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Role of Ca^{2+}-ATPase inhibitors in activation of cytosolic phospholipase A_2 in human polymorphonuclear neutrophils

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Abstract

In the present study, we investigated the involvement of Ca^{2+}-signaling and protein kinases in the effect of Ca^{2+}-ATPase inhibitors on the activation of cytosolic phospholipase A_2 ($cPLA_2$) in human polymorphonuclear neutrophils. We found that activity and mobility on electrophoresis gels of the $cPLA_2$ protein were significantly increased by f-Met–Leu–Phe (fMLP), 12-myristate 13-acetate (PMA) and the Ca^{2+}-ATPase inhibitors, thapsigargin and cyclopiazonic acid. This effect was completely suppressed by staurosporine. Calphostin C partially inhibited the fMLP- and PMA-induced $cPLA_2$ activation, but had no influence on thapsigargin- and cyclopiazonic acid-treated cells. Thapsigargin and cyclopiazonic acid also showed no effect on protein kinase C activity. However, the thapsigargin- and cyclopiazonic acid-induced $cPLA_2$ activation was completely inhibited by the tyrosine kinase inhibitor, erbstatin, and Ca^{2+} chelator, EGTA. In addition, the $cPLA_2$ activity was reduced after pretreatment with the mitogen-activated protein kinase kinase inhibitor PD98059. The arachidonic acid release was significantly reduced in cells pretreated with the $cPLA_2$ inhibitor, $AACOCF_3$. Furthermore, we found that the human neutrophil $cPLA_2$ cDNA contain a Ca^{2+}-dependent-lipid binding domain which shares homology to several other enzymes such as protein kinase C and phospholipase C. Our results suggest that tyrosine kinases and the MAP kinase cascade are involved in Ca^{2+}-ATPase inhibitor-induced activation and phosphorylation of $cPLA_2$. Protein kinase C is not required in this event.

Keywords: Mitogen-activated protein kinase; Tyrosine kinase; Protein kinase C; Ca^{2+}; Cytosolic phospholipase A_2; Polymorphonuclear neutrophil

1. Introduction

Phospholipase A_2 is the enzyme regulating the release of arachidonic acid from the *sn*-2 position of glycerol in membrane phospholipids in most cell types. Arachidonic acid functions as a precursor of prostaglandins, thromboxanes and leukotrienes. These factors play an important role in numerous physiological and pathophysiological processes.

PLA_2 can be subdivided into two isoforms, a low molecular weight (14 kDa) secret phospholipase A_2 ($sPLA_2$) and a high molecular weight (85 kDa) cytosolic phospholipase A_2 ($cPLA_2$) (Kim et al., 1991). Both have been purified, cloned and sequenced in several cell lines (Clark et al., 1991; Sharp et al., 1991; De Carvalho et al., 1996). $sPLA_2$ requires millimolar concentrations of Ca^{2+} for activation, whereas $cPLA_2$ requires micromolar concentrations of Ca^{2+} for activation and translocation from the cytosol to membranes, where its substrate phospholipid is localized (Lin et al., 1993; Schievella et al., 1995; Bauldry et al., 1996). Recent studies have concentrated on identifying the protein kinase that may be involved in the activation of $cPLA_2$. It was reported that $cPLA_2$ is phosphorylated in collagen- and thrombin-stimulated human platelets independent of protein kinase C (PKC) and mitogen-activated protein (MAP) kinase (Hasegawa et al., 1991). In macrophages, activation of $cPLA_2$ by MAP kinase through PKC-dependent and -independent pathways has been reported (Qiu and Leslie, 1994; Withnall et al., 1995). In the Chinese hamster (CHO) cell line, $cPLA_2$ can be phosphorylated and activated by MAP kinase but not by PKC (Lin et al., 1993). These observations suggest that multiple signaling pathways are involved in the activation of $cPLA_2$. Mobilization of intracellular Ca^{2+} by Ca^{2+}-ATPase leads to an increase of arachidonic acid release. (Kruger et al., 1995; Rider et al., 1996; Alonso Torre and Garcia Sancho, 1997; Heemskerk et al., 1997). However,

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the mechanisms underlying this event remained unknown. In our study, we used four agonists that have previously been shown to activate PKC or to mobilize intracellular Ca^{2+} in different manners: the inhibitors of endoplasmic and sarcoplasmic reticulum Ca^{2+}-ATPase, thapsigargin and cyclopiazonic acid, which cause the membrane of intracellular Ca^{2+} stores to be leaky, irreversibly inhibit Ca^{2+}-ATPase and mobilize Ca^{2+} without increasing the level of inositol phosphates (Begum et al., 1993); the receptor agonist f-Met–Leu–Phe (fMLP), which increases $[Ca^{2+}]_i$ due to extracellular Ca^{2+} influx and mobilization of intracellular Ca^{2+} and activates PKC; the PKC activator 12-myristate 13-acetate (PMA), which functions independently of receptors and without mobilization of Ca^{2+} (Dang et al., 1995).

In the present study, we investigated the mechanisms by which the mobilization of intracellular Ca^{2+} and activation of protein kinases (such as tyrosine kinases and MAP kinases) induced by Ca^{2+}-ATPase inhibitors affect on the activation of $cPLA_2$, in order to better understand the signal transduction steps leading to activation of this enzyme in human neutrophils.

2. Materials and methods

2.1. Materials

f-Met–Leu–Phe (fMLP), 12-myristate 13-acetate (PMA), thapsigargin, cyclopiazonic acid, staurosporine, 2-mercaptoethanol, nonidet P-40, sodium orthovanadate, phenylmethylsufonyl fluoride, leupeptin, aprotinin, calphostin C, arachidonic acid, monoclonal anti-phosphotyrosine-agarose and rabbit anti-mouse IgG were purchased from Sigma. Ficoll–Paque plus and Dextran T-500 were purchased from Pharmacia Biotech. The Plasmid Miniprep kit was purchased from Genomed. The Original TA Cloning kit was purchased from Invitrogen. The QI-Aquick PCR purification kit and QIAquick gel extraction kit were from QIAGEN. The RNA zol™B RNA isolation kit was from BIOTECX BULLETIN. Monoclonal anti-MAP kinase antibody, Taq DNA polymerase, restriction enzyme, RNAase inhibitor, DNA marker 1 kb and deoxyribonuclease I were purchased from GIBCO BRL. The reverse transcription-polymerase chain reaction (RT-PCR) kit was from Stratagene. $cPLA_2$ primers were synthesized by TIB MOLBIOL. The ^{T7}Sequencing™ kit was from Pharmacia Biotech. 1-Palmitoyl-[2-^{14}C]arachidonoyl-phosphatidycholine (50 mCi/mmol), [^{14}C]arachidonic acid (54 m Ci/mmol) and [α-^{35}S]dCTP were purchased from Dupont NEN. [γ-^{32}P]ATP(5000 μCi/mmol) was from Amersham Life Science.

2.2. Isolation of human neutrophils

Human neutrophils were obtained from the peripheral blood of normal volunteers anticoagulated with heparin-sodium. The platelets and mononuclear cells were removed by gradient centrifugation with Ficoll–Paque, and the remaining red blood cells were lysed by hypotonic shock as described (Liang and Huang, 1995). The neutrophils were suspended in phosphate-buffered saline (PBS buffer) without Ca^{2+}/Mg^{2+}, but containing 0.25% bovine serum albumin and 0.1% glucose (pH 7.4).

2.3. Cytosolic phospholipase A_2 activity assay

After stimulation of neutrophils with different agonists, cells were pelleted and resuspended in lysis buffer containing 50 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and an antiprotease cocktail consisting of leupeptin 100 μg/ml, pepstatin 100 ng/ml, aprotinin 100 μg/ml, phenylmethylsufonyl fluoride 2 mM, benzamidine 100 μM, iodoacetamide 1 mM, and chymostatin 100 μg/ml. The cell mixture was lysed by sonication. The lysate was then centrifuged at $1000 \times g$ for 5 min to remove nuclei and debris. The samples were collected by further centrifugation at $100{,}000 \times g$ for 1 h at 4°C. The pellet was used to measure membrane-associated $cPLA_2$ activity. The substrate 1-palmitoyl-[2-^{14}C]arachidonoyl-phosphatidycholine was dried under N_2 and then resuspended to a final concentration of 20 μM 1-palmitoyl-[2-^{14}C]arachidonoyl-phosphatidycholine in 20 mM HEPES (pH 7.4) containing 200 μM Triton X-100, 250 μg/ml bovine serum albumin, and 70% glycerol. The solution was sonicated to form mixed micelles, and then 100 μl of cell suspension (from 5×10^6 neutrophils), 2 mM dithiothreitol and 5 mM $CaCl_2$ were added together. The mixture was incubated at 37°C for 30 min in a shaker. The reaction was stopped by the addition of 500 μl methanol containing 25 μg free fatty acid. The lipids were extracted by the method of Bligh and Dyer (Bligh and Dyer, 1959). The thin-layer chromatography (TLC) plate was developed with ether/petroleum ether/acetic acid in a 20/80/0.1 ratio. The free fatty acid fractions were recovered after TLC on silica-gel G plates. The spots were visualized with I_2 vapor, scraped off and counted in a scintillation counter.

2.4. Measurement of MAP kinase activity

Human neutrophils (5×10^6 cells/ml) were labeled with 5 μM Fura-2/AM and incubated with different concentrations of agonists and inhibitors for appropriate time intervals. After stimulation, the cells were centrifuged at 1200 rpm for 5 min at 4°C, and the pellet was resuspended in lysis buffer containing 1 mM phenylmethylsufonyl fluoride, 2 mM EDTA, 2 mM EGTA, 25 mM β-glycerophosphate, 0.2 mM sodium orthovanadate, 25 mM sodium fluoride, 10 μg/ml leupeptin, 1 μg/ml aprotinin and 1% NP-40 in 50 mM Tris–HCl, 75 mM NaCl (pH 7.5) (Grinstein and Furuya, 1992). The solution was kept on ice for 20 min, and was then centrifuged at 15,000 rpm at 4°C

for 10 min. The supernatant was used for the kinase activity assay. MAP kinase activity was measured using a synthetic peptide TH (KQAEAVSPR), which has been shown to be a specific substrate for MAP kinase (Gonzalez et al., 1991; Northwood et al., 1991). The reaction mixture consisted of 10 μl cell lysate, 5 μl 25 mM HEPES (pH 7.4), 20 mM $MgCl_2$ and 50 μM [γ-^{32}P]ATP (10 μCi/nmol). The reactions were terminated after 30 min at 25°C by addition of 45% formic acid containing 25 mM ATP. The phosphorylated synthetic peptide was isolated by applying 25 μl of the reaction mixture to Whatman P-81 phosphocellulose paper. The filters were washed three times with 0.5% phosphoric acid and rinsed with 90% ethanol. The radioactivity was determined by liquid scintillation counting. MAP kinase activity is expressed as picomoles ^{32}P incorporated per 10^6 neutrophils in 1 min (pmol/min/10^6 neutrophils).

2.5. Measurement of PKC activity in human neutrophils

Neutrophils (10^7 cells/ml) were incubated at 37°C with different agonists for appropriate times at effective concentrations. The reactions were stopped by placing the tubes in ice for 5 min. The cells were washed twice with PBS and resuspended in 50 μl reaction buffer containing 137 mM NaCl, 5.4 mM KCl, 0.3 mM sodium phosphate, 0.4 mM potassium phosphate, 1 mg/ml glucose, 20 mM HEPES (pH 7.2), 10 mM $MgCl_2$, 25 mM β-glycerophosphate, 5 mM EGTA, 2.5 mM $CaCl_2$, with [γ^{32}P]ATP 100 μM (5000 cpm/pmol), 300 μM peptide (VRKRTLRRL) (Winitz et al., 1994), and 50 μg/ml digitonin. The reactions were terminated after 15 min at 30°C with 10 μl 25% trichloroacetic acid. Aliquots (45 μl) of the acidified reaction mixtures were spotted on 2 × 2-cm squares of phosphocellulose (Whatman P-81). These squares were washed three times with 75 mM phosphoric acid and finally washed with 75 mM sodium phosphate (pH 7.5), using washing volumes of 500 ml each. The squares were dried by air and the radioactivity of each square was measured by liquid scintillation counting. The PKC activity is expressed as picomoles ^{32}P incorporated per 10^7 neutrophils in 1 min.

2.6. Immunoprecipitation and Western blot

After stimulation, 5×10^6 neutrophils were suspended in 100 μl of lysis buffer (containing 150 mM NaCl, 1 mM EGTA, 10 mM sodium pyrophosphate, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM, phenylmethylsufonyl fluoride 2 mM, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μM pepstain, 10 μM glycerol, 50 mM HEPES–Na, and 1% SDS, pH 7,4) and boiled for 5 min. Then, 1 ml of ice-cold lysis buffer containing 1% nonidet P-40 was added and the cells were sonicated and sedimented by centrifugation for 5 min at 5000 rpm. The supernatant was placed in a new tube, and 5 μl agarose-conjugated monoclonal anti-phosphotyrosine was added to the supernatant, which had been previously blocked for at least 1 h with 10% bovine serum albumin in 50 mM HEPES–Na, pH 7.5. After incubation of the mixture at 4°C for 2 h, the beads were washed twice with 50 mM HEPES–Na containing 150 mM NaCl pH 7.5 and twice with 50 mM HEPES–Na containing 0.5 mM LiCl, pH 7.5. The washed beads were boiled for 5 min in Laemmli sample buffer and rapidly sedimented. The supernatants were used for electrophoresis on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked by incubation for 2 h in TBST buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween-20, 2% bovine serum albu-

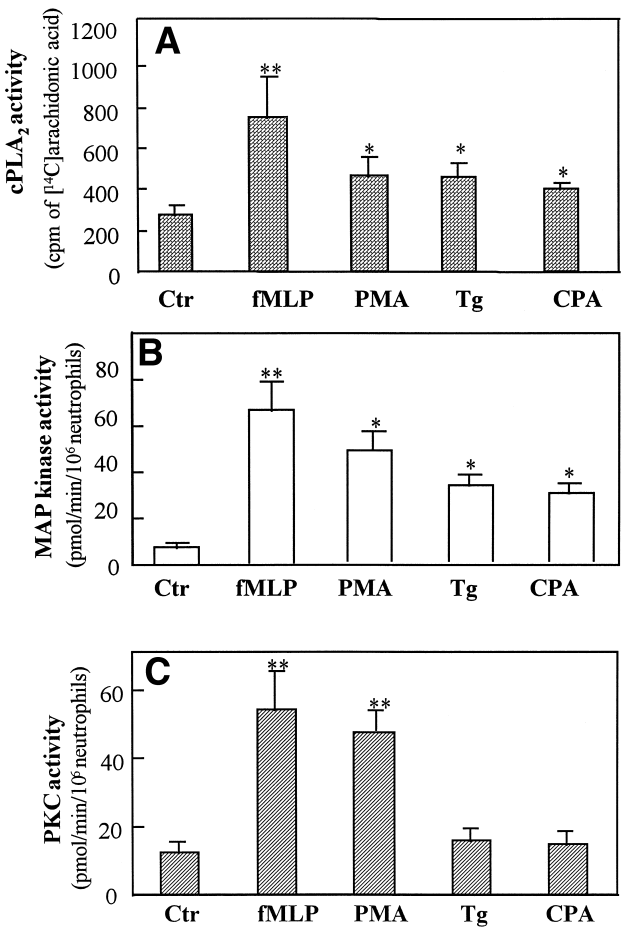

Fig. 1. Effects of fMLP, PMA, thapsigargin and cyclopiazonic acid on activation of PKC, MAP kinase and $cPLA_2$ in human neutrophils. Neutrophils were adjusted to 2×10^7 cells/ml and treated with fMLP (100 nM for 5 min), PMA (100 nM for 10 min), thapsigargin (Tg) (1 μM for 10 min) and cyclopiazonic acid (CPA) (2.5 μM for 15 min), respectively. (a) $cPLA_2$ activity was measured by using 1-palmitoyl-[2-^{14}C]arachidonoyl-phosphatidycholine as substrate. (b) The synthetic peptide KQAEAVSPR was used as a MAP kinase substrate. (c) The peptide VRKRTLRRL was used as specific substrate for PKC. The enzyme activity assay was described in Section 2. Values are presented as means ± S.D. from four separate experiments. $^*P < 0.05$ as compared with the control.

min) and was then incubated with monoclonal anti-MAP kinase antibodies. Afterwards, the blot was washed with TBST buffer three times for 10 min and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG for 60 min at room temperature. The membrane was washed three times for 10 min with a substrate buffer (100 mM Tris pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$), and then 5-bromo-4-chloro-3-indolyl-phosphate/4-nitroblue tetrazolium chloride (NBT/BCIP) was added to the substrate buffer and the membrane was incubated for 1 h at room temperature.

2.7. Measurement of [^{14}C]arachidonic acid release from human neutrophils

The [^{14}C]arachidonic acid release assay was done as described earlier (Krump et al., 1995). Briefly, neutrophils were adjusted to 5×10^6 cells/ml and labelled with [^{14}C]arachidonic acid (0.1 μCi/ml) for 20 min in PBS buffer with 0.25% bovine serum albumin and 0.1% glucose at 37°C. The free [^{14}C]arachidonic acid was removed by washing twice with PBS buffer and the cells were resuspended in PBS buffer (5×10^6 cells/ml). After stimulation of neutrophils with different agonists with or without pretreatment with the $cPLA_2$ inhibitor $AACOCF_3$, the lipids were extracted by the method of Bligh and Dyer (Bligh and Dyer, 1959). The samples were spotted on the TLC plates with standard arachidonic acid. The plate was developed in the solvent mixture diethylester:*n*-hexane:acetic acid (50:50:1). Afterwards the TLC plate was dried by air and exposed to iodine vapor. The band of arachidonic acid was cut out, added to 10 ml scintillation fluid and counted for radioactivity.

2.8. Molecular characterization of Ca^{2+}-dependent lipid-binding (CaLB) domain by using RT-PCR and cDNA sequencing analysis

Total RNA was isolated from human neutrophils and reversibly transcribed to cDNA. The primer pairs were designed according to the published cytosolic phospholipase A_2 sequence of a human U_{937} cell line sense: 5′-ATGTCATTTATAGATCC and anti-sense: 5′-GCTCTAG-AACCATTTCAGTG. There primers amplify the first 400 nucleotides of $cPLA_2$ including the CaLB domain sequence. The primers for amplification of the whole $cPLA_2$ sequence are sense: 5′-AAAACTG-CAGATGTCATT-TATAGATCC and anti-sense: 5′-ACACGTCGACC-TATGCTTTGGGTT. The cDNA from human neutrophils and the standard $cPLA_2$ plasmid DNA were used as template. The PCR reaction was held at 94°C for 45 s, 60°C for 45 s, 72°C for 1 min and 35 cycles. The PCR products were purified and cloned into the PCR™II vector from Invitrogen. After transformation, plasmid DNA preparation, and restriction analysis, the sequence reaction was done by using the ^{T7}Sequencing™ kit from Pharmacia Biotech according to the manufacturers instructions.

3. Results

The neutrophils were treated with fMLP (100 nM, 1 min), PMA (100 nM, 5 min), thapsigargin (1 μM, 10 min)

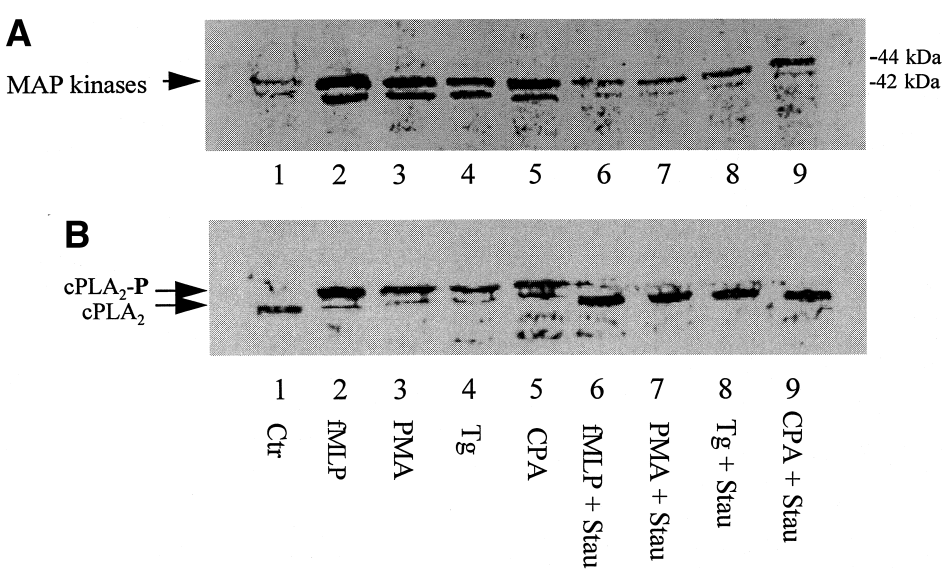

Fig. 2. Immunoprecipitation and Western blot analysis of MAP kinase and $cPLA_2$ phosphorylation in human neutrophils. Neutrophils were stimulated with 100 nM fMLP for 1 min, 100 nM PMA for 5 min, thapsigargin (Tg) 1 μM for 10 min or cyclopiazonic acid (CPA) 2.5 μM for 15 min, respectively. In some experiments, neutrophils were pretreated with 300 nM staurosporine for 10 min. The whole cell lysate was divided into two parts. One part of the lysate (30 μg protein) was immunoprecipitated with monoclonal anti-phosphotyrosine-agrose. After the beads were boiled and centrifuged, the supernatant was loaded onto a 10% SDS-PAGE gel. After electrophoresis and blotting, the membrane was analyzed by Western blot using monoclonal anti-MAP kinase antibodies (a). The other part was subjected to electrophoresis on a 7.5% SDS-PAGE gel. After electrophoresis, the proteins were transferred to nitrocellulose membranes and immunoblotted with a polyclonal anti-cytosolic phospholipase A2 antibody (b).

Table 1
Effects of different protein kinase inhibitors on $cPLA_2$ activity

Stimuli	$cPLA_2$ activity (cpm of [^{14}C]AA)				
	Without inhibitor	Staurosporine (300 nM)	Calphostin C (500 nM)	Erbstatin (5 μM)	PD98059 (2 μM)
Control	276 ± 47	218 ± 65	272 ± 51	257 ± 56	245 ± 89
fMLP (100 nM)	752 ± 197^{aa}	321 ± 136^{b}	545 ± 123^{b}	435 ± 178^{b}	412 ± 121^{b}
PMA (100 nM)	469 ± 58^{a}	237 ± 42^{b}	377 ± 83^{b}	385 ± 102^{b}	385 ± 92^{b}
Thapsigargin (1 μM)	497 ± 147^{a}	290 ± 91^{b}	478 ± 114^{b}	256 ± 67^{bb}	289 ± 87^{bb}
Cyclopiazonic acid (2.5 μM)	433 ± 132^{a}	279 ± 94^{b}	452 ± 121	287 ± 56^{bb}	247 ± 56^{bb}
PMA (100 nM) + Thapsigargin (1 μM)	779 ± 241^{aa}	336 ± 85^{bb}	637 ± 197^{b}	518 ± 178^{b}	481 ± 148^{b}

After treatment of human neutrophils with various agonists (with or without inhibitors), the cells were pelleted and resuspended in lysis buffer. The cell mixture were lysed by sonication and the cell membranes were prepared as described in Section 2.
1-Palmitoyl-[2-^{14}C]arachidonoyl-phosphatidycholine was used as substrate to measure membrane-associated $cPLA_2$ activity.
The data (means ± S.D.) represent duplicate determinations from five separate experiments.
$^{a}P < 0.05$ vs. control.
$^{aa}P < 0.01$ vs. control.
$^{b}P < 0.05$ vs. without inhibitor.
$^{bb}P < 0.01$ vs. without inhibitor.

and cyclopiazonic acid (2.5 μM, 15 min), respectively. The used concentrations and stimulation times for each agonist were selected according to previous results (Zhang et al., 1998) as being able to effectively activate human neutrophils. To determine whether the phosphorylation of $cPLA_2$ alters its activity under the influence of agonists, an in vitro liposome assay was used for measuring [^{14}C]arachidonic acid release from 1-palmitoyl-[2-^{14}C]prachidonoyl-phosphatidy-choline. We found that the $cPLA_2$ activity present in the lysates derived from thapsigargin-, cyclopiazonic acid-, fMLP- and PMA-treated neutrophils was significantly increased (Fig. 1a). This phenomenon was paralleled by an increase in MAP kinase activity in response to these four agonists (Fig. 1b). However, PKC activity was increased in fMLP- and PMA-treated neutrophils, but not in thapsigargin- and cyclopiazonic acid-treated neutrophils (Fig. 1c).

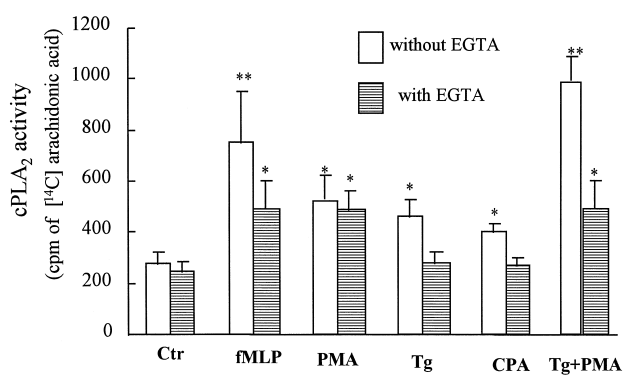

Fig. 3. Influence of Ca^{2+} on $cPLA_2$ activation. Neutrophils were pretreated with or without EGTA 2 mM for 15 min, and then stimulated with 100 nM fMLP for 1 min, 100 nM PMA for 5 min, thapsigargin (Tg) 1 μM for 10 min or cyclopiazonic acid (CPA) 2.5 μM for 15 min. The $cPLA_2$ activity was measured as described in Section 2. The data are presented as means ± S.D. from four separate experiments. $^{*}P < 0.05$ vs. control, $^{**}P < 0.01$ vs. control.

The cell lysate of each reaction was divided into two parts. One part was immunopreciptated with monoclonal anti-phosphotyrosine, separated by electrophoresis on 10% polyacrylamide gels and immunoblotted with monoclonal anti-MAP kinases. The other part was used for gel-mobility shift Western blot analysis, using polyclonal anti-$cPLA_2$ antibody. We found that after stimulation of neutrophils, the 42- and 44-kDa MAP kinase proteins were strongly phosphorylated at their tyrosine residues, an effect that could be reversed by staurosporine (Fig. 2a). The 85-kDa $cPLA_2$ protein was observed as a double band: the upper band had a slightly reduced mobility compared with that of the same protein isolated from unstimulated neutrophils because of agent-mediated protein phosphorylation. Pre-

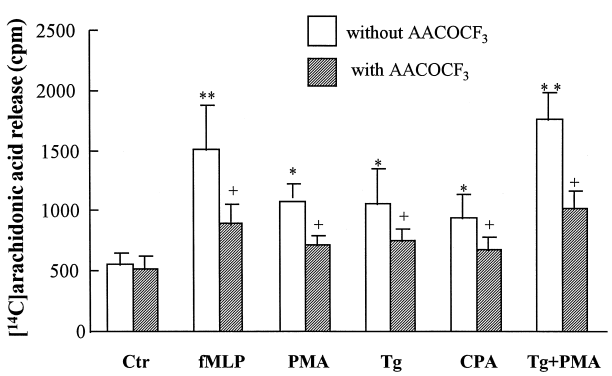

Fig. 4. Effect of $AACOCF_3$ on arachidonic acid release in human neutrophils. The cells were adjusted to 5×10^6 cells/ml and labeled with [^{14}C]arachidonic acid (0.25 μCi/ml). The neutrophils were stimulated with different agents for the given times. For the inhibition reaction the cells were pretreated with 20 μM $AACOCF_3$ for 15 min. The reactions were stopped with 3.8 ml ice-cold stop solution (methanol: chloroform:acetic acid—50:25:1). Lipid extraction and TLC analysis were done as described in Section 2. Results are presented in means ± S.D. for five separate experiments. $^{*}P < 0.05$ compared with control, $^{**}P < 0.05$ compared with the group without pretreatment with $AACOCF_3$.

treatment of cells with 300 nM staurosporine at 37°C for 10 min eliminated the tyrosine phosphorylation of MAP kinase as well as the migration of the $cPLA_2$ protein in the slow migrating band; the $cPLA_2$ rotein was now found in the more rapidly migrating band (Fig. 2b).

Furthermore, our results showed that pretreatment of neutrophils with the non-specific protein kinase inhibitor staurosporine (300 nM for 10 min) completely suppressed the $cPLA_2$ activity induced by these four agonists. Calphostin C (500 nM for 10 min), a specific PKC inhibitor partially inhibited fMLP- or PMA-induced $cPLA_2$ activity but had no influence on thapsigargin- or cyclopiazonic acid-induced $cPLA_2$ activity. However, thapsigargin- and cyclopiazonic acid-induced $cPLA_2$ activity was abolished when the tyrosine kinase inhibitor erbstatin was used (Table 1). These results were similar to those for the effect of the three inhibitors on MAP kinase activity in response to thapsigargin, cyclopiazonic acid, PMA and fMLP (Zhang et al., 1998). The MAP kinase kinase (MEK) inhibitor PD98059 (5 μM for 10 min) significantly reduced $cPLA_2$ activity in response to all the agonists (Table 1). This indicates that MAP kinase pathway is involved in thapsigargin- and cyclopiazonic acid-induced $cPLA_2$ activity. Our result also showed that coincubation with thapsigargin and PMA together induced a greater $cPLA_2$ activity in neutrophils than either agent alone (Table 1).

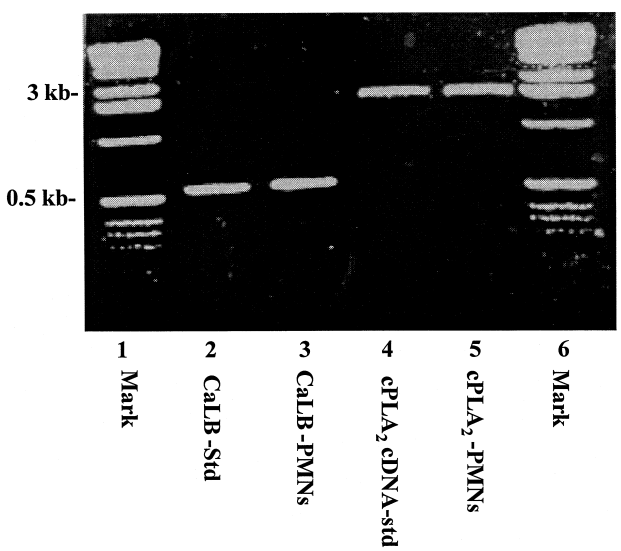

Fig. 5. RT-PCR analysis of human neutrophil $cPLA_2$. Total RNA from human neutrophils was isolated. After reverse transcription, the cDNA was used as template for PCR under conditions of 94°C denaturation 45 s, 60°C annealing 45 s, 72°C extension 1.5 min, and 35 cycle. Line 1. and Line 6. 1 kb DNA marker, Line 2. PCR with Ca^{2+}-dependent lipid-binding (CaLB) domain primer (sense: 5′-AAAACTGCAG-ATGTCATTTATAGATCC, anti-sense: 5′-GCTCTAGAACCATTTCAGTG), Line 4. PCR with whole $cPLA_2$ cDNA primer (sense: 5′-AAAACTGCAGATGTCATTTATAGATCC, anti-sense:5′-ACACGTCGACCTATGCTTTGGGTT), Line 3. and Line 5. PCR with standard $cPLA_2$ cDNA as template, using the CaLB domain primer and whole $cPLA_2$ cDNA primer, respectively.

Pretreatment of neutrophils with the Ca^{2+} chelator EGTA (2 mM for 15 min) completely abolished thapsigargin- and cyclopiazonic acid-induced $cPLA_2$ activity, partially inhibited the effect of fMLP and had no influence on PMA-induced $cPLA_2$ activity (Fig. 3).

Although the activation of $cPLA_2$ in response to the four agonists caused significant activation of $cPLA_2$, the release arachidonic acid from human neutrophils was not completely inhibited by $AACOCF_3$, a specific inhibitor of $cPLA_2$ (Fig. 4).

In addition, by using RT-PCR and cDNA sequence analysis we found that the first 400 base pairs of $cPLA_2$ cDNA contained the sequence of a Ca^{2+}-dependent lipid-binding (CaLB) domain that shared 100% homology to the $cPLA_2$ of human U_{937} monocytic cells (Fig. 5).

4. Discussion

In the present study, we investigated the role of $cPLA_2$ activated by Ca^{2+}-ATPase inhibitors in human neutrophils. We found that the thapsigargin-, cyclopiazonic acid-, fMLP- and PMA-induced activation of MAP kinase and $cPLA_2$ was accompanied by a parallel increase in the phosphorylation of these enzymes (Fig. 1a, 1b and Fig. 2). The activation and phosphorylation of $cPLA_2$ were measured by in vitro liposome assay and gel-mobility shift Western blot. The $cPLA_2$ activity present in the lysates derived from either thapsigargin-, cyclopiazonic acid-, fMLP- or PMA-treated neutrophils was significantly increased compared with that from untreated cells. This indicates that the activation of $cPLA_2$ is caused by protein phosphorylation. The $cPLA_2$ protein isolated from stimulated neutrophils migrated as a double band (Fig. 2b), but most of the protein migrated in the upper band, unlike the protein isolated from unstimulated cells. Pretreatment with staurosporine resulted in the loss of the more slowly migrating band, indicating that this band was phosphorylated $cPLA_2$ protein. These results suggest that all the four agonists used in the present study induce phosphorylation of $cPLA_2$ in human neutrophils. However, the PKC activity remained unchanged in thapsigargin- and cyclopiazonic acid-treated neutrophils (Fig. 1c). Interestingly, pretreatment of neutrophils with staurosporine eliminated the $cPLA_2$ activity, whereas pretreatment with calphostin C partially inhibited the $cPLA_2$ activity in response to fMLP and PMA, but had no effect on $cPLA_2$ activity in response to thapsigargin- or cyclopiazonic acid (Table 1). The thapsigargin- and cyclopiazonic acid-induced $cPLA_2$ activity was abolished completely by erbstatin. Erbstatin was also found to completely inhibit the thapsigargin- and cyclopiazonic acid-induced MAP kinase activity under our experimental conditions (Zhang et al., 1998). This inhibitory effect of staurosporine and calphostin C on $cPLA_2$ activity stimulated by fMLP, PMA, thapsigargin and cyclopiazonic acid mirrors our results regarding activation of the MAP

kinases (Zhang et al., 1998). Lin et al. (Lin et al., 1993) have reported that MAP kinases are serine/threonine protein kinases, and thus phosphorylation of $cPLA_2$ occurs at the serine-505 residue. Our results showed that the changes in the phosphorylation and activity of PKC and MAP kinase occurred in parallel with the translocation of $cPLA_2$ and the of arachidonic acid release (Figs. 1 and 2). This event was inhibited in cells pretreated with staurosporine, calphostin C and PD 98059 (Table 1). So, we assume that the PKC-pathway as well as MAP kinase pathway is involved in $cPLA_2$ activation in human neutrophils.

Thapsigargin and cyclopiazonic acid are inhibitors of endoplasmic and sarcoplasmic reticulum Ca^{2+}-ATPase and cause leakage in membrane of intracellular Ca^{2+} stores, irreversibly inhibit Ca^{2+}-ATPase and mobilize Ca^{2+} without increasing the level of inositol phosphates. This sustained increase in $[Ca^{2+}]_i$ is thought to be due to the activation of a store-operated Ca^{2+} entry pathway (SOCP) as a consequence of the emptying of the intracellular Ca^{2+} stores (Begum et al., 1993). Our results showed that thapsigargin and cyclopiazonic acid did not influence on PKC activity (Fig. 1c). These results suggest that the thapsigargin- and cyclopiazonic acid-induced increase in $cPLA_2$ activity is due to the mobilization of intracellular Ca^{2+} and the subsequent activation of tyrosine kinases and MAP kinases. These events finally lead to the activation of $cPLA_2$ in human neutrophils. However, PKC was not required for the thapsigargin- and cyclopiazonic acid-induced activation of $cPLA_2$. That Ca^{2+} signal leads to activation of tyrosine kinases has recently also been reported in human platelets (Vosters et al., 1991), vascular endothelial cells (Fleming et al., 1995), mast cells (Rider et al., 1996; Zhang et al., 1997) and fibroblasts (Chao et al., 1992). A more recent study shows (Chao et al., 1997) that thapsigargin stimulates Src tyrosine kinase and subsequently activates Raf-1 and MAP-kinase in H19-7 cells. One group has shown (Della Rocca et al., 1997) the involvement of Ca^{2+}-dependent tyrosine kinases such as Pyk in the activation of the MAP kinase cascade, which provides an explanation for the dependence of tyrosine kinase on an increase in Ca^{2+} levels. Furthermore, the involvement of tyrosine kinases and the MAP kinase cascade in the activation of $cPLA_2$ in human neutrophils has been reported by several groups (Fouda et al., 1995; Nahas et al., 1996a,b; Hazan et al., 1997; Coffer et al., 1998).

In fact, $cPLA_2$ contains consensus phosphorylation sites for MAP kinases and for PKC (Alvarez et al., 1991; Clark et al., 1991). In addition to phosphorylation, intracellular Ca^{2+} has been reported to be an important stimulator of $cPLA_2$ activity (Kramer et al., 1991; Xing and Mattera, 1992). Clark et al. reported that the U_{937} cell line contains a Ca^{2+}-dependent lipid-binding domain region with homology to several other Ca^{2+}-dependent-lipid binding proteins, including PKC, p65, GTPase-activating proteins and phospholipase C (Clark et al., 1991; Schievella et al., 1995). We demonstrated in the present study that the $cPLA_2$ cDNA in human neutrophils also contains a Ca^{2+}-dependent lipid-binding domain that initiates the translocation of $cPLA_2$ from the cytosol to the membrane. This may explain the regulatory function of the intracellular Ca^{2+} level on the activation of $cPLA_2$ in human neutrophils. Our observation that PMA, a well-known Ca^{2+}-independent activator of PKC, consistently increased MAP kinase and $cPLA_2$ activity suggests that, beside the Ca^{2+}-dependent lipid-binding domain, a second Ca^{2+}-independent regulatory domain must be present for $cPLA_2$ activity. This observation confirms the results of previous studies demonstrating that Ca^{2+} may not be required for the catalytic activity of $cPLA_2$ (Wijkander and Sundler, 1992; Nalefski et al., 1994).

The observation that the thapsigargin-induced stimulation of $cPLA_2$ activity could be further enhanced by the addition of PMA suggests that the mobilization of intracellular Ca^{2+} and the activation of protein kinases have a synergistic effect on the activation of $cPLA_2$. Preincubation of neutrophils with the protein kinase inhibitor staurosporine caused inhibition of the agonist-stimulated phosphorylation and activation of $cPLA_2$.

$AACOCF_3$ is a trifluoromethyl ketone analogue of arachidonic acid in which the COOH group is replaced by $COCF_3$. It was found to be a tight- and slow-binding inhibitor of human $cPLA_2$. Thus $AACOCF_3$ inhibits $cPLA_2$ by direct binding to the enzyme rather than by decreasing the fraction of enzyme bound to the substrate interface (Riendeau et al., 1994). We found that pretreatment of neutrophils with $AACOCF_3$ significantly but not completely inhibited fMLP-, PMA-, thapsigargin- or cyclopiazonic acid-induced arachidonic acid release. This indicates that $cPLA_2$ is major pathway for arachidonic acid release in the presence of micromolar amounts of intracellular Ca^{2+} in human neutrophils. But this is not the only pathway. $sPLA_2$, phospholipase D and phospholipase C have been also reported to be involved in arachidonic acid release from human neutrophils (Nakashima et al., 1995; Tithof et al., 1996; Fujita et al., 1996; Bauldry and Wooten, 1997).

In summary, Ca^{2+}-ATPase inhibitors such as thapsigargin and cyclopiazonic acid lead to the activation of $cPLA_2$ in human neutrophils, a process which is due to the mobilization of intracellular Ca^{2+} following activation of tyrosine kinases and MAP kinases. PKC activity is not affected. Furthermore, our results indicate that the activation of $cPLA_2$ in neutrophils in response to various stimuli is regulated by multiple pathways. It may occur through both PKC-dependent/-independent mechanisms. It can be stimulated by Ca^{2+}-mediated tyrosine kinases and the MAP kinase pathway. However, there is also a PMA-sensitive Ca^{2+}-independent pathway. MAP kinases appear to be common mediators of the activation of $cPLA_2$ in response to receptor-/non-receptor-mediated stimulation or Ca^{2+} mobilization. An increase in the intracellular Ca^{2+} level and protein kinase activity synergistically induces the

phosphorylation and activation of $cPLA_2$ in human neutrophils.

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