Expressed on a cell-number basis, neutrophils were found to possess the highest level of activity among each of the leucocyte populations

Table 4 Distribution of *ecto*-PAPase among sorted blood leucocytes

*Cells were selected on the basis of antigen expression and light scattering characteristics. Analysis after sorting revealed that over 97% of the cells within each population met the threshold criteria for selection. *Ecto*-PAPase was determined using 20 μ M diC₈PA and 2.5×10^4 sorted cells/assay. Each value is the mean $\pm$ S.D. of three determinations.

Sorted cell population	Fluorescence/antigen*	DiC ₈ PA hydrolysis (pmol/h per 2.5×10^4 cells)
Neutrophils	CD11b/CD15	99 ± 4
Natural killer cells	CD56	60 ± 2
Monocytes	CD11b	45 ± 7
Cytotoxic T-cells	CD8	40 ± 7
B-lymphocytes	CD19	33 ± 6
Helper T-cells	CD4	17 ± 4

Figure 4 Sephadex G-100 gel filtration of PAPase in phosphonate-1-solubilized extracts of intact neutrophils

PAPase eluted after excluded proteins (V_0), near the calibrated elution position of BSA (results not shown). Fractions 7–11 were pooled, concentrated and used for further analyses.

examined (Table 4). Natural killer leucocytes (selected on the basis of reactivity with anti-CD56 monoclonal antibody) and monocytes (identified by light scattering properties and the presence of CD11b) possessed about half as much activity as neutrophils. CD4⁺ (helper) T lymphocytes had the lowest levels of activity. CD8⁺ (cytotoxic) T cells and CD19⁺ B cells expressed activities between that of CD4⁺ T cells and neutrophils.

Solubilization of *ecto*-PAPase

Incubation of intact neutrophils (or neutrophil plasma membranes) with phosphonate 1 liberated most of the cell-associated PAPase activity into the supernatant. Phosphonate-1-mediated enzyme solubilization was temperature-dependent, with little

Figure 5 Electrophoretic analysis of solubilized neutrophil plasma membrane PAPase

These silver-stained gels were run in parallel with gels that had been renatured as described in the text. After processing, slices of the unstained gels were removed from the areas indicated by the square brackets and analysed for PAPase activity. Numbers to the right of each bracket indicate radioactivity released (c.p.m. above background) during incubation of the renatured gel slice with substrate. (A) shows the results from three separate crude extracts. In (B), activity from one extract was further analysed to establish its mobility in relation to the position of three prestained molecular-mass markers, fumarase (55 300 Da), lactate dehydrogenase (42 700 Da) and triosephosphate isomerase (35 200 Da) in the unstained gel.

release of activity at 4 °C. At 37 °C, $75.9 \pm 7.1\%$ of the cell-associated PAPase activity (mean $\pm$ S.D.) was released during a 15 min incubation of intact cells with the analogue (100 μ g/ml); higher concentrations caused almost complete release, but resulted in cellular disruption. Cells treated with phosphonate 1 at a concentration of 100 μ g/ml retained their ability to exclude Trypan Blue dye (results not shown), even though their *ecto*-PAPase activity was lost, suggesting that neither permeabilization nor membrane solubilization was required for release of activity. Phosphonate-1-solubilized supernatants were concentrated 10–50-fold using an Amicon B15 osmotic concentrator with nearly complete (> 95%) retention of PAPase activity. Concentrates prepared in this manner also effectively dephosphorylated PNPP, and this activity was optimal at neutral pH (results not shown). When concentrated extracts were resolved by Sephadex G-100 gel filtration, PAPase activity was eluted in a broad peak after the excluded proteins (Figure 4). Concentrated pooled

Table 5 Isolation of neutrophil plasma-membrane PAPase

PAPase was purified from sonicated neutrophils as described in the Experimental section. The results are from one purification procedure, which was repeated twice.

	PAPase activity (nmol/min)	Protein (mg)	Specific activity (nmol/min per mg of protein)	Purification (fold)	Yield (%)
Cell sonicate	956	922	1.036	1	
Plasma membranes	391	40.8	9.58	9.25	40.8
Solubilized extract	332	12.6	26.34	25.42	34.7
Heparin-Sepharose CL6B	39	0.54	72.22	69.71	4.0
Sephacryl S-300	19	0.099	191.91	185.24	2.0
DEAE-Sepharcel	1.41	0.012	117.5	113.41	0.15

material retained its ability to hydrolyse phosphatidic acid, but activity against PNPP was completely lost (results not shown).

Purification of plasma-membrane PAPase

Isolated plasma membranes were further purified in order to exclude potential interference by PAPases contained in the neutrophil cytosol. Incubation of plasma membranes with phosphonate 1 under the conditions given above resulted in near-complete liberation of associated PAPase activity; activity in these detergent-free extracts was recovered in a single peak which eluted in a similar position to that of extracts of intact cells after Sephadex G-100 gel filtration (results not shown). Neutrophil plasma membrane extracts contained several proteins detectable in silver-stained gels (Figure 5). Although the pattern of silver-stained proteins varied noticeably between different extracts, in each case activity was recovered in renatured gel slices from approximately the same area (Figure 5A). Activity consistently localized approximately midway between lactate dehydrogenase and fumarase, indicating an approximate molecular mass of 45 000–52 000 kDa (Figure 5B).

Table 5 shows PAPase recovery and specific activity after each step of purification. Solubilization of plasma membranes with phosphonate 1 resulted in a 2.5-fold increase in specific activity and 85% recovery. Subsequent purification procedures resulted in substantial decreases in recovery, but a purification of over 100-fold was obtained. Addition of detergent (0.1–1.0% Triton X-100) did not improve recovery. In contrast, isolation procedures carried out in detergent-free conditions consistently resulted in higher specific activities. Marked precipitation of extracted proteins was observed when solubilized plasma membrane extracts were initially dialysed in detergent-free conditions. However, PAPase activity did not precipitate, and the resulting specific activity of the dialysed extracts was increased by 25–30% (results not shown). Of several procedures evaluated in preliminary experiments, optimal results were obtained using heparin-Sepharose chromatography, which resulted in a 2.7-fold increase in specific activity. Increased recoveries of PAPase were obtained with cation-exchange and hydroxyapatite chromatography, but little increase in specific activity was gained, since, similar to other proteins contained in the extracts, PAPase did not bind tightly to these supports under the conditions used (results not shown). Sephacryl S-300 gel filtration of fractions obtained from heparin-Sepharose chromatography increased specific activity 2.6-fold and removed many of the contaminants

Figure 6 Electrophoretic analysis of plasma-membrane extracts at various stages of PAPase purification

Solubilized extracts of neutrophil plasma membranes were treated as indicated in Table 4. Silver-stained SDS gels of active fractions recovered from heparin–Sepharose, gel-filtration and anion-exchange chromatography are shown in lanes 1, 2 and 3 respectively. After purification, a single faint band was recovered in the area of the gel expected to contain PAPase activity, indicated by the arrow.

which had been detected by silver staining (Figure 6), but a single prominent band at the location identified by the in-gel phosphatase assays was not consistently observed. Although anion-exchange chromatography resulted in a substantial loss of activity and decreased specific activity, the process effectively removed many of the contaminants remaining after gel filtration. Concentrated anion-exchange fractions possessed PAPase activity (Table 5) and resulted a single faint band on silver-stained gels in the region where PAPase activity in crude extracts was recovered (Figure 6).

In the experiments presented above, PAPase was purified from extracts prepared from isolated neutrophil plasma membranes. We utilized the in-gel phosphatase assay to determine the profile of activity released upon exposure of intact cells to the phosphonate 1, since the in-gel assay provides a unique method to characterize activity in crude extracts. In order to increase sensitivity, assays were carried out at pH 6.2, since our previous results demonstrated enhanced catalytic activity of *ecto*-PAPase at decreased pH (Figure 1d). As shown in Figure 7(a), the activity released from intact cells had a similar distribution to that of activity released from isolated membranes, with a major peak of activity recovered in fractions containing proteins with a molecular mass of 43–55 kDa. In subsequent experiments, prestained molecular-mass markers were added to extracts before separation in 10 cm × 10 cm gels, rather than mini-gels, in order to assess the molecular-mass range of the enzyme involved more precisely. Again, most PAPase activity was recovered from gel slices between the 43 and 55 kDa markers (Figure 7b), indicating an approximate molecular mass of 45–52 kDa for neutrophil *ecto*-PAPase.

DISCUSSION

Perry et al. provided the first evidence for the presence of a unique phosphatidic acid phosphohydrolase on the outer surface of neutrophil plasma membranes [9]. The present report extends these findings and provides information pertaining to the identity and characteristics of this enzyme. The implication by Perry et al. that plasma membrane PAPase is an *ecto*-enzyme was based on the finding that the substrate was hydrolysed by intact cells. In addition, the hydrolysis product, [^{32}P]P_i, was exclusively released to the extracellular medium. Xie and Lou reported that part of the PAPase activity of cultured keratinocytes was solubilized when intact cells were incubated with either papain or trypsin, supporting their conclusion that the active site of this enzyme was

Figure 7 In-gel phosphatase assay of extracts of intact neutrophils

Intact cells were incubated with phosphonate 1 to liberate *ecto*-PAPase under conditions that did not disrupt or permeabilize cells. Extracts were concentrated and resolved by SDS/PAGE on (a) 6 × 8-cm mini-gels or, for better resolution, (b) on 10 × 10-cm standard sized gels. Gels were renatured and sectioned as described in the Experimental section. In these experiments, assays of PAPase activity were carried out in 75 mM citrate buffer at pH 6.2. The position of the excised slices is compared with the migration of pre-stained molecular-mass markers run concurrently. The results illustrated were from 4 separate experiments.

also located extracellularly [10]. Our previous studies have shown that neutrophils [25,26], like other cell types [14–17,27–31], possess at least two distinct PAPase activities that are readily distinguished by their pH profiles, sensitivity to *N*-ethylmaleimide and cation dependence. Unlike the cytosolic enzyme, PAPase in isolated neutrophil plasma membranes hydrolysed long-chain, unsaturated phosphatidic acid only in the presence of detergent [25,26]. This activity was neither Ca^{2+} - nor Mg^{2+} -dependent and was resistant to inhibition with 25 mM *N*-ethylmaleimide. In contrast, cytosolic activity was Mg^{2+} -dependent and was completely abolished by *N*-ethylmaleimide at concentrations of less than 5.0 mM. Unlike activity confined to the plasma membrane, cytosolic PAPase effectively hydrolysed dioleoyl phosphatidic acid in the absence of detergent [26], similar to results reported by other workers [31]. The cytosolic enzyme displayed a well-defined optimum at pH 6.9; very little activity was observed at pH values of 5.7 or less. In contrast, in the presence of detergent, plasma membrane PAPase hydrolysed dioleoyl phosphatidic acid most efficiently under acidic conditions [26,27]. The pH profile of this activity was similar to that of the *ecto*-PAPase described in the present report. It is therefore possible that both the detergent-dependent and detergent-independent activities of neutrophil plasma-membrane PAPase result from the same enzyme which requires detergent to hydrolyse long-chain substrates but hydrolyses more soluble, short-chain substrates in aqueous solution without detergent. Studies are presently being carried out to characterize more fully the purified *ecto*-enzyme in relation to the plasma-membrane PAPases described previously.

With respect to enzymes described previously, there has been considerable difficulty defining the molecular mass of *N*-ethylmaleimide-insensitive PAPases to date, reflecting the fact that PAPase has been an extremely difficult protein to purify. Kanoh et al. [14] initially reported a molecular mass of 83 kDa for PAPase isolated from porcine thymus, based on the recovery of a single band on SDS/PAGE of isolated material. However, these authors recently advanced the possibility that a 35 kDa rather than an 83 kDa polypeptide accounted for the activity within the purified preparation [32]. Using a similar extraction method to isolate *N*-ethylmaleimide-insensitive PAPase from rat liver, Fleming and Yeaman [16] also attributed enzymic activity to an 83 kDa protein in resolved extracts. However, these authors were unable to refute the possibility that activity resided in 35 kDa and 54 kDa polypeptides which also appeared in SDS gels of the extracts. Waggoner et al. used a novel extraction method and isolation procedure to purify *N*-ethylmaleimide-insensitive PAPase from rat liver [15]. The enzyme was recovered in both an anionic and cationic form, with approximate molecular masses of 53 and 51 kDa respectively. Although enzyme activity of the resolved proteins was not shown directly, treatment of isolated fractions with *N*-glycanase F converted both the putative anionic and cationic forms of the enzyme into a single band of 28 kDa. However, these conclusions have recently been questioned by Siess and Hofstetter [17], who used a novel antibody bridge to assess the distribution of PAPases in SDS gels of rat liver extracts. These experiments indicated a molecular size of 31 kDa for rat liver PAPase, despite the fact that the preparations used were not treated to remove carbohydrates.

The in-gel phosphatase assay described herein may provide a method to resolve this controversy and explore the size distribution of PAPases in crude extracts. Our results with neutrophil plasma membrane extracts, using this assay, most closely parallel those of Waggoner et al., obtained with purified rat liver extracts [15]. In preliminary experiments, however, we were not able to immunoprecipitate material solubilized from neutrophilic leucocytes with an antibody against rat liver PAPase provided by

D. N. Brindley of the University of Alberta (D. English, unpublished work). We have not, as yet, applied the in-gel analysis to fractions treated with *N*-glycanase F or other enzymes, but it is possible that differences in glycosylation account for many of the contrasts found in previous studies. In addition, heterogeneous glycosylation may be the cause of the relatively wide bands of activity we observed, in addition to minor variations between preparations. Since recovery of enzyme activity was low, our results must be assessed with caution, but the data clearly indicate the presence of a renaturable PAPase in extracts of neutrophil plasma membranes with a molecular mass of approximately 50 kDa. Similar activity was recovered in supernatants of intact cells, extracted in a manner that did not result in cell disruption or increase cell permeability. Since direct comparison of purified material may yield confusing or misleading results, experiments have been undertaken to characterize rat liver extracts and extracts of other tissues using the in-gel phosphatase assay. This analysis may allow us to assess the relationship of neutrophil plasma-membrane PAPase to previously isolated enzymes more closely, and to determine if differences in methods of extraction or protein isolation account for differences in the molecular masses reported to date.

In summary, neutrophil *ecto*-PAPase displays characteristics similar to those of previously characterized plasma membrane PAPases, but the relationship of these activities remains to be defined. Regardless of the identity of the enzymes, however, our results indicate that a substantial portion of the PAPase activity associated with neutrophil plasma membranes is capable of hydrolysing exogenous substrate. While PAPase on the inner surface of the plasma membrane potentially modulates important aspects of phosphatidic-acid-dependent signal transduction pathways [1,33,34], the physiological significance of the *ecto*-enzyme is unknown. Such an enzyme may effectively limit the responses of cells to either phosphatidic acid or lysophosphatidic acid, released *in vivo* by cell disruption, or generated at the surface of cells in nearby tissues by the activation of certain metabolic pathways. In addition, *ecto*-PAPase may modulate the association of exogenous phosphatidic acid with specific cellular binding sites [4] and consequently exert an important influence in modulating responses of cells to this phospholipid in biological systems.

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