Phospholipase D is involved in cytosolic phospholipase A_2-dependent selective release of arachidonic acid by fMLP-stimulated rat neutrophils

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Abstract When rat polymorphonuclear neutrophils (PMN) were treated with *N*-formyl-Met-Leu-Phe (fMLP), the release of arachidonic acid in preference to other fatty acids was observed. Levels of arachidonic acid reached a plateau within 5 min, and were accompanied by an ~4-fold increase in in vitro phospholipase (PL) A_2 and PLD activities in PMN lysates. Treatment of PMN with ethanol (an inhibitor of PLD-mediated phosphatidic acid formation), propranolol (a phosphatidic acid phosphatase inhibitor), or 4-bromophenacylbromide (a PLA_2 inhibitor), each suppressed fMLP-stimulated arachidonate release. Treatment with RHC-80267 (a diacylglycerol lipase inhibitor), however, had no such effect. The cytosolic PLA_2 ($cPLA_2$) inhibitor, arachidonoyl trifluoromethyl ketone, suppressed PLA_2 activity in PMN homogenates and arachidonate release by fMLP-treated PMN. These results suggest that fMLP-elicited arachidonate release is mediated by $cPLA_2$ but not diacylglycerol lipase, and that the activation of $cPLA_2$ is downstream of the PLD-dependent signaling pathway.

Key words: Phospholipase A_2; Phospholipase D; Neutrophil; Formyl-Met-Leu-Phe; Arachidonic acid; Rat

1. Introduction

The 85-kDa cytosolic phospholipase A_2 ($cPLA_2$), an arachidonic acid-preferential PLA_2 isozyme, is distributed throughout a wide variety of animal tissues and cells. It preferentially hydrolyses phospholipids containing arachidonic acid at the *sn*-2 position, translocates from the cytosol to intracellular membrane compartments in response to a submicromolar order of intracellular Ca^{2+} concentration, and is activated by phosphorylation via mitogen-activated protein (MAP) kinase (reviewed in [1–3]). On the basis of these features, $cPLA_2$ is believed to regulate arachidonic acid release by mammalian cells in response to stimuli that mobilize intracellular Ca^{2+} and activate MAP kinase.

The production of phosphatidic acid (PA) from phospholipase D (PLD)-catalyzed hydrolysis of phosphatidylcholine is a rapid and widespread response of cells stimulated with diverse agonists, and is implicated in a broad spectrum of physiological and pathological processes, such as exocytosis, mitogenesis, oncogenesis and inflammation (reviewed in [4]). There are also several lines of evidence to suggest that arachidonic acid release correlates with PLD activation. Arachidonic acid release by activated cells is reported to be accompanied by the increase in PLD activity and inhibition of PLD by ethanol is associated with the decrease in arachidonic acid release [5–10]. To account for these observations, some investigators have proposed that the PA generated by the PLD reaction is converted to diacylglycerol (DG) by PA phosphatase, and that arachidonic acid is released from DG by DG lipase [6,7]. In contrast to this 'indirect' model for arachidonic acid release via DG, other investigators have proposed that arachidonic acid is 'directly' released from phospholipids by PLA_2 [8–10]. In the latter case, certain product(s) of the PLD-dependent signaling pathway must act as modulator(s) to regulate PLA_2 activity.

Several reports have shown that activation of PLD is indispensable for several cellular responses in polymorphonuclear neutrophils (PMN) [11–15]. A number of agonists have also been reported to activate $cPLA_2$, leading to arachidonic acid release in PMN [16–20]. Therefore PMN represent a useful model system for addressing the functional relationship between $cPLA_2$ and PLD after stimulation with a variety of stimuli. We now report that the selective release of arachidonic acid, which is predominantly regulated by $cPLA_2$, is attenuated by inhibiting the PLD-dependent signaling pathway in *N*-formyl-Met-Leu-Phe (fMLP)-stimulated rat PMN, thereby providing evidence for a functional correlation between PLD and $cPLA_2$.

2. Materials and methods

2.1. Materials

Female Sprague-Dawley rats (weighing 190–220 g) were obtained from Japan Clear (Tokyo, Japan). Histopaque 1077, fMLP, cytochalasin B, Na_3VO_4, 4-bromophenacylbromide (*p*BPB), 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, and acid phosphatase (from white potato, type VII) were purchased from Sigma (St. Louis, MO). Arachidonyl trifluoromethyl ketone ($AACOCF_3$) was purchased from Calbiochem-Novabiochem International (San Diego, CA). 9-Anthryldiazomethane and RHC-80267 were purchased from Funakoshi (Tokyo, Japan). 2-Decanoyl-1-(*O*-(11-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*S*-indacene-3-propionyl)amino)undecyl)-*sn*-

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Abbreviations: $cPLA_2$, cytosolic phospholipase A_2; PLD, phospholipase D; fMLP, *N*-formyl-Met-Leu-Phe; PMN, polymorphonuclear neutrophils; $AACOCF_3$, arachidonyl trifluoromethyl ketone; PA, phosphatidic acid; PEt, phosphatidylethanol; PC, phosphatidylcholine; *p*BPB, 4-bromophenacylbromide; DG, diacylglycerol; MAP kinase, mitogen-activated protein kinase; HBSS, Hanks' balanced salt solution; 1-BODIPY-PC, 2-decanoyl-1-(*O*-(11-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*S*-indacene-3-propionyl)amino)undecyl)-*sn*-glycero-3-phosphocholine

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glycero-3-phosphocholine (1-BODIPY-PC) is a product of Molecular Probes (Eugene, OR).

2.2. Preparation of PMN

Preparation of PMN was principally according to a method reported by Bøyum [21]. Rats were injected intraperitoneally with 25 ml of 3% (w/v) sodium caseinate. After 16 h, 15 ml of Hanks' balanced salt solution (HBSS) containing 1 mM EDTA (HBSS/EDTA) was injected intraperitoneally, and peritoneal cells were collected by centrifugation at $200\times g$ for 10 min at 15°C. After two washes, the cells were resuspended in HBSS containing 0.5 mM EDTA and 1.25 mg/ml BSA (HBSS/BSA) at 1.5×10^7 cells/ml. Mononuclear cells were removed by step-wise density gradient centrifugation with Histopaque 1077 at $500\times g$ for 30 min at 15°C. The resulting pellet containing PMN was recovered, and the remaining erythrocytes were removed by washing with 155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA, pH 7.2. The cells were washed twice, resuspended in HBSS/BSA and kept on ice until use. The purity of the PMN was checked by Giemsa staining, and their viability was determined by trypan blue dye exclusion. PMN with a purity of $90.8\pm0.7\%$ (mean ± S.E.M.; $n=7$) were used in this study.

2.3. Stimulation of PMN

To a suspension of 1.25×10^7 cells/ml PMN, 1.26 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.41 mM $MgSO_4$ and 5 μg/ml cytochalasin B were added, and the mixture was preincubated for 5 min at 37°C. PMN were then stimulated by 0–1250 nM fMLP and incubated for up to 15 min at 37°C. The effects of the pretreatment with various inhibitors on PMN were determined, as described in the appropriate figure legends. Reactions were stopped by dipping the tubes into an ice-water bath.

2.4. Fatty acid analysis

0.5 nmol of *n*-heptadecanoic acid and 2.5 nmol of 2,6-bis(1,1-dimethylethyl)-4-methylphenol were added as an internal standard and anti-oxidant, respectively, followed by the addition of 1.25 ml of Dole's reagent [22]. Free fatty acids were extracted into the *n*-heptadecanoic acid layer, dried under a nitrogen stream and then converted into fluorescent derivatives by the addition of 100 μl of 0.1 mg/ml 9-anthryldiazomethane dissolved in methanol/ethyl acetate (9:1, v/v), followed by incubation for 1 h in the dark at room temperature. Free fatty acids were analyzed on a Capcell Pak C18 column (AG120, 4.6×250 mm, S-5 μm; Shiseido, Tokyo, Japan) at 35°C, using a solvent system of acetonitrile/2-propanol/water (80:16:4, v/v) at a flow rate of 1.2 ml/min. The 9-anthryldiazomethane derivatives were detected with the aid of a fluorescence detector (excitation at 365 nm and emission at 412 nm).

2.5. Preparation of homogenates of fMLP-stimulated PMN

PMN, adjusted to 3.75×10^7 and 1.25×10^8 cells/ml for assaying PLA_2 and PLD activities, respectively, were resuspended in 20 mM Tris-HCl, pH 7.4, containing 2 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, and 1 mM PMSF at 4°C. The cells were then disrupted using a Model UR-20P sonicator (TOMY, Tokyo, Japan) at 20 W for 3 s. Any undisrupted cells and cell debris were removed by centrifugation at $18\,000\times g$ for 1 min at 4°C. Supernatants were recovered and used for PLD assay. For PLA_2 assay, this fraction was further centrifuged at $100\,000\times g$ for 60 min at 4°C to obtain a cytosol fraction.

2.6. PLA_2 assay

PLA_2 activity was assayed by measurement of free arachidonic acid released [23]. The reaction mixture consisted of 100 mM Tris-HCl, pH 9.0, 4 mM $CaCl_2$, 1 mg/ml BSA, 12.5 μM 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, and PMN homogenate soluble fraction (9.4×10^5 cell equivalents). After incubation for 20 min at 37°C, the released arachidonic acid was extracted, and levels determined using high performance liquid chromatography (HPLC), as described above.

2.7. Phosphatase treatment of PMN homogenate

PMN homogenates were prepared by sonication in cell lysis buffer composed of 20 mM Tris-HCl, pH 7.4, 2 mM dithiothreitol, 1 mM EDTA and 1 mM EGTA. Then the homogenates were incubated for 10 min at 37°C with either 18 units/ml acid phosphatase or 1 mM Na_3VO_4 in 50 mM 2-[*N*-morpholino]ethanesulfonic acid (pH 6.0) and 1 mM $MgCl_2$. After the incubation, $cPLA_2$ activity was assessed as described above.

2.8. PLD assay

PLD activity was assayed principally according to a method reported by Jones et al. [24]. Briefly, 12.5 μl of reaction mixture, composed of 60 μM 1-BODIPY-PC, 0.12 mM *n*-octyl-glucopyranoside, 96 mM NaCl, 15 mM 2-[*N*-morpholino]ethanesulfonic acid (pH 6.0), 6 mM piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] (pH 6.5), 3 mM EGTA, 0.6 mM EDTA, 24 mM glycerol-2-phosphate (pH 7.0), 0.6 mM dithiothreitol and 5 μl of PMN homogenates, was incubated for up to 30 min at 30°C. The reaction was stopped by dipping the tubes into an ice-cold water bath. Then 5 μl of the reaction mixture was spotted onto silica gel plates (TLC plates silica gel 60 without fluorescent indicator, Art. 5641; Merck) and developed with chloroform/methanol/water/acetic acid (65:35:4:1, v/v). The spots were visualized with the aid of UV lamp, and spots corresponding to PA and phosphatidylethanol (PEt) were scraped off and extracted with 1.5 ml of ethanol. After removal of the silica gel by centrifugation, the fluores-

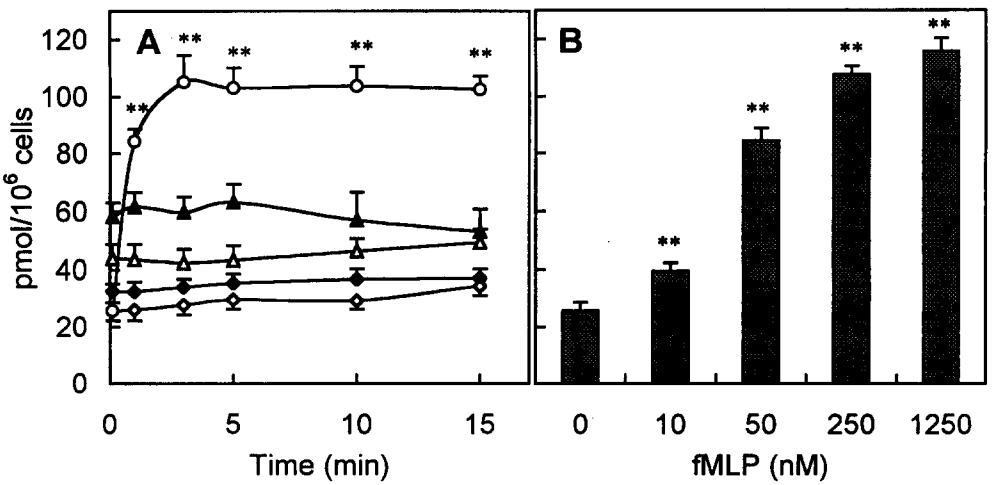
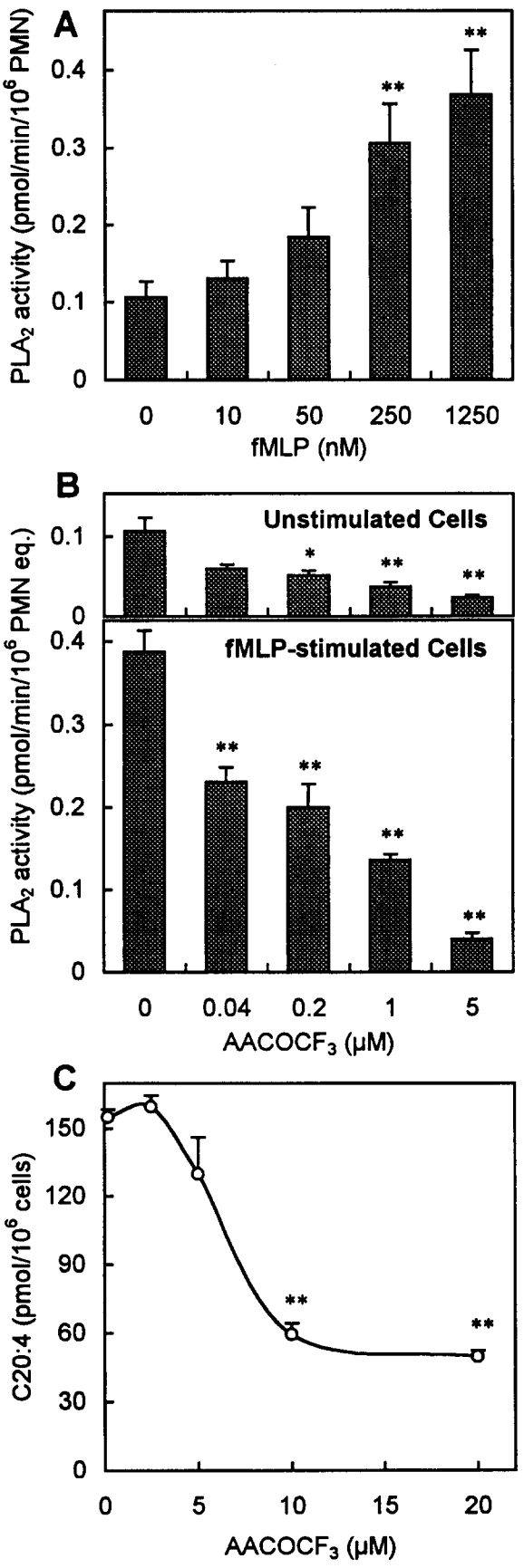


Fig. 1. Fatty acid release by fMLP-stimulated PMN. A: PMN were incubated in the presence of 50 nM fMLP for the indicated periods and the amounts of free fatty acids, including arachidonic acid (open circles), linoleic acid+myristic acid (open triangles), oleic acid (open squares), palmitoic acid (closed triangles) and stearic acid (closed squares) were quantified, as described in Section 2. B: PMN were incubated with the indicated concentrations of fMLP for 1 min at 37°C, and the levels of free arachidonic acid were quantified. Data are expressed as the mean ± S.E.M. of six independent experiments, each of which was carried out in duplicate. $^{**}P<0.01$ versus without fMLP.



cence intensity of the reaction products was determined using a fluorescence spectrophotometer at an excitation wavelength of 507 nm and emission wavelength of 514 nm.

Fig. 2. PLA_2 activity in 100 000 × *g* supernatants of PMN lysates. A: Activation of PLA_2 activity by fMLP. PLA_2 activity in the 100 000 × *g* supernatants of PMN homogenates, which were prepared from PMN that had been stimulated for 1 min with various concentrations of fMLP, were assayed as described in Section 2. Data are expressed as the mean ± S.E.M. of five independent experiments, each of which was carried out in duplicate. $^{**}P < 0.01$ versus without fMLP. B: Effect of $AACOCF_3$ on PLA_2 activity. 100 000 × *g* supernatants of homogenates prepared from PMN before and after stimulation for 1 min with 1250 nM fMLP were incubated with the indicated concentrations of $AACOCF_3$ for 10 min at 37°C, and then assayed for PLA_2 activity. Data are expressed as the mean ± S.E.M. of three independent experiments, each of which was carried out in duplicate. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus without $AACOCF_3$. C: Effect of $AACOCF_3$ on the release of arachidonic acid by fMLP-stimulated PMN. PMN, pretreated with the indicated concentrations of $AACOCF_3$ for 20 min at 37°C, were incubated with 50 nM fMLP for 5 min at 37°C. The free fatty acids released were analyzed. Data are expressed as the mean ± S.E.M. of five independent experiments, each of which was carried out in duplicate. $^{**}P < 0.01$ versus without $AACOCF_3$.
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2.9. Statistical analysis

Data are expressed as the mean ± S.E.M. of more than three independent experiments, each of which was carried out in duplicate. Statistical analysis was performed using an unpaired Student's *t*-test.

3. Results

3.1. Free fatty acid release in fMLP-stimulated PMN

When PMN were stimulated with 50 nM fMLP, levels of free arachidonic acid increased, reaching a maximum and plateauing at 103.7 ± 6.9 pmol/10^6 cells after 3 min (Fig. 1A). In contrast, levels of other fatty acids, including linoleic, myristic, oleic, palmitoic and stearic acids, did not change appreciably. The release of arachidonic acid was dependent upon the concentration of fMLP, having a minimal effect at 10 nM, and causing a maximal release of 115.6 ± 4.8 pmol/10^6 cells at 1250 nM (Fig. 1B). No appreciable increase in fatty acids other than arachidonic acid was observed at any fMLP concentration (data not shown).

3.2. PLA_2 activity in fMLP-stimulated PMN

PLA_2 activity in the 100 000 × *g* supernatant of PMN homogenate increased following stimulation with fMLP, from a basal release of 0.10 ± 0.02 pmol of arachidonic acid/min/10^6 cell equivalents to 173% at 0.17 ± 0.04 and 347% at 0.35 ± 0.05 pmol/min/10^6 cell equivalents at 50 and 1250 nM fMLP, respectively (Fig. 2A). Thus, activation of PLA_2 activity in the 100 000 × *g* supernatant of PMN homogenate paralleled the increase in arachidonic acid release (Fig. 1B). PLA_2 activity in the 100 000 × *g* supernatants of homogenates prepared from unstimulated and fMLP-stimulated PMN (Fig. 2B) were inhibited by the $cPLA_2$-specific inhibitor, $AACOCF_3$ [25], in a similar dose-dependent manner: PLA_2 activity in the 100 000 × *g* supernatants of unstimulated PMN homogenates fell from 0.10 to 0.048 (47%), and to 0.022 (21%) pmol/min, and that of fMLP-stimulated PMN homogenates fell from 0.36 to 0.19 (52%), and to 0.037 (10%) pmol/min at 0.2 and 5 μM $AACOCF_3$, respectively. Thus, PLA_2 activity in the PMN homogenates is most probably attributable to $cPLA_2$.

The increased $cPLA_2$ activity in fMLP-stimulated PMN returned to the basal level when PMN homogenates were treated with acid phosphatase; $cPLA_2$ activities in lysates of

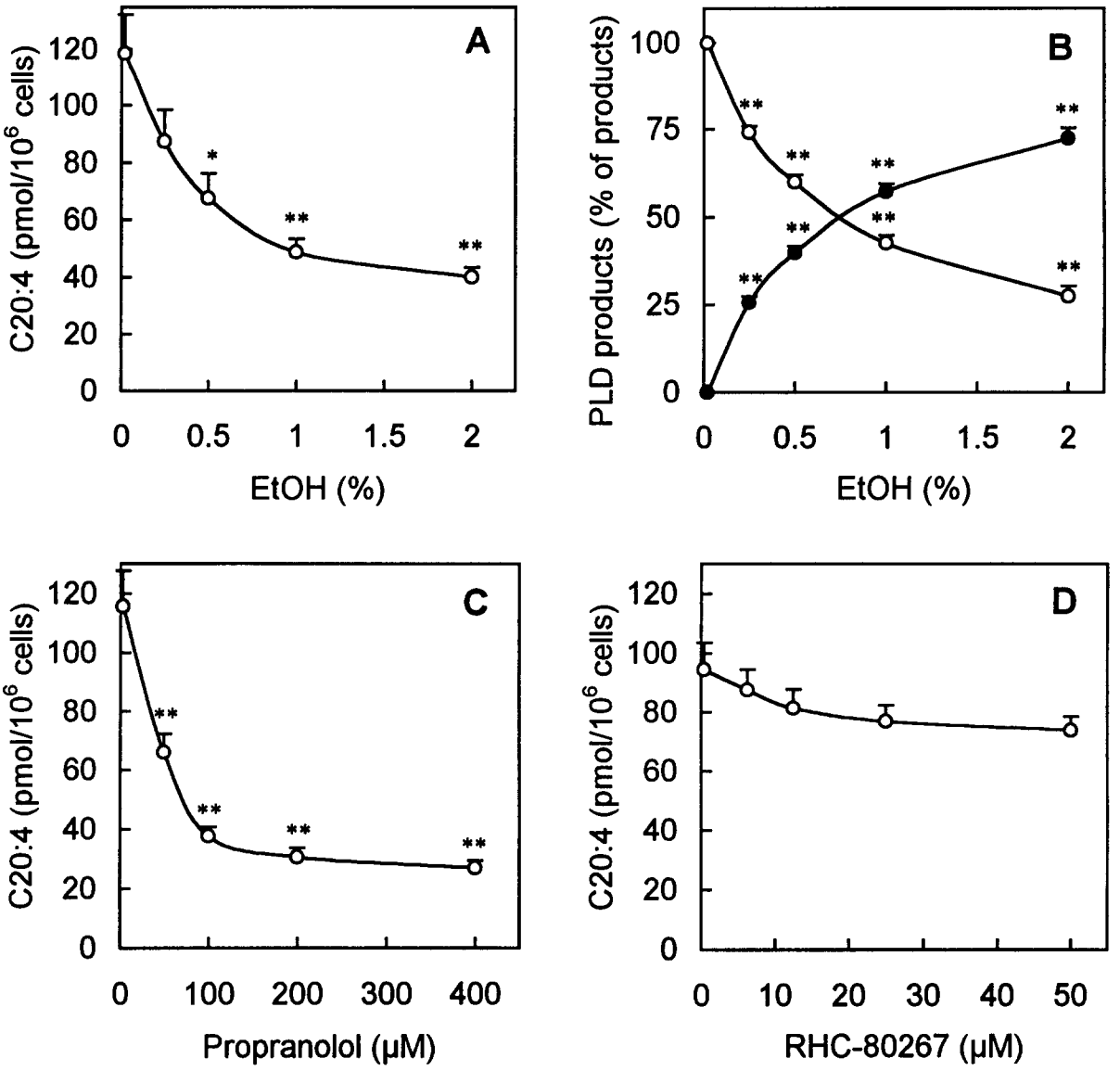

Fig. 3. Effects of inhibitors of the PLD pathway on arachidonic acid release by fMLP-stimulated PMN. PMN were pretreated with the indicated concentrations of ethanol (A, B), propranolol (C) and RH-80267 (D) for 5 min at 37°C and incubated with 50 nM fMLP for 5 min at 37°C. Arachidonic acid release by cells (A, C, D) and PLD activity in cell lysates as assessed by the formation of phosphatidic acid (open circles) and PEt (closed circles) (B) were quantified as described in Section 2. Data are expressed as the mean ± S.E.M. of six (A and C), five (B) and four (D) independent experiments, each of which was carried out in duplicate. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus without each inhibitor.

unstimulated cells, those activated with 1250 nM fMLP, and those activated with fMLP and then treated with acid phosphatase were 0.139 ± 0.009, 0.287 ± 0.031 ($P < 0.01$ versus unstimulated cells), and 0.153 ± 0.051 ($P < 0.05$ versus fMLP-activated cells without phosphatase treatment) pmol/min/10^6 cell equivalents ($n = 3$), respectively. These results imply that the increase in $cPLA_2$ activity in fMLP-stimulated PMN results from the phosphorylation, as has been reported [1–3,19].

3.3. PLD activity in fMLP-stimulated PMN

PLD activity in the PMN homogenates was measured by assessing the conversion of fluorescence-labeled PC (1-BODIPY-PC) to 1-BODIPY-PA. PLD activity in unstimulated PMN, with the production of 0.008 ± 0.001 pmol of PA/min/10^6 cell equivalents ($n = 5$), increased to 234% with 0.019 ± 0.003 ($n = 5$; $P < 0.05$), and to 289% with 0.024 ± 0.004 ($n = 5$; $P < 0.01$) pmol/min/10^6 cell equivalents after stimulation with 10 and 50 nM fMLP, respectively. When 2% ethanol, which has often been used to assess the involvement of PLD in cellular functions by inhibiting PA production with a reciprocal formation of PEt through transphosphatidylation [11–15], was added to the PLD assay mixture, both 1-BODIPY-PEt and 1-BODIPY-PA were formed with a ratio of 7:3. Under this assay condition, production of 1-BODIPY-PEt and 1-BODIPY-PA increased in parallel in response to the incremental concentrations of fMLP (data not shown).

3.4. Effect of inhibitors

Ethanol reduced arachidonic acid release by fMLP-stimulated PMN, with an IC_{50} of 0.3% (Fig. 3A). $cPLA_2$ activity in the 100 000 × g supernatant of fMLP-stimulated PMN homo-

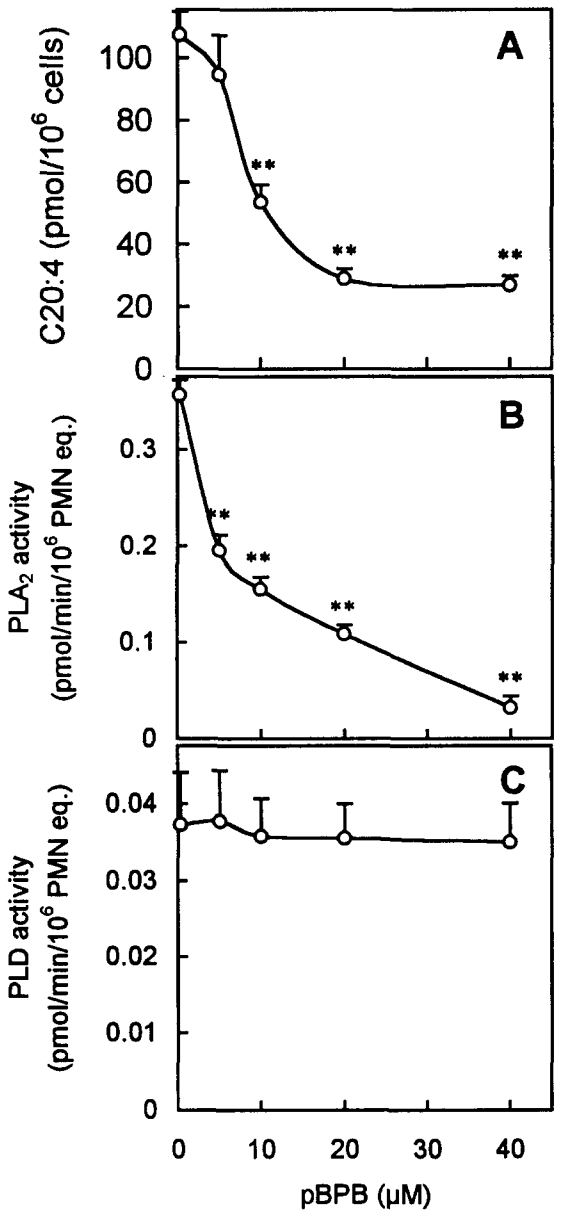

Fig. 4. Effects of *p*BPB on arachidonic acid release and in vitro PLA_2 and PLD activity. PMN were pretreated with the indicated concentrations of *p*BPB for 10 min at 37°C, and incubated with 50 nM fMLP for 5 min at 37°C. The free fatty acids released (A), PLA_2 activity (B) and PLD activity (C) were measured as described in Section 2. Data are expressed as the mean ± S.E.M. of three (B) and five (A and C) independent experiments, each of which was carried out in duplicate. $^{**}P < 0.01$ versus without *p*BPB.

genates decreased from 0.368 ± 0.050 pmol/min/10^6 cell equivalents ($n = 4$) to 0.226 ± 0.019 ($P < 0.05$) and 0.174 ± 0.017 pmol/min/10^6 cell equivalents ($P < 0.01$) when the cells were treated with fMLP in the presence of 0.5 and 1% ethanol, respectively. $cPLA_2$ activity in homogenates of PMN exposed to fMLP in the presence of 1% ethanol, which was comparable to that in homogenates of unstimulated PMN, was insensitive to acid phosphatase treatment (data not shown). The ethanol-induced inhibition of arachidonic acid release and in vitro $cPLA_2$ activity paralleled that of PLD activity in fMLP-stimulated PMN homogenates: ethanol prevented PA formation with an IC_{50} of 0.5%, accompanied by reciprocal formation of PEt (Fig. 3B). Propranolol, which inhibits the hydrolysis of PA to DG [26], also reduced arachidonic acid release by fMLP-stimulated PMN, with an IC_{50} of 25 μM (Fig. 3C). Neither ethanol nor propranolol, at the concentrations used here, affected $cPLA_2$ activity appreciably when added directly to the $cPLA_2$ assay mixture (data not shown). In contrast, RHC-80267, a DG lipase inhibitor [27], exhibited only a minimal suppressive effect on arachidonic acid release by fMLP-stimulated PMN (Fig. 3D). The non-specific PLA_2 inhibitor, *p*BPB [2], inhibited both arachidonic acid release by fMLP-stimulated PMN (Fig. 4A) and PLA_2 activity in the homogenate of fMLP-stimulated PMN (Fig. 4B), with a similar IC_{50} of 7 μM, and without any appreciable effect on PLD activity (Fig. 4C). The $cPLA_2$-specific inhibitor, $AACOCF_3$ [25], inhibited arachidonic acid release by fMLP-stimulated PMN with an IC_{50} of 7 μM (Fig. 2C). These data suggest that fMLP-stimulated arachidonic acid release is mediated by $cPLA_2$ but not DG lipase, and that the activation of $cPLA_2$ is downstream of the PLD-dependent signaling pathway.

4. Discussion

The release of arachidonic acid in preference to other fatty acids has been shown to occur in several cells such as A23187-stimulated rabbit alveolar macrophages [28], thrombin-stimulated human platelets [29], and A23187-stimulated human PMN [30,31]. To determine whether or not arachidonic acid is released selectively, it is important to discover what types of phospholipase(s) are involved. In the work presented here, we have quantified the absolute amounts of each fatty acid released from rat PMN upon stimulation with fMLP, by means of fluorescence-based HPLC, and have confirmed the strict specificity of fatty acid liberation for arachidonic acid. This arachidonic acid release was suppressed significantly by $cPLA_2$ inhibitors, including *p*BPB and $AACOCF_3$. These observations suggest that $cPLA_2$, an arachidonoyl-preferential PLA_2 isozyme, is the predominant enzyme that regulates arachidonic acid release by fMLP-stimulated rat PMN. This is consistent with recent reports that demonstrate the involvement of $cPLA_2$ in the arachidonate metabolism of human neutrophils [16–20].

We have shown here that when PMN were stimulated with fMLP, both $cPLA_2$ and PLD activity in the homogenates increased in parallel, and were accompanied by a concomitant arachidonic acid release. The latter was reduced by the addition of either ethanol, which inhibits PA formation through transphosphatidylation [11–15], or $AACOCF_3$ [25] and *p*BPB [2], as inhibitors of $cPLA_2$ and PLA_2s, respectively, but not by RHC-80267, a DG lipase inhibitor [27]. These results suggest that in rat PMN PLD is functionally linked to $cPLA_2$, but not to DG lipase. Propranolol, generally used as a PA phosphatase inhibitor [26], also suppressed arachidonic acid release, suggesting that certain products generated after the PLD/PA phosphatase pathway are involved in $cPLA_2$ activation. Since DG, a product of the PLD/PA phosphatase pathway, is a potent activator of protein kinase C [32], subsequent kinases, such as MAP kinase, may be responsible for $cPLA_2$ activation. A correlation between PLD and the *ras*/MAP kinase pathway has been reported [33]. Indeed, the 3.5-fold increase in in vitro $cPLA_2$ activity in PMN observed following stimulation with fMLP is consistent with the general understanding

that $cPLA_2$ activity is augmented several-fold by MAP kinase-dependent phosphorylation [1–3], and with the observation that fMLP-induced MAP kinase activation in human neutrophils leads to $cPLA_2$ phosphorylation and activation [19]. This speculation is further supported by the observation that increased $cPLA_2$ activity was reversed by treatment of the PMN homogenate with acid phosphatase.

Whereas $AACOCF_3$ completely inhibited $cPLA_2$ activity in vitro, it inhibited arachidonic acid release by fMLP-stimulated PMN only partially ($\sim$60%), even when used in excess amounts. One of the explanations for this discrepancy is that type II secretory PLA_2, which is stored in PMN granules and is released by exocytosis upon cell activation [34,35], may also contribute, at least in part, to arachidonate release by PMN. Indeed, several pharmacological studies have demonstrated the suppression of arachidonate metabolism in PMN by type II PLA_2 inhibitors [36–38], and Wijkander et al. [20] have recently demonstrated the existence of a positive feedback regulation of stimulus-induced arachidonate metabolism that involves both $cPLA_2$ and type II PLA_2 in human neutrophils. However, the strict specificity of fatty acid liberation for arachidonic acid by fMLP-stimulated rat PMN contradicts the fact that type II PLA_2 cleaves *sn*-2 fatty acids from dispersed phospholipids without selectivity. Alternatively, the partial suppression of arachidonic acid release by $AACOCF_3$, as well as requirement of approximately 10-fold higher concentrations of $AACOCF_3$ for suppression of arachidonic acid release by these cells than that of in vitro $cPLA_2$ activity, may be due to its limited accessibility to $cPLA_2$ inside the intact cells, as has been pointed out in other studies [25,39].

Taken together, our results have demonstrated that $cPLA_2$ activation is also regulated by a PLD-dependent mechanism, which makes the understanding of the regulation of this particular PLA_2 isozyme during cell activation more complex. This finding argues against the previously proposed route for the supply of arachidonic acid via the PLD/PA phosphatase/DG lipase pathway [6,7]. Although the molecular entity and the regulation of PLD in PMN are unknown, recent cDNA cloning of a PLD isoform, which is PC-specific and is activated by PIP_2 and small G protein ARF [40], will provide new insight into the regulatory mechanism of $cPLA_2$.

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