THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 1, Issue of January 2, pp. 606–612, 1998

*Printed in U.S.A.*

# Secretory Phospholipase $A_2$ Activates the Cascade of Mitogen-activated Protein Kinases and Cytosolic Phospholipase $A_2$ in the Human Astrocytoma Cell Line 1321N1*

(Received for publication, August 8, 1997, and in revised form, September 26, 1997)

**Marita Hernández, Silvia López Burillo, Mariano Sánchez Crespo‡, and María Luisa Nieto**

*From the Instituto de Biología y Genética Molecular, Universidad de Valladolid-Consejo Superior de Investigaciones Científicas, 47005 Valladolid, Spain*

**The biological effects of type IIA 14-kDa phospholipase $A_2$ ($sPLA_2$) on 1321N1 astrocytoma cells were studied. $sPLA_2$ induced a release of [$^3$H]arachidonic acid ([$^3$H]AA) similar to that elicited by lysophosphatidic acid (LPA), a messenger acting via a G-protein-coupled receptor and a product of $sPLA_2$ on lipid microvesicles. In contrast, no release of [1-$^{14}$C]oleate could be detected in cells labeled with this fatty acid. As these findings pointed to a selective mechanism of [$^3$H]AA release, it was hypothesized that $sPLA_2$ could act by a signaling mechanism involving the activation of cytosolic $PLA_2$ ($cPLA_2$), *i.e.* the type of $PLA_2$ involved in the release of [$^3$H]AA elicited by agonists. In keeping with this view, stimulation of 1321N1 cells with $sPLA_2$ elicited the decrease in electrophoretic mobility that is characteristic of the phosphorylation of $cPLA_2$, as well as activation of p42 mitogen-activated protein (MAP) kinase, c-Jun kinase, and p38 MAP kinase. Incubation with $sPLA_2$ of quiescent 1321N1 cells elicited a mitogenic response as judged from an increased incorporation of [$^3$H]thymidine. Attempts to correlate the effect of extracellular $PLA_2$ with the generation of LPA were negative. Incubation with pertussis toxin prior to the addition of either $sPLA_2$ or LPA only showed abrogation of the response to LPA, thus suggesting the involvement of pertussis-sensitive $G_i$-proteins in the case of LPA. Treatments with inhibitors of the catalytic effect of $sPLA_2$ such as *p*-bromophenacyl bromide and dithiothreitol did not prevent the effect on $cPLA_2$ activation. In contrast, preincubation of 1321N1 cells with the antagonist of the $sPLA_2$ receptor *p*-aminophenyl-α-D-mannopyranoside-bovine serum albumin, blocked $cPLA_2$ activation with a $EC_{50}$ similar to that described for the inhibition of binding of $sPLA_2$ to its receptor. Moreover, treatment of 1321N1 cells with the MAP kinase kinase inhibitor PD-98059 inhibited the activation of both $cPLA_2$ and p42 MAP kinase produced by $sPLA_2$. In summary, these data indicate the existence in astrocytoma cells of a signaling pathway triggered by engagement of a $sPLA_2$-binding structure, that produces the release of [$^3$H]AA by activating the MAP kinase cascade and $cPLA_2$, and leads to a mitogenic response after longer periods of incubation.**

Phospholipases $A_2$ (phosphatide *sn*-2-acylhydrolases, EC 3.1.1.4) from mammalian tissues play a role in physiological functions such as defense mechanisms and the production of bioactive lipids (1–3). In the last years, purification and molecular cloning of phospholipases $A_2$ ($PLA_2$)[1] has allowed the characterization of several enzymes displaying significant differences in both structural and functional properties. On the one hand, the 14-kDa type IIA $PLA_2$ ($sPLA_2$) behaves as an acute phase protein whose production is induced in a variety of immunoinflammatory conditions, *e.g.* rheumatoid arthritis and endotoxemia (4–8), although its causal role in these conditions has not been ascertained, and there is no clear evidence about its involvement in the release of arachidonic acid elicited by agonists. Recent studies have shown the ability of $sPLA_2$ to promote mitogenesis by acting on a cell surface receptor (9, 10) and the appearance of chronic epidermal hyperplasia and hyperkeratosis similar to those observed in human dermopathies in mice hyperexpressing the human type IIA $PLA_2$ gene (11). A similar histological picture accompanied by inflammatory changes is produced by injection of $sPLA_2$ in the skin of experimental animals (12, 13). In addition, $sPLA_2$ may initiate cell activation because of its ability to generate the lipid mediator lysophosphatidic acid (14).

On the other hand, cytosolic phospholipase $A_2$ ($cPLA_2$) plays a central role in the release of arachidonic acid (AA) triggered by growth factors and neurotransmitters (15–17), and contains the consensus primary sequence (Pro-Leu-Ser-Pro) for phosphorylation by mitogen-activated protein (MAP) kinases, which play an important role in its regulation (18–20). Since $sPLA_2$ is an ectoenzyme that first encounters the outer leaflet of the lipid bilayers, two means of interaction leading to cell signaling should be considered. (i) $sPLA_2$ might interact with a binding structure on the outer leaflet of the cell membrane, or (ii) $sPLA_2$ might generate both unesterified fatty acid and lysophospholipid, *e.g.* lysophosphatidate (LPA) and lysophosphatydylcholine, which could act on signaling either as cofactors for protein kinase C or, in the case of LPA, by acting on specific receptors. This poses as a likely possibility that $sPLA_2$ might ultimately lead to the activation of $cPLA_2$ by eliciting a signaling cascade mimicking the usual transducing mechanism conveyed by the physiological activators of this enzyme. In this connection, it should mentioned that cross-talk between $cPLA_2$

* This study has been supported by Grant PB94-1114 from Comisión Interministerial de Ciencia y Tecnología. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Instituto de Biología y Genética Molecular, Facultad de Medicina, 47005 Valladolid, Spain. Tel.: 34-83-423273; Fax: 34-83-423085; E-mail: mscres@wamba.cpd.uva.es.

[1] The abbreviations used are: $PLA_2$, phospholipase $A_2$; AA, arachidonic acid; BPB, *p*-bromophenacyl bromide; BSA, bovine serum albumin; $cPLA_2$, cytosolic phospholipase $A_2$; DTT, dithiothreitol; ERK, extracellular signal-regulated kinase; GST, glutathione *S*-transferase; JNK, c-Jun N-terminal kinase; LPA, lysophosphatidic acid; MAP, mitogen-activated protein; mannose-BSA, *p*-aminophenyl-α-D-mannopyranoside-BSA; MEK, mitogen-activated protein kinase kinase; PAGE, polyacrylamide gel electrophoresis; PTX, pertussis toxin; $sPLA_2$, secretory phospholipase $A_2$.

and $sPLA_2$ has been suggested in signal transduction events in polymorphonuclear leukocytes and macrophages (21, 22), and a recent study in neural cells has shown a complex interplay between neurotransmitter-activated $cPLA_2$ and $sPLA_2$ (23).

$cPLA_2$ is expressed in human astrocytes of the gray matter (24), and, in a recent study, we have observed coupling of this enzyme to the activation of both muscarinic and thrombin receptors in the 1321N1 astrocytoma cell line (25, 26). This cell line displays thrombin and muscarinic $M_3$ receptors, and its pattern of responses elicited by ligand binding includes activation of phospholipases $A_2$, C, and D (25–32) and induction of AP-1 transcriptional activity (30, 31). 1321N1 astrocytoma cells express high amounts of $cPLA_2$, and they do not contain $sPLA_2$. Thus, this cell line is a good model to study the biochemical responses elicited by exogenously added $sPLA_2$.

## EXPERIMENTAL PROCEDURES

*Materials*—Plasma from patients with septicemia was obtained from venous blood anticoagulated with heparin. [9,10-$^3$H]Myristic acid (53 Ci/mmol), [1-$^{14}$C]oleate (53, 9 mCi/mmol), and [$^3$H]arachidonic acid (100 Ci/mmol) were from Amersham International, Bucks, United Kingdom. Essentially fatty acid-free BSA was from Miles Laboratories. Reagent for the measurement of proteins according to the method of Bradford (33) was purchased from Bio-Rad. Heparin-agarose type I, *p*-aminophenyl-α-D-mannopyranoside-BSA (mannose-BSA), and porcine pancreatic $PLA_2$ were from Sigma. A C127 mouse fibroblast line stably transfected with the coding sequence of type IIA $PLA_2$ from human placenta (34) was used as a source of human recombinant type IIA $PLA_2$. Rabbit polyclonal anti-$cPLA_2$ antibody was obtained as described (35). Mouse monoclonal anti-MAP kinase antibody reacting with both p42 and p44 MAP/ERK was from Zymed Laboratories Inc., San Francisco, CA. Rabbit polyclonal anti-p38 MAP kinase antibody was from Santa Cruz Biotechnology Inc., Santa Cruz, CA. Monoclonal anti-phosphotyrosine antibody clone 4G10 was from Upstate Biotechnology, Lake Placid, NY. The MAP kinase kinase (MEK) inhibitor PD-98059 was a gift from Dr. Alan R. Saltiel (Parke Davis Pharmaceutical Research, Ann Arbor, MI) (36). The p38 MAP kinase inhibitor SB 203580 was a gift from Dr. John C. Lee (SmithKline Beecham Pharmaceuticals, King of Prussia, PA) (37). Glutathione *S*-transferase (GST) fusion protein with amino acids 1–223 of the N-terminal portion of c-Jun protein (a kind gift of Dr. Carmen Caelles, Instituto de Investigaciones Biomédicas, Madrid, Spain) was expressed in bacteria using a pGEX-2T plasmid (Pharmacia Biotech Inc.) and purified with glutathione-agarose beads from Sigma.

*Purification of $sPLA_2$*—$sPLA_2$ was purified from both plasma of patients with septicemia and culture medium according to the protocol described in Ref. 38. Briefly, heparin-agarose was used to bind $sPLA_2$ from plasma. Fractions showing $PLA_2$ activity in the [1-$^{14}$C]oleate-labeled *Escherichia coli* assay were concentrated and loaded into a HiLoad Superdex 75 column (Pharmacia LKB, Uppsala, Sweden). Fractions containing $PLA_2$ after this step were made in 0.1% trifluoroacetic acid, and applied into a $C_1/C_8$ reverse-phase FPLC column (ProRPC HR 5/2, Pharmacia LKB). Fractions showing $PLA_2$ activity were pooled and evaporated to dryness in a Speed-Vac concentrator. Human recombinant type IIA phospholipase $A_2$ was purified from cultures at superconfluence of line C127 mouse fibroblasts stably transfected with the coding sequence of type IIA $PLA_2$ from human placenta (34).

*Assay of $sPLA_2$ Activity*—The assay was carried out in a total volume of 0.1 ml, according to the procedure of Elsbach *et al.* (39). Samples were incubated with ≈5,000 dpm of [1-$^{14}$C]oleate-labeled autoclaved *E. coli* of a K12 strain, containing 10–20 nmol of phospholipid, as assessed by the measurement of phospholipid-associated phosphate. The assay medium contained 0.1 M Tris/HCl, 1 mg/ml fatty acid-free BSA, and 0.5 mM $CaCl_2$, pH 7.4. The reaction proceeded for 30 min and was stopped by addition of 0.04 ml of ice-cold 2 N HCl and 0.02 ml of 10% BSA, followed by centrifugation for 5 min at 13,000 rpm in an Eppendorf microcentrifuge. The radioactivity released into the supernatant was assayed by liquid scintillation counting.

*Cell Culture and Metabolic Labeling of 1321N1 Cells*—1321N1 astrocytoma cells were cultured in Dulbecco's modified Eagle's medium containing 5% fetal calf serum at 37 °C in an atmosphere containing 5% $CO_2$. Labeling with [$^3$H]AA was performed in cells in monolayer that had been deprived of fetal calf serum for 16 h to render them quiescent. Labeling with [$^3$H]AA was carried out for 2 h in the presence of 0.3 μCi of [$^3$H]AA/ml. Labeling with 0.3 μCi/ml [1-$^{14}$C]oleate and 1 μCi/ml [$^3$H]myristic acid was carried out under similar conditions but increasing the labeling period to overnight incubation. After labeling, cells were washed at 37 °C four or five times with medium, and finally allowed to equilibrate at 37 °C before addition of agonists or vehicle solution. The release of labeled [$^3$H]AA and [1-$^{14}$C]oleic acid acid was assessed in 0.2-ml aliquots of culture medium. Production of LPA was assessed from the incorporation of [$^3$H]myristic acid into phosphatidic acid and was separated from the label incorporated in other phospholipid classes by two-dimensional chromatography using a system of solvents consisting of chloroform/methanol/28% ammonium hydroxide (6:4:1; v/v/v) in the first dimension and chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1; v/v/v) in the second dimension (40). Experiments were carried out with triplicate samples.

*Measurement of DNA Synthesis Reinitiation*—Quiescent 1321N1 cells were treated in serum-free Dulbecco's modified Eagle's medium for 24 h with different agonists in the presence of 0.5 μCi/ml [$^3$H]thymidine. At the end of this period, the incubation was terminated with three washes with ice-cold 0.1 M $MgCl_2$, and the radioactivity incorporated into the trichloroacetic acid-precipitable fraction measured.

*Immunoblots of $cPLA_2$, p42 MAP Kinase, and Immunoprecipitated p38 MAP Kinase*—Cell lysates from preconfluent 1321N1 cells were loaded into a 10% SDS-PAGE gel, and transferred to polyvinyldifluoride membrane (Immobilon P, Millipore Corp., Bedford, MA) using a liquid transfer module from CBS Laboratories. The membranes were blocked with dry milk for 2 h, washed with Tris-buffered saline, and used for immunoblot using a rabbit polyclonal anti-$cPLA_2$. When the purpose of the experiments was the detection of p42 MAP kinase, a semidry transfer system was used and the membrane was incubated with mouse monoclonal antibody. This was followed by incubation with sheep anti-mouse IgG-horseradish peroxidase-conjugated antibody, and detection with the Amersham ECL system. For detection of tyrosine phosphorylation of p38 MAP kinase, the endogenous kinase was immunoprecipitated from cell lysates using anti-p38 MAP antibody. The immune complex was recovered using Gammabind G-Sepharose. After washing three times with Nonidet-P-40-buffer and twice with LiCl buffer, the beads were resuspended in Laemmli sample buffer and subjected to SDS-PAGE. The extent of tyrosine phosphorylation of the p38 MAP kinase immunoprecipitated was determined by immunoblot with anti-phosphotyrosine mouse monoclonal antibody.

*Assay of JNK Activity*—To obtain the substrate for the kinase assay as a GST-c-Jun fusion protein, the procedure of Smith and Corcoran (41) was followed. For this purpose, transformed XL1-blue cells containing a pGEX-2T plasmid encoding residues 1–223 of the N-terminal portion of c-Jun protein were grown in LB/ampicillin medium. The expression of the fusion protein was induced by addition of 1 mM isopropyl-1-thio-β-D-galactoside. Cells were lysed using a probe sonicator and the fusion protein purified with glutathione-agarose beads. The cytosolic extracts for the kinase assay were obtained from the lysis of $5 \times 10^6$ 1321N1 cells in 200 μl of a medium containing 25 mM Hepes, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 100 mM orthovanadate, 20 mM β-glycerophosphate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin, pH 7.7. After centrifugation at 12,000 rpm at 4 °C, the supernatant was diluted in 600 μl of the above mentioned medium without NaCl, and mixed with 10 μg of GST-c-Jun protein and glutathione-agarose beads. The mixture was incubated under continuous shaking for 3–5 h at 4 °C, and then washed to remove the fraction not associated to the glutathione-agarose beads. The kinase reaction was carried out with 20 mM ATP and 5 μCi of [γ-$^{32}$P]ATP in a volume of 30 μl. The reaction was diluted in buffer and centrifugated to discard supernantant and then boiled in Laemmli SDS sample buffer and DTT. Phosphorylated GST-c-Jun was resolved by 10% SDS-PAGE and detected by autoradiography. Quantitation of the phosphorylation was carried out by densitometric scanning.

## RESULTS

*$sPLA_2$ Produces [$^3$H]AA Release and Mitogenesis in 1321N1 Astrocytoma Cells, but Does Not Release [1-$^{14}$C]Oleic Acid*—Incubation of 1321N1 cells with $sPLA_2$ at concentrations of 10 ng to 0.4 μg induced the release of [$^3$H]AA into the medium (Table I, Fig. 1*A*). This release was similar to that produced by agonists acting on membrane receptors on this cell line, namely carbachol (25), thrombin (26), and LPA (Fig. 1*B*). Astrocytes labeled with [1-$^{14}$C]oleic acid were treated with $sPLA_2$ under the same conditions used for the assay of [$^3$H]AA release. As shown in Table I, no significant release of [1-$^{14}$C]oleic acid was observed. Since $sPLA_2$ produces mitogenesis in astrocytes (9), we addressed whether this response was also elicited in quies-

TABLE I

*Effect of $sPLA_2$ on [$^3$H]AA and [1-$^{14}$C]oleate release by 1321N1 cells*

1321N1 cells were labeled and incubated for 45 min at 37 °C with different concentrations of $sPLA_2$. At the end of this period, the radioactivity contained in 0.2 ml of cell medium was assayed. The radioactivity incorporated in cell phospholipids under the conditions used for labeling was ≈120,000 dpm for [$^3$H]AA and ≈16,000 for [1-$^{14}$C]oleate. Results represent mean ± S.E. of three independent experiments in triplicate.

| $sPLA_2$ | [$^3$H]AA | [1-$^{14}$C]Oleate |
|---|---|---|
| *μg/ml* | *dpm* | *dpm* |
| 0 | 876 ± 123 | 289 ± 23 |
| 0.01 | 1435 ± 345 | 267 ± 32 |
| 0.1 | 2139 ± 197 | 278 ± 56 |
| 0.4 | 2646 ± 424 | 301 ± 79 |

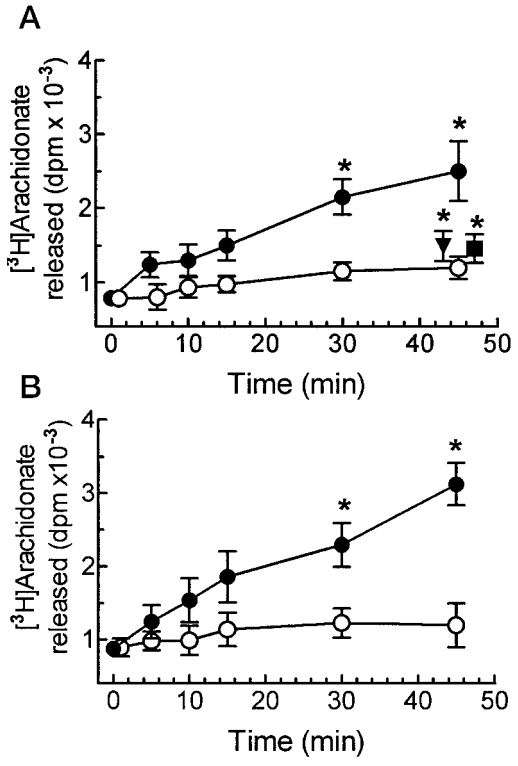


FIG. 1. **Release of [$^3$H]AA in response to $sPLA_2$ and LPA.** 1321N1 cells were incubated in the presence of 0.1 μg/ml $sPLA_2$ (*A*) or 0.2 μM LPA (*B*) for the times indicated, and then the release of [$^3$H]AA assayed in the culture medium. *Closed circles* indicate cells incubated in the presence of agonist. *Open circles* indicate cells incubated in the presence of vehicle. Data represent mean ± S.E. of six independent experiments in duplicate. *, $p < 0.05$ as compared with control cells incubated for the same time. The *closed triangle* in *A* indicates cells preincubated for 30 min with 25 μM PD-98959 prior to the addition of $sPLA_2$. The *closed square* in *A* indicates cells pretreated with 25 μM SB 203580 under identical conditions. The release of [$^3$H]AA in the presence of these drugs has been compared with that produced by $sPLA_2$ in the absence of these compounds.

TABLE II

*Incorporation of [$^3$H]thymidine into acid-precipitable material*

Results are expressed as percent of the response elicited by 10% FCS (83,000 dpm). Data represent mean ± S.E. of three experiments with duplicate samples.

| Stimulus | [$^3$H]Thymidine |
|---|---|
| | % |
| None | 12 ± 4 |
| Serum | 100 ± 0 |
| Thrombin (0.5 unit/ml) | 45 ± 13 |
| $sPLA_2$ (0.1 μg/ml) | 51 ± 7 |

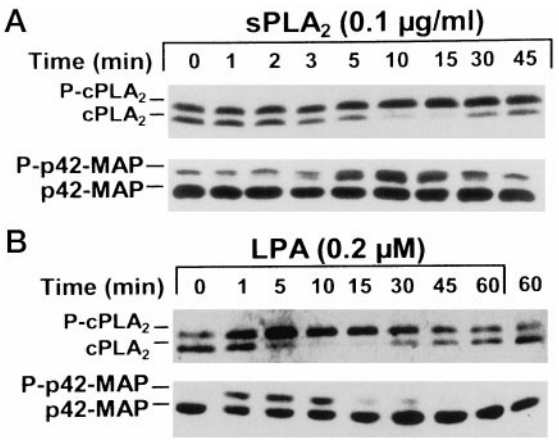


FIG. 2. **Activation of $cPLA_2$ and p42 MAP kinase by $sPLA_2$ (*A*) and LPA (*B*).** Cells were stimulated with 0.1 μg/ml $sPLA_2$ purified from patients with septicemia or 0.2 μM LPA, and at the times indicated washed and then lysed in Laemmli's buffer. About 50 μg of protein from the same samples were processed separately by SDS-PAGE (10% gel) with prolonged electrophoresis to separate the phosphorylated (*P-cPLA$_2$, P-p42-MAP*) from the nonphosphorylated forms (*cPLA$_2$, p42-MAP*) of $cPLA_2$ and p42 MAP. The proteins were electrophoretically transferred to polivinyldifluoride membranes, and immunoblotting was carried out with anti-$cPLA_2$ antibody or with anti-MAP/ERK antibody.

cent 1321N1 cells, using 10% fetal calf serum as a positive control and thrombin as a prototypic mitogenic agonist of this cell line (42). As shown in Table II, $sPLA_2$ behaved as a mitogenic agonist somewhat more potent than thrombin.

*$sPLA_2$ Induces the Phosphorylation of Both $cPLA_2$ and MAP Kinases*—Since $cPLA_2$ is the most specific enzyme that releases arachidonate from phospholipids, and $sPLA_2$ does not display selectivity for any unsaturated fatty acid on the *sn*-2 position of phospholipids (43, 44), $sPLA_2$ responses might *a priori* reflect either a direct consequence of its catalytic activity or recruitment of the arachidonate-selective enzyme $cPLA_2$. Considering that 1321N1 cells contain $cPLA_2$ as the main $PLA_2$ activity detected in cell-free homogenates and the implication of this activity in the mobilization of [$^3$H]AA produced by receptor stimulation (25, 26), we hypothesized that activation $cPLA_2$ could explain the release of [$^3$H]AA triggered by $sPLA_2$. The increase in catalytic activity of the 85-kDa $PLA_2$ has been linked to phosphorylation of the enzyme, which results in reduced mobility upon electrophoresis. As shown in Fig. 2*A*, 0.1 μg/ml $sPLA_2$ purified from the plasma of patients with septicemia induced phosphorylation of $cPLA_2$. The activation of this protein shows a time course that clearly precedes [$^3$H]AA release. Maximal amount of P-$cPLA_2$ was achieved within 10–15 min and was maintained up to 30 min after cellular stimulation. Interestingly, phosphorylation of the p42 MAP kinase preceded $cPLA_2$ phosphorylation, since it was near maximal values at 5 min (Fig. 2*A*). *In vitro* kinase assay of c-Jun kinase activity in lysates from cells stimulated with $sPLA_2$ showed an increase of the activity that peaked about 10 min after addition of $sPLA_2$ (Fig. 3*A*). Blotting with anti-phosphotyrosine antibody of the immunoprecipitate obtained with anti-p38 MAP antibody in lysates from 1321N1 cells, showed an increase of tyrosine phosphorylation of p38 MAP kinase of about 4-fold, 2 min after addition of $sPLA_2$ (Fig. 3*B*), thus suggesting that $sPLA_2$ activates all the subgroups of the MAP kinase family following different time courses.

We also investigated the effect of human recombinant $sPLA_2$ isolated from permanent transfected C127 fibroblasts. Stimulation of astrocytes with concentrations of human recombinant $sPLA_2$ above 0.1 μg/ml also resulted in a shift of the electrophoretic mobility of $cPLA_2$ (Fig. 4). Similarly, the addition of type I $PLA_2$ (pancreatic $PLA_2$, 0.8–8 μg/ml) to 1321N1 astro-

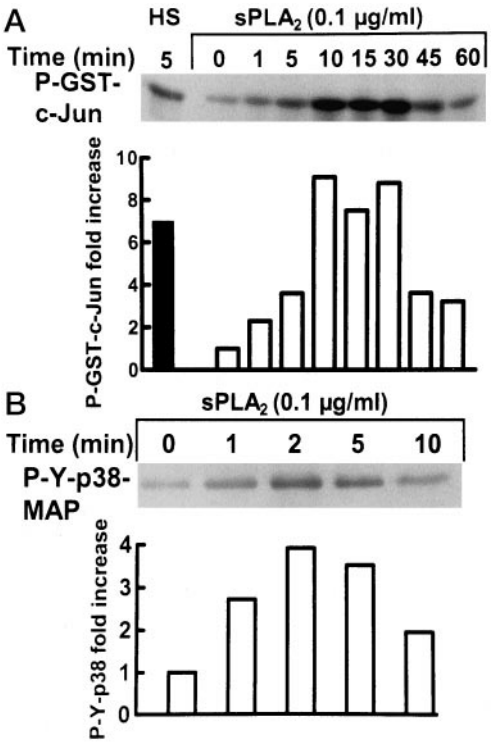


FIG. 3. **Effect of $sPLA_2$ on c-Jun kinase activity 2nd on p38 MAP kinase phosphorylation.** 1321N1 cells were incubated with $sPLA_2$ for the times indicated, and at the end of these periods, cell lysates were collected and used for *in vitro* assay of c-Jun kinase activity using GST-c-Jun protein and glutathione-agarose beads. Heat shock (*HS*) was used as a positive control for c-Jun kinase activation. This was carried out by incubating the cells for 2 min in medium preheated at 45 °C, followed by incubation at 37 °C. Quantitative analysis of the phosphorylation of GST-c-Jun was obtained by densitometric scanning and is expressed as fold-increase of basal activity (*A*). The experiment shown in *B* was carried out by immunoprecipitation of p38 MAP kinase from cell lysates, SDS-PAGE separation of the immunoprecipitate, and blotting with anti-phosphotyrosine antibody. The histogram shows the densitometric scanning of the blots. *P-Y-p38-MAP* indicates p38 MAP kinase phosphorylated in tyrosine.

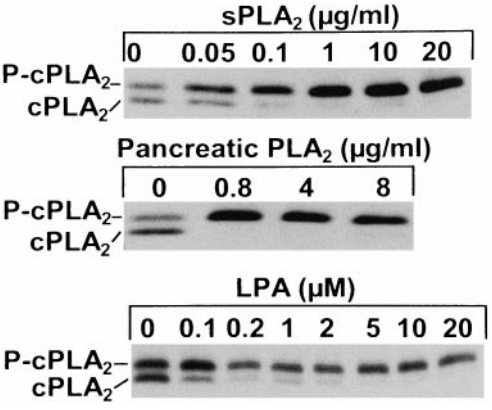


FIG. 4. **Activation of $cPLA_2$ by pancreatic $PLA_2$, recombinant human $sPLA_2$, and LPA: dose dependence.** 1321N1 cells were incubated with different concentrations of agonists for 15 min. At the end of these periods, the cell lysate was processed. Immunoblots are representative of at least three blots with identical pattern.

cytoma cells also increased the amount of P-$cPLA_2$ detected upon electrophoresis (Fig. 4). To confirm that the observed increase of the $cPLA_2$ phosphorylation was due to $sPLA_2$ rather than linked to a possible lipopolysaccharide contamination in the $sPLA_2$ preparation from septic patients, we treated our cells with 10 $\mu$g/ml lipopolysaccharide. SDS-PAGE revealed that lipopolysaccharide is not able to induce $cPLA_2$ phosphorylation (data not shown), thus ruling out the view that the observed activation of $cPLA_2$ could be due to contamination by lipopolysaccharide. Having established that addition of either of the two secreted forms of $PLA_2$ induced phosphorylation of $cPLA_2$, we hypothesized two possible mechanisms either a direct action of $sPLA_2$ on its receptor or an indirect effect through lipid mediators generated as a consequence of its catalytic activity.

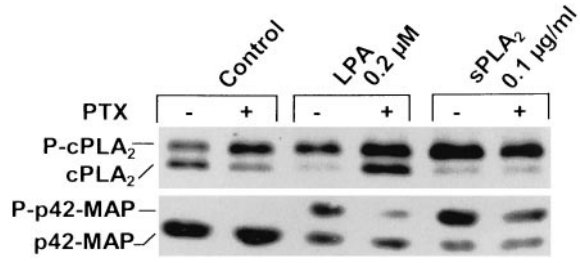


FIG. 5. **Effect of PTX on the activation of $cPLA_2$ and p42 MAP kinase elicited by LPA and $sPLA_2$.** 1321N1 cells were incubated overnight with 100 ng/ml PTX, and then stimulated with vehicle or the indicated concentrations of agonists for 15 min. At the end of this period, the cell lysate was obtained and used to separate, by electrophoresis, the phosphorylated and nonphosphorylated forms of $cPLA_2$ and p42 MAP. Immunoblot is representative of two blots with identical trend.

*$sPLA_2$ Elicits Its Effect Independently of Lysophosphatidate Formation*—Since LPA is a mitogenic agonist (reviewed in Ref. 45) and a product of $sPLA_2$ (14), we put forward the hypothesis that $sPLA_2$ could elicit its effect via the formation of this lipid mediator that acts via the interaction with a G-protein-coupled receptor. To check this notion, we first looked at the effect of LPA. As shown in Fig. 1*B*, a concentration of LPA as low as 0.2 $\mu$M induced [$^3$H]AA release. To determine the time course of LPA-induced phosphorylation of $cPLA_2$, astrocytes were exposed to 0.2 $\mu$M LPA for 0–60 min. As shown in Fig. 2*B*, the response is already evident by 5 min and is fully developed by 10 min. $cPLA_2$ band-shift induced by LPA was preceded by p42 MAP kinase phosphorylation, which was already significant at 1 min and maximal at 5 min (Fig. 2*B*). Fig. 4 shows the dose-dependent effect of LPA. Since both $sPLA_2$ and LPA produced a similar pattern of activation, this finding could be considered as an initial hint that LPA could be involved in the mediation of $sPLA_2$ effect.

It has been shown that the LPA-induced MAP kinase activation is sensitive to pertussis toxin inhibition (46, 47), thus indicating a critical role for a pertussis toxin-sensitive $G_i$-protein. On this basis, if $sPLA_2$ were acting through LPA generation, $cPLA_2$ and MAP kinase activation in response to $sPLA_2$ should show identical sensitivity to PTX. Then, in astrocytes preincubated with or without 100 nM PTX, we looked at the effect of $sPLA_2$ on the phosphorylation of p42 MAP kinase and $cPLA_2$. Whereas overnight incubation of 1321N1 cells with PTX inhibited the LPA-induced shift in electrophoretic mobility of both $cPLA_2$ and p42 MAP kinase, this treatment did not affect the ability of $sPLA_2$ to phosphorylate $cPLA_2$ or p42 MAP kinase (Fig. 5). This suggests not only that LPA is not involved in the cellular response to $sPLA_2$, but also that $sPLA_2$ acts through a pathway independent of $G_i$-proteins.

Attempts to demonstrate formation of LPA by $sPLA_2$ were carried out by labeling the phospholipid pools with [$^3$H]myristic acid and analysis of the cellular culture medium. The lipid fraction was analyzed by a two-dimensional TLC system, which allows LPA to be separated from other polar lipids with a high degree of resolution. However, upon $sPLA_2$ treatment, [$^3$H]LPA accumulation was not detected, even though a high concentration of lipid-free BSA was added to the medium to trap LPA

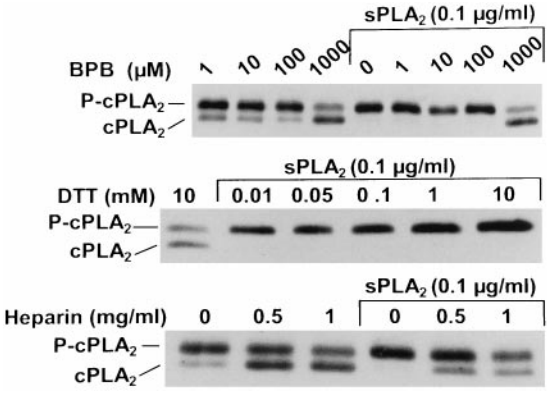


FIG. 6. **Effect of BPB, DTT, and heparin on the activation of $cPLA_2$ by $sPLA_2$.** BPB was added to the cells in the range of concentrations shown, 10 min before the addition of $sPLA_2$. In the case of experiments with DTT, the enzyme was incubated with the indicated concentrations of DTT for 30 min at room temperature, and then added to the cells to reach a final 100-fold dilution of DTT, and the indicated concentration of $sPLA_2$. The *left lane* shows the effect of the maximal concentration of DTT on 1321N1 cells. Heparin was added to 1321N1 cells 10 min before $sPLA_2$.

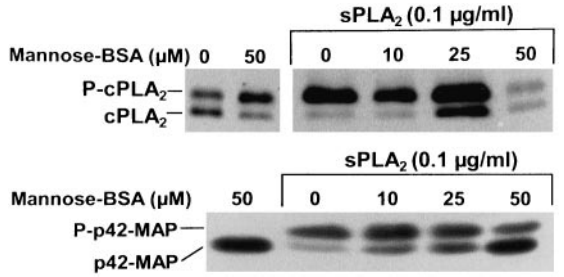


FIG. 7. **Effect of mannose-BSA on the activation of $cPLA_2$ and p42 MAP kinase elicited by $sPLA_2$.** 1321N1 cells were incubated for 10 min with the indicated concentrations of mannose-BSA prior to the addition of $sPLA_2$. This immunoblot is representative of three blots with identical pattern.

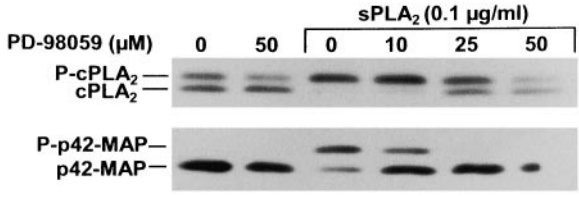


FIG. 8. **Effect of the MEK inhibitor PD-98059 on the activation of $cPLA_2$ and p42 MAP kinase elicited by $sPLA_2$.** 1321N1 cells were incubated for 10 min with different concentrations of PD-98059 prior to the addition of $sPLA_2$. About 10 min after addition of $sPLA_2$, the cell lysate was collected for immunoblotting. This immunoblot is representative of two blots with identical trend.

because of its strong binding to albumin (48).

*Inactivation of $sPLA_2$ Catalytic Activity Does Not Block the Ability to Induce Phosphorylation of $cPLA_2$*—As we failed to find accumulation of LPA or any other fatty acid but [$^3$H]AA in the cell culture medium, we addressed the possibility of regarding $sPLA_2$ as the direct responsible for $cPLA_2$ phosphorylation. To determine whether blockade of $sPLA_2$ catalytic activity may affect its ability to induce $cPLA_2$ activation, the actions of known $sPLA_2$ inhibitors were examined. We first looked at the effect of *p*-bromophenacyl bromide (BPB), a compound that inactivates the enzyme by alkylating a histidine residue located in the active site (49). Pretreatment of $sPLA_2$ for 30 min with different doses of the inhibitor resulted in a dose-dependent lose of its catalytic activity on the *E. coli* membrane system, reaching a complete blockage at 100 $\mu$M. However, even in the presence of this doses of BPB, the $cPLA_2$ band-shift induced by $sPLA_2$ was not affected (Fig. 6). It should be noted that 1 mM BPB (but not the other doses) alters agonist-induced $cPLA_2$ band-shift, thus suggesting a toxic effect of this compound, which we could confirm by the appearance of the cell culture; this agrees with the report by Lister *et al.* (50), who have suggested that the inhibitory effects of high concentrations of BPB is nonspecific, as it is due to the hydrophobicity of the compound. Incubation of $sPLA_2$ for 30 min with the thiol reagent DTT, dramatically reduced the catalytic activity of this enzyme (50% with 0.1 mM, 90% with 1 mM, and 100% with 10 mM); however, this treatment did not affect the ability of $sPLA_2$ to phosphorylate $cPLA_2$ upon addition to 1321N1 cells (Fig. 6). Taken together, the above results show that both $cPLA_2$ phosphorylation and AA mobilization induced by $sPLA_2$ are events independent of the catalytic activity of the enzyme.

*Compounds Blocking Binding of $sPLA_2$ to Cell Membrane Surface Inhibit the Ability to Phosphorylate $cPLA_2$*—In contrast to the aforementioned data, previous treatment of 1321N1 cells with mannose-BSA prior to $sPLA_2$ addition blocked $cPLA_2$ band-shift (Fig. 7) with an $EC_{50}$ similar to that described for the inhibition of binding of $sPLA_2$ to its receptor (51). The same effect was observed when the same samples were used to study the effect on p42 MAP kinase band-shift (Fig. 7). Since it has been described that $sPLA_2$ may trigger mast cell activation through binding of its heparin-binding domain to the cell surface (52), the effect of exogenous heparin on $sPLA_2$-induced $cPLA_2$ phosphorylation was also tested. As shown in the *lower panel* in Fig. 6, concentrations of heparin similar to those active on mast cells inhibited the $cPLA_2$ band-shift, without affecting significantly $sPLA_2$ catalytic activity on [1-$^{14}$C]oleate-labeled autoclaved *E. coli* (data not shown). All these findings suggesting that both $cPLA_2$ and p42 MAP kinase activation can be explained by interaction of $sPLA_2$ with a binding structure on 1321N1 cell surface.

*Blockade of MAP/ERK Kinase Activation Inhibits $cPLA_2$ Phosphorylation and [$^3$H]AA Release*—As shown previously, prolonged SDS-PAGE and immunoblotting of 1321N1 cell lysates, with a monoclonal antibody that recognizes an epitope found in both the 42- and 44-kDa isoforms of MAP/ERK kinases, only showed positive staining of a 42-kDa protein in resting cells, suggesting that this is the main isoform of the ERK subfamily of MAP kinases expressed in 1321N1 cells. Preincubation of 1321N1 cells with the compound PD-98059 (36), which inhibits MAP/ERK kinase activation by interfering with the upstream kinase MEK, inhibited both $cPLA_2$ and p42 MAP kinase activation over the same range of concentrations (Fig. 8), as well as the release of [$^3$H]AA (Fig. 1*A*), thus suggesting the involvement of the MAP/ERK subgroup of MAP kinases in the phosphorylation of $cPLA_2$ elicited by $sPLA_2$. Pretreatment of the cells with the p38 inhibitor SB 203580 at the concentration of 25 $\mu$M also caused inhibition of [$^3$H]AA release (Fig. 1*A*), thus suggesting that this subfamily of MAP kinases could be involved in the pathway for $cPLA_2$ activation elicited by $sPLA_2$.

## DISCUSSION

We have selected 1321N1 astrocytoma cells to study the effect of $sPLA_2$ because these cells do not express this enzyme, but do contain high amounts of $cPLA_2$, which is the form of enzyme most usually involved in the release of [$^3$H]AA coupled to receptor stimulation. Analysis of the physiological effects of $sPLA_2$ indicates several possible mechanisms through which they might be exerted. One of them takes into account the lysophospholipids formed as a consequence of the catalytic properties of the enzyme. In this connection, analysis of the involvement of LPA is of central importance, since this is a

multifunctional signaling phospholipid that elicits cell responses by binding to a receptor, which couples to both PTX-sensitive and PTX-insensitive G-proteins ($G_i$ and $G_q$, respectively) to trigger various effector pathways. At least four G-protein-mediated signaling pathways have been identified in the action of LPA (revised in Ref. 45): (i) stimulation of phospholipases C, D, and $A_2$ (this report); (ii) inhibition of adenylyl cyclase; (iii) activation of Ras and the downstream Raf/MAP kinase pathway; and (iv) protein-tyrosine phosphorylation. This is relevant to the present study since LPA is detected in human serum at concentrations in the range 2–70 $\mu$M (45–47), and the effect of $sPLA_2$ on platelets incubated with lipid microvesicles has been related to the production of LPA (14). Some of our findings agree with this mechanism of signaling in view of the ability of exogenous LPA to trigger biochemical signals in 1321N1 cells resembling a portion of the effect of $sPLA_2$; however, a careful appraisal of the results shows the existence of several differences, *e.g.* the involvement of a PTX-sensitive G-protein in LPA signaling, which is not involved in $sPLA_2$ signaling. Moreover, direct attempts to assay LPA formation upon $sPLA_2$ did not show the production of this mediator. Generation of unesterified fatty acids by $sPLA_2$ could be another mechanism through which this enzyme conveys cell responses. This point is a matter of considerable debate, since there is a number of mammalian cells where there has not been possible to trigger AA release by $sPLA_2$ (38, 53, 54), unless a membrane rearrangement of phospholipids is produced (55). Separation by TLC of cell-associated lipids and assay of supernatants of cells in culture stimulated with $sPLA_2$ showed no evidence of unesterified [1-$^{14}$C]oleate, but did show [$^3$H]AA. Since unlike $cPLA_2$, $sPLA_2$ does not have a preferential effect on AA-containing membrane phospholipids as compared with those containing other fatty acids, our results should be explained on the basis of the activation by $sPLA_2$ of a selective mechanism for AA release that would implicate a signaling cascade leading to $cPLA_2$ activation. Selective release of AA by $sPLA_2$ has already been reported in mice bone marrow mast cells (56). In this study, concentrations of $\approx$1 $\mu$g/ml $PLA_2$ from different sources, including human recombinant type IIA $sPLA_2$ and *Naja naja* type I $PLA_2$, elicited the release of AA in a similar way to that observed in response to immunological challenge by specific antigen. Since other unsaturated fatty acids were not detected in the supernatant, this finding also points to the recruitment by $sPLA_2$ of a selective mechanism of AA release.

The characterization of the binding site in cell membrane involved in the triggering of the response to $sPLA_2$ herein described requires a detailed discussion in view of the different structures that could be involved. Thus, there is some evidence associating many effects of $sPLA_2$ to the activation of a membrane surface receptor, which shows significant homology with the macrophage mannose-binding receptor (6, 7, 51), and is also activated by the pancreatic type of $PLA_2$, thus suggesting that endogenous $PLA_2$(s) might be its physiological ligands. In fact, stimulation of prostaglandin synthesis by pancreatic type $PLA_2$ acting through a receptor-binding reaction has been shown in rat mesangial cells (57) and in mouse osteoblastic cells (58). Moreover, inflammatory factors stimulate expression of type IIA $PLA_2$ in astrocytes in culture (59), and brain tissue is rich in N-type $PLA_2$ receptors (60). However, previous reports do not support the involvement of $PLA_2$ receptors in our system, since unlike the rabbit receptor (10), the human 180-kDa receptor expressed in COS cells binds neither type IIA $PLA_2$ nor mannose-BSA (61). Interaction of $sPLA_2$ with heparan sulfate proteoglycans is another possibility, in view of a recent report where $sPLA_2$ expressed endogenously and anchored on cell surfaces via its C-terminal heparin-binding domain was shown to be involved in the biosynthesis of prostaglandins elicited by growth factors and cytokines (62). Our attempts to unveil the binding structure by using pharmacological agents such as heparin and mannose-BSA have shown inhibition by either compound, thus suggesting more than one binding structure or, alternatively, a scarce selectivity for these compounds. Therefore, additional studies of binding and receptor expression are required to characterize these structures more precisely.

Irrespective of the nature of the membrane structure involved in $sPLA_2$ binding, the overall response induced by $sPLA_2$ in 1321N1 cells is in keeping with a mechanism dependent on the occupancy of the physiological binding sites for secreted $PLA_2$. Little is known about the biochemical signaling triggered by $sPLA_2$ receptor binding. Murakami *et al.* (52) have proposed the involvement of protein-tyrosine phosphorylation reactions in $sPLA_2$-mediated mast cell activation in view of the blockade of the response by inhibitors of this reactions such as genistein and herbimycin A. In view of the important interactions of protein-tyrosine phosphorylation signaling and activation of the MAP kinase cascade, this report agrees with our finding of the activation of the MAP kinase cascades by $sPLA_2$, including p42 MAP kinase, p38, and c-Jun kinase. Based on the different time courses of the activation of these kinases, and the results obtained with the MEK inhibitor PD-98959 as well as with SB 203580, which inhibits p38 MAP kinase activity, our data suggest the implication of both kinases in the signaling pathway leading to [$^3$H]AA release, although characterization of the actual kinase(s) implicated in $cPLA_2$ phosphorylation requires additional studies. Studies on the regulation of $cPLA_2$ have stressed the requirement of $Ca^{2+}$-dependent translocation to the cell membrane for elicitation of its catalytic effect (63). In keeping with this mechanism, we have observed that both pancreatic type $PLA_2$ and type IIA $sPLA_2$ elicit $Ca^{2+}$ mobilization in fura-2-labeled 1321N1 cells[2] showing a pattern similar to that elicited by LPA, thus suggesting a mechanism of action compatible with the occupancy of a binding site. However, since mechanisms other than $Ca^{2+}$ mobilization have been implicated in the translocation of $cPLA_2$ (25, 64), we cannot establish a direct link between $Ca^{2+}$ mobilization and the activation of $cPLA_2$ as yet.

As to the pathophysiological significance of our findings, it should be pointed out that the effect of $sPLA_2$ has been obtained with concentrations of enzyme below those detected in human plasma in a number of clinical conditions, including septic shock (65), salicylate intoxication (66), and severe *Plasmodium falciparum* malaria (67).

In summary, our data show that $sPLA_2$ elicits biochemical signaling in 1321N1 astrocytoma cells by a mechanism that is best explained by interaction with a membrane receptor similar to the macrophage mannose receptor or, alternatively, via engagement of heparan sulfate proteoglycans. The set of responses observed includes phosphorylation of $cPLA_2$, most probably involving the p42 MAP kinase route, release of AA, and mitogenesis. These findings might be of interest to explain some of the controversial findings regarding release of AA by $sPLA_2$.

*Acknowledgments*—We thank Dr. G. Bereziat for providing the transfected fibroblast cell line, Dr. Victor Calvo for help in assaying MAP kinase, Dr. J. Castañeda for selecting patients with multiple organ failure and high levels of $sPLA_2$ to obtain plasma, Dr. Joan Heller Brown for providing the 1321N1 cell line, and Dr. Carmen Caelles for providing the GST-c-Jun fusion protein.

[2] M. Hernández, S. López Burillo, M. Sánchez Crespo, and M. L. Nieto, unpublished results.

REFERENCES


1. Dennis, E. A., Rhee, S. G., Billah, M. M., and Hannun, Y. A. (1991) *FASEB J.* **5,** 2068–2077
2. Mayer, R. J., and Marshall, L. A. (1993) *FASEB J.* **7,** 339–348
3. Serhan, C. N., Haeggstrom, J. Z., and Leslie, C. C. (1996) *FASEB J.* **10,** 1147–1158
4. Vadas, P., Wasi, S., Movat, H., and Hay, J. H. (1981) *Nature* **293,** 583–585
5. Vadas, P. (1984) *J. Lab. Clin. Med.* **104,** 873–881
6. Chilton, F. (1996) *J. Clin. Invest.* **97,** 2161–2162
7. Hack, C. E., Wolbink, G. J., Schalwijk, C., Speijer, H., Hermens, W. T., and van den Bosch, H. (1997) *Immunol. Today* **18,** 111–115
8. Jacques, C., Bériziat, G., Humbert, L., Olivier, J. L., Corvol, M. T., Masliah, J., and Berenbaum, F. (1997) *J. Clin. Invest.* **99,** 1864–1872
9. Arita, H., Hanasaki, K., Hanako, T., Oda, S., Teraoka, H., and Matsumoto, K. (1991) *J. Biol. Chem.* **266,** 19139–19141
10. Lambeau, G., Ancian, P., Barhanin, J., and Lazdunski, M. (1994) *J. Biol. Chem.* **269,** 1575–1578
11. Grass, D. S., Felkner, R. H., Chiang, M. Y., Wallace, R. E., Nevalainen, T. J., Bennet, C. F., and Swanson, M. E. (1996) *J. Clin. Invest.* **97,** 2233–2241
12. Pruzanski, W. P., Vadas, P., and Fornasier, V. (1986) *J. Invest. Dermatol.* **86,** 380–382
13. Nair, X., Nettleton, D., Clever, D., Tramposch, K. M., Ghosh, S., and Franson, R. C. (1993) *Inflammation* **17,** 205–215
14. Fourcade, O., Simon, M. F., Viode, C., Rugani, N., Leballe, F., Ragab, A., Fournie, B., Sarda, L., and Chap, H. (1995) *Cell* **80,** 919–927
15. Leslie, C. C., Voelker, D. R., Channon, J. Y., Wall, M. M., and Zelarney, P. T. (1988) *Biochim. Biophys. Acta* **963,** 476–492
16. Clark, J. D., Lin, L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N., and Knopf, J. L. (1991) *Cell* **65,** 1043–1051
17. Sharp, J. D., White, D. L., Chiou, X. G., Goodson, T., Gamboa, G. C., McClure, D., Burgett, S., Hoskins, J., Skatrud, P. L., Sportsman, J. R., Becker, G. W., Kang, L. H., Roberts, E. F., and Kramer, R. (1991) *J. Biol. Chem.* **266,** 14850–15853
18. Davis, R. J. (1993) *J. Biol. Chem.* **268,** 14553–14556
19. Cobb, M. H., and Goldsmith, E. J. (1995) *J. Biol. Chem.* **270,** 14843–14846
20. Lin, L. L., Wartmann, M., Lin, A. Y., Knopf, J. L., Seth, A., and Davis, R. J. (1993) *Cell* **72,** 269–278
21. Wijkander, J., O'Flaherty, J. T., Nixon, A. B., and Wykle, R. L. (1995) *J. Biol. Chem.* **270,** 26543–26549
22. Balsinde, J., and Dennis, E. A. (1996) *J. Biol. Chem.* **271,** 6758–6765
23. Kolko, M., DeCoster, M. A., Rodríguez de Turco, E. B., and Bazán, N. G. (1996) *J. Biol. Chem.* **271,** 32722–32728
24. Stephenson, D. T., Manetta, J. V., White, D. L., Chiou, X. G., Cox, L., Gitter, B., May, P. C., Sharp, J. D., Kramer, R. M., and Clemens, J. A. (1994) *Brain Res.* **637,** 97–115
25. Bayón, Y., Hernández, M., Alonso, A., Núñez, L., García-Sancho, J., Leslie, C. C., Sánchez Crespo, M., and Nieto, M. L. (1997) *Biochem. J.* **323,** 281–287
26. Hernández, M., Bayón, Y., Sánchez Crespo, M., and Nieto, M. L. (1997) *Biochem. J.*, **328,** 263–269
27. Orellana, S. A., Solski, P. A., and Heller Brown, J. (1985) *J. Biol. Chem.* **260,** 5236–5239
28. Martinson, E. A., Goldstein, D., and Brown, J. H. (1989) *J. Biol. Chem.* **264,** 14748–14754
29. Trejo, J. A., and Brown, J. H. (1991) *J. Biol. Chem.* **266,** 7876–7882
30. LaMorte, V. J., Kennedy, E. D., Collins, L. R., Goldstein, D., Harootunian, A. T., Brown, J. H., and Feramisco, J. R. (1993) *J. Biol. Chem.* **268,** 19411–19415
31. Martinson, E. A., Trilivas, I., and Brown, J. H. (1990) *J. Biol. Chem.* **265,** 22282–22287
32. Nieto, M. L., Kennedy, E., Goldstein, D., and Heller Brown, J. (1994) *Mol. Pharmacol.* **46,** 406–413
33. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
34. Pernas, P., Masliah, J., Olivier, J. L., Salvat, C., and Bereziat, G. (1991) *Biochem. Biophys. Res. Commun.* **178,** 1298–1305
35. deCarvalho, M. S., McCormack, F. X., and Leslie, C. C. (1993) *Arch. Biochem. Biophys.* **306,** 534–540
36. Dudley, D. T., Pang, L., Decker, S.. J., Bridges, A. J., and Saltiel, A. R.. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 7686–7689
37. Lee, J. C., and Young, P. R. (1996) *J. Leukocyte Biol.* **59,** 152–157
38. Gijón, M. A., Pérez, C., Méndez, E., and Sánchez Crespo, M. (1995) *Biochem. J.* **306,** 167–175
39. Elsbach, P., Weiss, J., Franson, R. C., Beckerdite-Quagliata, S., Schneider, A., and Harris, L. (1979) *J. Biol. Chem.* **254,** 11000–11009
40. Tokomura, A., Harada, K., Fukuzawa, K., and Tsukatani, H. (1986) *Biochim. Biophys. Acta* **875,** 31–38
41. Smith, D. B., and Corcoran, L. M. (1994) in *Current Protocols in Molecular Biology* (Ausubel, F. A., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) pp. 16.7.1–16.7.7, John Wiley & Sons, New York
42. Post, G. R., Collins, L. R., Kennedy, E. D., Moskowitz, S. A., Aragay, A. M., Goldstein, D., and Brown, J. H. (1996) *Mol. Biol. Cell* **7,** 1679–1690
43. Seilhamer, J. J., Pruzanski, W., Vadas, P., Plant, S., Miller, J. A., Kloss, J., and Johnson, L. K. (1989) *J. Biol. Chem.* **264,** 5335–5338
44. Kramer, R. M., Hession, C., Johansen, B., Hayes, G., McGray, P., Chow, E. P., Tizard, R., and Pepinsky, R. B. (1989) *J. Biol. Chem.* **264,** 5768–5775
45. Moolenaar, W. H. (1995) *J. Biol. Chem.* **270,** 12949–12952
46. Tigyi, G., Henschen, A., and Miledi, R. (1991) *J. Biol. Chem.* **266,** 20602–20609
47. Tigyi, G., and Miledi, R. (1992) *J. Biol. Chem.* **267,** 21360–21367
48. Eichholtz, T., Jalink, K., and Moolenaar, W. H. (1993) *Biochem. J.* **291,** 677–680
49. Volwerk, J. J., Pieterson, W. A., and de Haas, G. H. (1974) *Biochemistry* **13,** 1446–1454
50. Lister, M. D., Glaser, K. B., Ulevitch, R. J., and Dennis, E. A. (1989) *J. Biol. Chem.* **264,** 8520–8528
51. Nicolas, J.-P., Lambeau, G., and Lazdunski, M. (1995) *J. Biol. Chem.* **270,** 28869–28873
52. Murakami, M., Hara, N., Kudo, I., and Inonue, K. (1993) *J. Immunol.* **151,** 5675–5684
53. Riendau, D., Guay, J., Weech, P. K., Laliberté, F., Yergey, J., Desmarais, S., Perrier, H., Liu, S., Nicoll-Griffith, D., and Street, I. P. (1994) *J. Biol. Chem.* **269,** 15619–15624
54. Bartoli, F., Lin, H.-K., Gomashchi, F., Gelb, M. H., Jain, M. K., and Apitz-Castro, R. (1994) *J. Biol. Chem.* **269,** 15625–15630
55. Kudo, I., Murakami, M., Hara, S., and Inonue, K. (1993) *Biochim. Biophys. Acta* **1170,** 217–231
56. Fonteh, A. N., Bass, D. A., Marshall, L. A., Seeds, M., Samet, J. M., and Chilton, F. H. (1994) *J. Immunol.* **152,** 5438–5446
57. Kishino, J., Kawamoto, K., Ishizaki, J., Verheij, H. M., Ohara, O., and Arita, H. (1995) *J. Biochem.* (*Tokyo*) **117,** 420–424
58. Tohkin, M., Kishino, J., Ishizaki, J., and Arita, H. (1993) *J. Biol. Chem.* **268,** 2865–2871
59. Oka, S., and Arita, H. (1991) *J. Biol. Chem.* **266,** 9956–9960
60. Nicolas, J.-P., Lin, Y., Lambeau, G., Ghomashchi, F., Lazdunski, M., and Gelb, M. H. (1997) *J. Biol. Chem.* **272,** 7173–7181
61. Ancian, P., Lambeau, G., Mattéi, M.-G., and Lazdunski, M. (1995) *J. Biol. Chem.* **270,** 8963–8970
62. Murakami, M., Nakatani, Y., and Kudo, I. (1996) *J. Biol. Chem.* **271,** 30041–30051
63. Kramer, R. M., Roberts, E. F., Manetta, J., and Putnam, J. E. (1991) *J. Biol. Chem.* **266,** 5268–5272
64. Peppelenbosch, M. P., Qiu, R. G., de Vries-Smits, A. M., Tertoolen, L. G., de Laat, S. W., McCormack, F., Hall, A., Symons, M. H., and Bos, J. L. (1995) *Cell* **81,** 849–856
65. Green, J. A., Smith, G. M., Buchta, R., Lee, R., Ho, K. Y., Rajkovic, I. A., and Scott, K. F. (1991) *Inflammation* **15,** 355–367
66. Vadas, P., Schouten, B. D., Stefanski, E., Scott, K., and Pruzanski, W. (1993) *Crit. Care Med.* **21,** 1087–1091
67. Vadas, P., Taylor, T. E., Chimsuku, L., Goldring, D., Stefanski, E., Pruzanski, W., and Molyneux, M. E. (1993) *Am. J. Trop. Med. Hyg.* **49,** 455–459