The Role of Calcium and Phosphorylation of Cytosolic Phospholipase A2 in Regulating Arachidonic Acid Release in Macrophages*

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Arachidonic acid release is induced in macrophages with diverse agonists including calcium ionophores, phorbol myristate acetate (PMA), okadaic acid, and the phagocytic particle, zymosan, and correlates with activation of cytosolic phospholipase A2 (cPLA2). The role of calcium and phosphorylation of cPLA2 in regulating arachidonic acid release was investigated. Zymosan induced a rapid and transient increase in [Ca2+]i. This in itself is not sufficient to induce arachidonic acid release since ATP and platelet activating factor (PAF), agonists that induce transient calcium mobilization in macrophages, induced little arachidonic acid release. Unlike zymosan, which is a strong activator of mitogen-activated protein kinase (MAPK), ATP and PAF were weak MAPK activators and induced only a partial and transient increase in cPLA2 phosphorylation (gel shift). However, ATP or PAF together with colony stimulating factor-1 (CSF-1) synergistically stimulated arachidonic acid release. CSF-1 is a strong MAPK activator that induces a rapid and complete cPLA2 gel shift but not calcium mobilization or arachidonic acid release. Arachidonic acid release was more rapid in response to CSF-1 plus ATP or PAF than zymosan and correlated with the time course of the cPLA2 gel shift. Although low concentrations of ionomycin induced a lower magnitude of calcium mobilization than ATP, the response was more sustained resulting in arachidonic acid release. A23187 and ionomycin induced weak MAPK activation, and a partial and transient cPLA2 gel shift. The MAPK kinase inhibitor, PD 98059 suppressed A23187-induced MAPK activation and cPLA2 gel shift but had little effect on arachidonic acid release. These results indicate that in macrophages a transient increase in [Ca2+]i, and sustained phosphorylation of cPLA2 can act together to promote arachidonic acid release but neither alone is sufficient. A sustained increase in calcium is sufficient for inducing arachidonic acid release. However, PMA and okadaic acid induce arachidonic acid release without increasing [Ca2+]i, although resting levels of calcium are required, suggesting alternative mechanisms of regulation.

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The production of the proinflammatory lipid mediators, the eicosanoids (i.e. prostaglandins and leukotrienes), is dependent on the availability of the precursor, free arachidonic acid. The release of arachidonic acid from the sn-2 position of membrane phospholipid is a highly regulated process that occurs in response to cell activation. Phospholipase A2 (PLA2) enzymes, which cleave fatty acid from the sn-2 position of phospholipid, play a central role in controlling the release of arachidonic acid. The importance of the arachidonic acid-selective, 85-kDa cytosolic PLA2 (cPLA2) in mediating agonist-induced release of arachidonic acid is now well recognized (1, 2). cPLA2 is regulated post-translationally by both phosphorylation and calcium. Calcium plays a role by promoting binding of cPLA2 to membrane, which is mediated by a calcium-phospholipid-binding domain at the amino terminus of the enzyme (3–5). Treatment of cells with calcium-mobilizing agonists has been shown to induce binding of cPLA2 to nuclear membrane and endoplasmic reticulum (6–8). Stimulation of a variety of cell types with diverse agonists that induce arachidonic acid release also has been shown to promote serine phosphorylation of cPLA2 that is accompanied by an increase in cPLA2 activity and a decrease in electrophoretic mobility (gel shift) (1, 9, 10). cPLA2 can be phosphorylated by protein kinase C, p42/p44 mitogen-activated protein kinases (MAPK), or protein kinase A in vitro but only phosphorylation by MAPK results in a significant increase in cPLA2 activity and induces a cPLA2 gel shift (10, 11). MAPK phosphorylates cPLA2 at Ser-505 and phosphorylation of this site has been shown to be required for cPLA2-mediated arachidonic acid release in Chinese hamster ovary cells treated with a variety of agonists (12).

Current evidence indicates that cPLA2 phosphorylation at Ser-505 by MAPK in itself is not sufficient for arachidonic acid release (1). It has been shown in Chinese hamster ovary cells that phosphorylation of cPLA2 induced by PMA is not sufficient for inducing arachidonic acid release, but PMA acts synergistically with calcium ionophore (10). In macrophages, CSF-1 induces cPLA2 phosphorylation but not arachidonic acid release although it can act synergistically with calcium mobilizing agonists (13). It has been suggested that an increase in intracellular calcium concentration [Ca2+]i, but not phosphorylation of cPLA2, is essential for arachidonic acid release in rat liver macrophages (14). In platelets, thrombin-stimulated arachidonic acid release does not require phosphorylation of cPLA2 on Ser-505 (15). Although these studies indicate an
important role for an increase in \( [\text{Ca}^{2+}]_i \) in regulating arachidonic acid release, alternative mechanisms are indicated by results showing that cPLA2-mediated arachidonic acid release induced by okadaic acid occurs without an increase in \( [\text{Ca}^{2+}]_i \) (16). In light of these observations, mouse peritoneal macrophages were used as a model to investigate the role of calcium and phosphorylation of cPLA2 in regulating arachidonic acid release.

**EXPERIMENTAL PROCEDURES**

**Materials**—\([5,6,8,9,11,12,14,15-^3\text{H}]\)Arachidonic acid (100 Ci/mmol) and \([^{32}\text{P}]\)orthophosphoric acid were from NEN Life Science Products Inc. Pathogen-free female ICR mice (8 weeks old) were from Harlan Sprague-Dawley. Fura-2-AM, Fluo-3-AM, Quin-2-AM, and pluronic F-127 were from Molecular Probes. Anti-rabbit IgG horseradish peroxidase-linked F(ab\(^9\))\(^2\) fragment, \([^{32}\text{P}]\)ATP (3000 Ci/mmol), and the ECL detection kit for immunoblotting were from Amersham. Zymosan (yeast cell walls), A23187, phenylmethylsulfonyl fluoride, probenecid, leupeptin, aprotinin, and fetal bovine serum were from Sigma. Zymosan was prepared as described previously (9). Okadaic acid and PMA were from LC Services Co. Ionomycin was from Calbiochem-Novabiochem Co. CSF-1 was a gift from The Genetics Institute (Cambridge, MA). The MAPK kinase (MAPKK) inhibitor, PD 98059, was kindly provided by Dr. Alan Saltiel (Parke Davis Research Division, Warner Lambert Co.). PAF was from Biomol Research Labs, Inc. Sequencing grade, modified trypsin was obtained from Promega. Dulbecco’s modified Eagle’s medium (DME/M) and Hank’s balanced salts solution were from Whittaker Bioproducts. Glass coverslips (13 mm diameter) were from Fisher. Protein concentrations were determined using the BCA reagent from...
Pierce. Polyclonal antibody (11683) to recombinant human cPLA₂ was produced as described previously (17). MAPK antibodies to the p44 (C-16) and p44 (C-14) isoforms that were used for immunoprecipitation were obtained from Santa Cruz Biochemicals. Antibody that recognizes tyrosine-phosphorylated p42 and p44 MAPK was obtained from New England Biolabs.

Arachidonic Acid Release—Resident mouse peritoneal macrophages were isolated and labeled with [³H]arachidonic acid as described previously (9). After labeling, the cells were washed 3 times with DMEM and stimulated in 1 ml of DMEM with various agonists as indicated for the specific experiments. For some experiments the [³H]arachidonic acid-labeled macrophages were depleted of calcium by incubation in DMEM containing 7.5 mM EGTA and 40 μM Quin-2-AM for 60 min (18, 19). The cells were washed and then treated with agonists in fresh DMEM containing 7.5 mM EGTA. The medium was removed and centrifuged at 1400 × g in a Sorvall RT 6000 refrigerated centrifuge for 10 min, and the cells were scraped into 1 ml of 0.1% Triton X-100. The amount of [³H]arachidonic acid released into the medium was determined and expressed as a percentage of the total radioactivity (cell-associated plus medium). Results are expressed as mean ± S.D. (n = 3) of a representative experiment and were verified in three independent experiments.

Immunoblotting—After stimulation, the macrophages (6 × 10⁴/35-mm dish) were lysed on ice with 100 μl of lysis buffer A (50 mM HEPES, pH 7.4, 150 mM NaCl, 1.0 mM EGTA, 1.0 mM EDTA, 10% glycerol, 1% Triton X-100, 100 μM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 μM sodium fluoride, 300 μM p-nitrophenyl phosphate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1.0 mM phenylmethylsulfonyl fluoride). After incubation on ice for 30 min the lysates were centrifuged for 15 min. The supernatant was boiled for 5 min in Laemmli buffer (20). For analysis of MAPK using phosphospecific antibodies, samples (20–40 μg of protein) were run on 10% polyacrylamide gels and immunoblotted according to the manufacturers instructions. For analyzing cPLA₂ gel shift, samples (20 μg of protein) were resolved on 20-cm 10% SDS-polyacrylamide gels (1% bisacrylamide, pH 8.3) and then transferred to a nitrocellulose membrane. After blocking with 5% milk for 1–2 h, the membrane was incubated overnight at 4 °C with anti-cPLA₂ polyclonal antibody at 1:2000 dilution in 20 μl Tris, pH 7.6, 137 mM NaCl, and 0.05% Tween (TTBS buffer) containing 5% milk, followed by incubation with anti-rabbit IgG horseradish peroxidase antibody (1:5000 dilution in TTBS) for 30 min at 25 °C. The immunoreactive protein was detected using the Amersham ECL system.

Determination of Intracellular Calcium by Spectrofluorimetry—Macrophages were plated on coverslips (13 mm diameter) in 2 cm² wells (24-well plate) at a density of 1 × 10⁶ cells/well, and incubated for 3 h in a humidified atmosphere of 10% CO₂ in air at 37 °C. The cells were washed twice with Ca²⁺- and Mg²⁺-free Hank’s balanced salts solution to remove non-adherent cells and incubated overnight in DMEM containing 10% fetal bovine serum. After rinsing twice with phosphate-buffered saline, and once with phenol red-free DMEM containing 2.5 mM probenecid (medium A), the cells were incubated for 1 h at 37 °C in medium A containing 5 μM Fura-2-AM and 0.025% pluronic F-127. The coverslips were rinsed twice with phosphate-buffered saline and once with medium A, and kept in medium A in the dark at room temperature until used for measuring [Ca²⁺]ᵢ (within 1 h). Coverslips were placed in a diagonal position in a standard 1-cm square quartz cuvette containing 2 ml of Krebs-Ringer phosphate dextrose buffer (4.8 mM KCl, 0.93 mM CaCl₂, 1.2 mM MgSO₄, 3.1 mM NaH₂PO₄, 12.5 mM Na₂HPO₄, 120 mM NaCl, and 0.2% dextrose) containing 2.5 mM probenecid. The cuvette was fitted with a plastic O-ring to position the coverslip just above a magnetic stirring bar. The cuvette was then placed in a SLM 8000 nm C Photon Counting Spectrofluorimeter and maintained at 37 °C with continuous stirring. After equilibration for 5 min, the excitation ratio of 340/380 nm was recorded with an emission wavelength of 505 nm. [Ca²⁺]ᵢ was calculated according to the equation, [Ca²⁺]ᵢ = [Rᵢ × b × (Rᵢ–min/(Rᵢ–max–Rᵢ))], where Rᵢ is the ratio of fluorescence at 380 nm at 0 and saturating Ca²⁺ concentrations. The leakage of Fura-2 from the cells, which was determined by adding 4 mM MnCl₂ to the assay mixture, was found to be negligible.

Determination of Intracellular Calcium Changes by Confocal Microscopy—Macrophages were plated in a 24-well plate at a density of 1 × 10⁶/well, and loaded with 5 mM Fluo-3-AM under the same conditions as described above. After loading, the cells were incubated in 200 μl of
Krebs-Ringer phosphate dextrose buffer containing 2.5 mM probenecid and observed with a Bio-Rad Confocal microscope MIC 500 with Fitz filter. After the basal calcium image was taken, 200 μl of Krebs-Ringer phosphate dextrose containing agonists was added. Images were recorded every 10 s.

**MAPK Activity Assay**—Macrophages were plated at 10 × 10⁶ cells/35-mm dish and stimulated as described above. Cells were scraped on ice into 150 μl of lysis buffer: 20 mM Hepes, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1% Triton X-100, 10% glycerol, 200 mM sodium orthovanadate, 10 mM tetrasodium pyrophosphate, 100 mM sodium fluoride, 3 μM para-nitrophenyl fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Lysates were preincubated with 30 μl of protein A-Sepharose beads (1:1 in lysis buffer) for 15 min at 4 °C. Either p42 or p44 MAPKs were immunoprecipitated from the supernatants by incubation with 0.5 μg of antibody and 12 μl of protein A-Sepharose beads (1:1 in lysis buffer) for 2 h at 4 °C. The beads were washed twice with lysis buffer and twice with 20 mM Hepes, pH 7.6, containing 200 μM sodium vanadate, 20 mM magnesium chloride, and 2 mM diithiothreitol (kinase buffer). Reactions were carried out in 50 μl of kinase buffer containing 700 μM ATP, 200 μM epidermal growth factor receptor peptide 662–681 substrate (Macromolecular Resources, Fort Collins, CO), 50 μg/ml cAMP-dependent protein kinase inhibitor IP-20, and 20 μCi of [γ-32P]ATP and incubated at 30 °C for 30 min. Reactions were stopped by brief centrifugation and addition of 15 μl of 50% trichloroacetic acid. A portion of the supernatant (30 μl) was spotted onto phosphocellulose P-81 filter discs, which were then washed with three times with 75 mM phosphoric acid (5 min) and once with acetone, followed by Cerenkov counting.

**Two-dimensional Phosphopeptide Mapping of [32P]-Labeled cPLA₂** Tryptic Peptides—[32P]-Labeled cPLA₂ was prepared in the Sf9 baculove expression system as described previously (16). To prepare labeled cPLA₂ from macrophages, cells (40 × 10⁶ cells/75 cm² flask) were isolated and cultured overnight as described previously (9). Two flasks were used for each treatment. Cells were rinsed with phosphate-free minimal essential medium and then incubated for 5 h in 10 ml of phosphate-free minimal essential medium, containing 5% fetal bovine serum and [32P]orthophosphoric acid (0.2 mCi/ml). The labeled cells were treated with either vehicle (Me₂SO) or okadaic acid for 90 min. After stimulation, cells were rinsed with ice-cold phosphate-buffered saline then lysed in 500 μl of ice-cold lysis buffer. Labeled cPLA₂ was then immunoprecipitated from cell lysates using a 1:50 dilution of antiserum for macrophage lysates or a 1:7.5 dilution for Sf9 cell lysates as described previously (16). Immunoprecipitated cPLA₂ was separated on a 10% SDS-polyacrylamide gel, detected by autoradiography, eluted from the dried gel and precipitated from the gel elution buffer using trichloroacetic acid as described previously (16). Recombinant human cPLA₂ (5 μg) was added as carrier protein to the macrophage samples before trichloroacetic acid precipitation. Trichloroacetic acid precipitates were resuspended in 200 μl of freshly made 50 mM ammonium bicarbonate (pH 7.8–8.2), and trypsin was added at a ratio of 1:10 (trypsin:cPLA₂ by weight). The samples were incubated for 3 h at 37 °C and then washed by repeated addition of water and removal in a Speed Vac. Samples were resuspended in acetic acid, and then thin layer electrophoresis followed by ascending chromatography was carried out as described previously (16).

**RESULTS**

**Role of Calcium Mobilization in Regulating Arachidonic Acid Release**—Arachidonic acid release can be induced in mouse peritoneal macrophages by diverse agonists such as PMA, the calcium ionophore, A23187, the phosphatase inhibitor, okadaic acid, and the phagocytic stimulus, zymosan. This correlates with activation of cPLA₂ which becomes phosphorylated on serine residues resulting in an increase in its activity. Experiments were carried out to investigate the role of calcium in regulating arachidonic acid release. Macrophages were loaded with Fluo-3-AM to investigate the effect of agonists on changes in [Ca²⁺], by confocal microscopy. This technique allowed evaluation of individual cells in the population. The effects of the agonists that induce arachidonic acid release were compared with ATP, a known calcium mobilizing agonist in macrophages (22, 23). Zymosan and ATP both induced a rapid increase in [Ca²⁺], as evidenced by the increased fluorescence at 10 s (Fig. 1). The response of the population was heterogeneous, with smaller changes evident by increases in blue fluorescence to greater increases in [Ca²⁺], in the yellow to red range. By 50–90 s the [Ca²⁺], had diminished but still remained above control levels. As expected, the response to A23187 was rapid, but in contrast to zymosan and ATP, the increase in [Ca²⁺], was more sustained and had not diminished by 90 s. Neither okadaic acid nor PMA induced an increase in [Ca²⁺], (Fig. 1). The lack of effect with PMA and okadaic acid was confirmed by spectrofluorimetric measurements using Fura-2-AM loaded cells (data not shown).

Although PMA and okadaic acid induced arachidonic acid release without increasing intracellular calcium, it was possible that resting levels of [Ca²⁺], were required. Consequently, the effect of depleting the cells of calcium by including EGTA in the culture medium and loading the cells with Quin-2-AM on agonist-induced arachidonic acid release was investigated (Fig. 2). Chelating extracellular calcium by incubating the macrophages in medium containing EGTA significantly suppressed arachidonic acid release in response to all the agonists.
Loading the cells with Quin-2-AM in addition to chelating extracellular calcium with EGTA further suppressed arachidonic acid release in response to zymosan, A23187, and okadaic acid. The response to PMA was not further affected in the Quin-2-AM loaded cells beyond the suppression observed using EGTA alone. Since PMA and okadaic acid did not induce an increase in $[\text{Ca}^{2+}]_i$, the inhibition of arachidonic acid release by extracellular EGTA suggested that this treatment was depleting the resting levels of $[\text{Ca}^{2+}]_i$, and that this was required for arachidonic acid release. Incubation of the macrophages in medium containing EGTA was found to rapidly decrease resting levels of $[\text{Ca}^{2+}]_i$ when evaluated by confocal microscopy or by fluorescence changes in Fura-2-AM loaded cells (data not shown). These results suggest that although PMA and okadaic acid do not induce an increase in $[\text{Ca}^{2+}]_i$, maintaining the resting level of $[\text{Ca}^{2+}]_i$, is necessary for optimal arachidonic acid release.

**Neither a Transient Increase in Calcium nor cPLA$_2$ Phosphorylation Are Sufficient for Arachidonic Acid Release**—Experiments were carried out to determine the contribution of calcium mobilization and cPLA$_2$ phosphorylation in regulating arachidonic acid release. Agonists were used that either induce strong MAPK activation leading to cPLA$_2$ phosphorylation, but with no calcium mobilization, or conversely, that promote calcium mobilization but little MAPK activation. A comparison of the effect of ATP and PAF on the magnitude and time course of $[\text{Ca}^{2+}]_i$ mobilization measured spectrophotometrically in Fura-2-AM loaded macrophages is shown in Fig. 3. ATP induced an increase in $[\text{Ca}^{2+}]_i$, from 40 to 220 nM within 10 s but returned to baseline levels by 100 s. PAF also induced a increase in $[\text{Ca}^{2+}]_i$, to a slightly lower magnitude than ATP but the response was consistently more sustained (Fig. 3B). In contrast, CSF-1 did not promote an increase in $[\text{Ca}^{2+}]_i$, as measured both by confocal analysis (Fig. 1) and spectrofluorometry (data not shown).
Calcium ionophores A23187 and ionomycin promote relatively large amounts of arachidonic acid release. Ionomycin (which does not autofluoresce as is the case for A23187) was used to correlate the concentration dependence of [Ca\textsuperscript{2+}] mobilization versus arachidonic acid release in the macrophages (Fig. 6A). A concentration-dependent accumulation of arachidonic acid in the medium occurred with ionomycin from 5 to 500 nM that correlated with its ability to induce an increase in [Ca\textsuperscript{2+}], (Fig. 6B). Considerably more arachidonic acid was released with 20–50 nM ionomycin compared with ATP (or PAF) even though the magnitude of the calcium change at these concentrations was less than with ATP. However, the increase in [Ca\textsuperscript{2+}] with ionomycin was considerably more sustained than with ATP, which is consistent with the influx of extracellular Ca\textsuperscript{2+} triggered by the ionophore. Ionomycin also induced a partial gel shift of cPLA\textsubscript{2} (Fig. 6C). This is consistent with our previous observation that A23187 treatment induced an increase in cPLA\textsubscript{2} activity in macrophages which was reversed by phosphatase treatment, although the magnitude of cPLA\textsubscript{2} activation was less than with other arachidonic acid mobilizing agonists (PMA, zymosan, and okadaic acid) (9). Consequently the ability of A23187 to induce a cPLA\textsubscript{2} gel shift at various times after agonist treatment was determined and compared with the time course of arachidonic acid release (Fig. 7). A23187 induced a partial cPLA\textsubscript{2} gel shift by 5 min that increased slightly by 10 min, however, the gel shift was not complete and it was tran-
sient. Arachidonic acid release induced by A23187 was evident by 5 min and continued to accumulate in the medium up to 60 min. Although a cPLA2 gel shift is characteristic of phosphorylation by MAPK, we had previously reported that A23187 induced only a very low, inconsistent activation of MAPK (24). This was investigated in greater detail using a more specific and sensitive assay for measuring p42 and p44 MAPK activity which involved immunoprecipitating the specific kinases and measuring kinase activity in an in vitro assay using an epidermal growth factor receptor peptide substrate (Fig. 8A). Zymosan, PMA, and okadaic acid all induced a 25-fold or greater increase in p42 MAPK activity and a 5–12-fold increase in p44 MAPK activation. A23187 induced a relatively low increase in p42 MAPK activation but no detectable increase in p44 MAPK above unstimulated controls. The time course of p42/p44 activation was evaluated by Western blot analysis using an antibody specific for tyrosine-phosphorylated MAPK (Fig. 8B). Consistent with the results above, A23187 primarily activated p42 MAPK, and the response was weak compared with MAPK activation by PMA. In addition, MAPK activation by A23187 was transient and returned to near baseline by 30 min. The weak activation of p42 MAPK by A23187 could be quantitatively inhibited by the MAPKK inhibitor, PD 98059 (90 and 100% inhibition in two experiments). Consistent with this observation, PD 98059 inhibited the cPLA2 gel shift induced by A23187 (Fig. 9). However, arachidonic acid release induced by A23187 was only slightly inhibited by PD 98059 (15% inhibition at 10 μM PD90859). At 50 μM PD98059, arachidonic acid release was slightly enhanced. These results suggest that phosphorylation at Ser-505 is not necessary for arachidonic acid release in these cells when there is a sustained increase in [Ca2+]i.

Okadaic Acid Induces Phosphorylation of Unique Sites on cPLA2 in Macrophages—The results with PMA and okadaic acid suggest that alternative mechanisms can regulate arachidonic acid release in macrophages since these agonists act without increasing [Ca2+]i. We have previously reported that when cPLA2 is expressed in insect cells using baculovirus, cPLA2-mediated arachidonic acid release can be induced by okadaic acid and this occurs without an increase in [Ca2+]i (16). In the Sf9 model, okadaic acid induces a predominant increase in phosphorylation of cPLA2 on Ser-727. In the macrophage model, okadaic acid induced a greater decrease in electrophoretic mobility of cPLA2 than the characteristic shift due to phosphorylation of Ser-505 seen with other agonists such as PMA (Fig. 10). This suggests that okadaic acid induces phosphorylation of additional sites on cPLA2 in macrophages. Experiments were carried out to investigate whether okadaic acid induced phosphorylation of Ser-727 in macrophages. Cells were labeled with [32P]orthophosphate, stimulated with okadaic acid, and tryptic peptides of the immunoprecipitated 32P-labeled cPLA2 analyzed by two-dimensional phosphopeptide mapping. Three predominant cPLA2 phosphopeptides were evident from unstimulated macrophages (Fig. 11A). Analysis of 32P-labeled cPLA2 from okadaic acid-stimulated macrophages showed the appearance of new phosphopeptides, particularly peptide 1, and an increase in labeling of other peptides. The Ser-727-containing phosphopeptide from okadaic acid-treated Sf9 cells (peptide 1, Fig. 11C) was found to comigrate with peptide 1 from okadaic acid-treated macrophages (Fig. 11D). These results suggest that Ser-727 is phosphorylated on cPLA2 in response to okadaic acid in the macrophages. The sequence of the tryptic peptide containing Ser-727 is identical in cPLA2 from mouse and human allowing this comparison to be made.

**DISCUSSION**

Arachidonic acid plays an important role as a second messenger and as a precursor of inflammatory lipid mediators, consequently its levels in cells are tightly regulated. The results of this study confirm that arachidonic acid release can be regulated in an agonist-dependent manner by diverse mechanisms in mouse peritoneal macrophages. Previous work has shown that agonist-induced arachidonic acid release is largely a cPLA2-mediated process in these cells (25, 26). We have previously reported that a variety of agonists that induce arachidonic acid release in peritoneal macrophages activate cPLA2 by serine phosphorylation through a MAPK-dependent mechanism (9, 24). In this study, agonists that act by diverse mechanisms were used to investigate the contribution of calcium and cPLA2 phosphorylation on Ser-505 in regulating arachidonic acid release in this cell model. Phosphorylation of cPLA2 on Ser-505 was evaluated by determining the ability of agonists to induce a cPLA2 gel shift. Current evidence indicates that the gel shift is due to phosphorylation on Ser-505 and that the extent of the gel shift can be used to evaluate the stoichiometry of phosphorylation at this MAPK site (10). We previously sug-
FIG. 11. Comparison of two-dimensional tryptic phosphopeptide maps of 32P-labeled cPLA₂ from Sf9 cells and macrophages. Tryptic digests of immunoprecipitated, gel-purified cPLA₂ from unstimulated macrophages (MØ/US), okadaic acid-stimulated macrophages (MØ/OA), okadaic-stimulated Sf9 cells (Sf9/OA), or from both okadaic-stimulated Sf9 cells and okadaic acid-stimulated macrophages co-spotted on the same plate (Sf9/MØ/OA) were separated by two-dimensional phosphopeptide mapping as described under “Experimental Procedures.” Electrophoresis was run in the horizontal dimension with the anode on the left and chromatography was run in the vertical dimension. The sample origin was below the lower right-hand corner of the chromatographs (not shown).

suggested that the okadaic acid-induced gel shift of cPLA₂ in insect cells may be due to phosphorylation of Ser-727. However, recent data shows that expression in Sf9 cells of cPLA₂ containing the S727A mutation, but not cPLA₂ containing the S505A mutation, exhibits a gel shift in response to okadaic acid.

Using physiological agonists for macrophages, the results demonstrate that neither a transient increase in [Ca²⁺]ᵢ as induced by ATP, nor phosphorylation of cPLA₂ on Ser-505 alone, as induced by CSF-1, are sufficient for inducing arachidonic acid release. We have previously shown that CSF-1 increases phosphorylation and activity of cPLA₂ in macrophages but is not able on its own to induce arachidonic acid release (13). In the present study, CSF-1 was confirmed not to induce an increase in [Ca²⁺]ᵢ, but did promote a sustained, stoichiometric phosphorylation of cPLA₂ on Ser-505. The calcium-mobilizing agonists ATP and PAF were found to be weak MAPK activators and induced only a partial and transient phosphorylation of cPLA₂ on Ser-505. ATP induces a transient increase in [Ca²⁺]ᵢ, and this together with its weak ability to promote phosphorylation of cPLA₂ were insufficient to induce arachidonic acid release. However, the transient increase in [Ca²⁺]ᵢ together with sustained, stoichiometric phosphorylation of cPLA₂ on Ser-505 as induced by CSF-1 act together to synergistically promote arachidonic acid release. Zymosan induces both a transient increase in calcium and stoichiometric phosphorylation of cPLA₂ on Ser-505 and is a potent inducer of arachidonic acid release. In addition, the time course of arachidonic acid release correlated with the time course of cPLA₂ phosphorylation on Ser-505 which was very rapid for CSF-1 and delayed for zymosan.

Compared with ATP, PAF consistently induced a low level of arachidonic acid release. PAF led to a similar degree of MAPK activation and cPLA₂ phosphorylation as ATP but it induced a more sustained increase in [Ca²⁺]ᵢ. In macrophages, PAF has been reported to induce a biphasic increase in [Ca²⁺]ᵢ, an initial phase due to release from intracellular stores and a second phase, due to influx of extracellular calcium (27). The results suggest that the more sustained increase in calcium is contributing to the ability of PAF to induce a low level of arachidonic acid release. In several models, arachidonic acid release has been linked to the influx of extracellular calcium (28–31). From results using rat liver macrophages, it has been suggested that an increase in [Ca²⁺]ᵢ, but not cPLA₂ phosphorylation, is necessary for arachidonic acid release (14). However, our results show that cPLA₂ phosphorylation on Ser-505 is required in macrophages when there is a transient increase in [Ca²⁺]ᵢ, but that it is not essential when there is a sustained increase in [Ca²⁺]ᵢ from extracellular sources as induced by the calcium ionophores. It has been shown in mast cells that a larger fraction of cPLA₂ translocates to nuclear membrane in response to calcium ionophore, and it remains on the membrane longer than when there is only a transient increase in calcium as occurs with IgE/antigen (8). Although the calcium ionophores induce a partial, transient cPLA₂ gel shift, the results suggest that this phosphorylation contributes little to arachidonic acid release since inhibition of ionophore-induced MAPK activation and phosphorylation of cPLA₂ on Ser-505 by the MAPKK inhibitor has no effect on arachidonic acid release. However, our previous results have shown that CSF-1 can augment A23187-induced arachidonic acid release in the macrophages (13). This suggests that the phosphorylation of Ser-505 must be stoichiometric and more sustained as occurs with CSF-1 to augment A23187-induced arachidonic acid release. These results suggest that a sustained increase in intra-

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cellular calcium induced by A23187 may be sufficient for arachidonic acid release. Although we cannot rule out the possibility that A23187 induces phosphorylation of a site that does not lead to a gel shift but may contribute to activation, this is unlikely since we have previously shown that A23187 induces only a small increase (10–15%) in $^{32}$P labeling of cPLA$_2$ in the macrophages (9).

The results using PMA and okadaic acid demonstrate that arachidonic acid release can be induced in macrophages without an increase in [Ca$^{2+}$]$_i$. However, chelating extracellular and intracellular calcium did suppress arachidonic acid release by these agonists, suggesting that resting levels of calcium may be important. Both of these agonists induce stoichiometric phosphorylation of cPLA$_2$ on Ser-505 but since this is not sufficient for inducing arachidonic acid release without an increase in [Ca$^{2+}$]$_i$, other regulatory events are implicated. The ability of PMA on its own to induce arachidonic acid release is unique to certain cell types such as macrophages and neutrophils (9, 32). In many cell types it is only effective when combined with a calcium mobilizing agonist (10). The mechanisms involved in regulating PMA-induced arachidonic acid release in macrophages are currently unknown. In contrast, we have previously shown that okadaic acid predominantly induces phosphorylation of cPLA$_2$ on a novel site (Ser-727) when it is expressed in insect cells. Okadaic acid induces arachidonic acid release in the SF9 model without an increase in [Ca$^{2+}$]$_i$, (16). In the macrophage model, the gel shift pattern of cPLA$_2$ from okadaic acid-stimulated cells suggests that it is also phosphorylated on unique sites in addition to Ser-505. In addition, analysis of cPLA$_2$ from okadaic acid-stimulated macrophages by two-dimensional phosphopeptide mapping suggests that it is phosphorylated on Ser-727. Studies are underway to determine the functional relevance of phosphorylation on Ser-727.

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The Role of Calcium and Phosphorylation of Cytosolic Phospholipase A\textsubscript{2} in Regulating Arachidonic Acid Release in Macrophages

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