Secretory Phospholipase A₂ Activates the Cascade of Mitogen-activated Protein Kinases and Cytosolic Phospholipase A₂ in the Human Astrocytoma Cell Line 1321N1*

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The biological effects of type IIA 14-kDa phospholipase A₂ (sPLA₂) on 1321N1 astrocytoma cells were studied. sPLA₂ induced a release of [³H]arachidonic acid ([³H]AA) similar to that elicited by lysophosphatidic acid (LPA), a messenger acting via a G-protein-coupled receptor and a product of sPLA₂ on lipid microvesicles. In contrast, no release of [¹⁴C]oleate could be detected in cells labeled with this fatty acid. As these findings pointed to a selective mechanism of [³H]AA release, it was hypothesized that sPLA₂ could act by a signaling mechanism involving the activation of cytosolic PLA₂ (cPLA₂), i.e. the type of PLA₂ involved in the release of [³H]AA elicited by agonists. In keeping with this view, stimulation of 1321N1 cells with sPLA₂ elicited the decrease in electrophoretic mobility that is characteristic of the phosphorylation of cPLA₂, as well as activation of p42 mitogen-activated protein (MAP) kinase, c-Jun kinase, and p38 MAP kinase. Incubation with sPLA₂ of quiescent 1321N1 cells elicited a mitogenic response as judged from an increased incorporation of [³H]thymidine. Attempts to correlate the effect of extracellular PLA₂ with the generation of LPA were negative. Incubation with pertussis toxin prior to the addition of either sPLA₂ or LPA only showed abrogation of the response to LPA, thus suggesting the involvement of pertussis-sensitive G proteins in the case of LPA. Treatments with inhibitors of the catalytic effect of sPLA₂ such as p-bromophenacyl bromide and dithiothreitol did not prevent the effect on cPLA₂ activation. In contrast, preincubation of 1321N1 cells with the antagonist of the sPLA₂ receptor p-aminophenyl-a-D-mannopyranoside-bovine serum albumin, blocked cPLA₂ activation with an EC₅₀ similar to that described for the inhibition of binding of sPLA₂ to its receptor. Moreover, treatment of 1321N1 cells with the MAP kinase kinase inhibitor PD-98059 inhibited the activation of both cPLA₂ and p42 MAP kinase produced by sPLA₂. In summary, these data indicate the existence in astrocytoma cells of a signaling pathway triggered by engagement of a cPLA₂-binding structure, that produces the release of [³H]AA by activating the MAP kinase cascade and cPLA₂, and leads to a mitogenic response after longer periods of incubation.

Phospholipases A₂ (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 A₂ (PLA₂)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 PLA₂ (sPLA₂) 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 sPLA₂ 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 dermatophasies in mice hyperexpressing the human type IIA PLA₂ gene (11). A similar histological picture accompanied by inflammatory changes is produced by injection of sPLA₂ in the skin of experimental animals (12, 13). In addition, sPLA₂ may initiate cell activation because of its ability to generate the lipid mediator lysophosphatic acid (14).

On the other hand, cytosolic phospholipase A₂ (cPLA₂) 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 sPLA₂ 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) sPLA₂ might interact with a binding structure on the outer leaflet of the cell membrane, or (ii) sPLA₂ might generate both unesterified fatty acid and lysophospholipid, e.g. lysophosphatidate (LPA) and lysophosphatidylcholine, 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 sPLA₂ might ultimately lead to the activation of cPLA₂ 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 cPLA₂ and MAP kinases may ultimately lead to the activation of cPLA₂ 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 cPLA₂

1 The abbreviations used are: PLA₂, phospholipase A₂; AA, arachidonic acid; BPB, p-bromophenacyl bromide; BSA, bovine serum albumin; cPLA₂, cytosolic phospholipase A₂; 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-aminoenol-α-α-mannopyranoside-BSA; MEK, mitogen-activated protein kinase kinase; PAGE, polyacrylamide gel electrophoresis; PTX, pertussis toxin; sPLA₂, secretory phospholipase A₂.

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and sPLA₂ 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 cPLA₂ and sPLA₂ (23).

cPLA₂ 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 M₃ receptors, and its pattern of responses elicited by ligand binding includes activation of phospholipases A₂, C, and D (25–32) and induction of AP-1 transcriptional activity (30, 31). 1321N1 astrocytoma cells express high amounts of cPLA₂, and they do not contain sPLA₂. Thus, this cell line is a good model to study the biochemical responses elicited by exogenously added sPLA₂.

**EXPERIMENTAL PROCEDURES**

**Materials**—Plasma from patients with septicemia was obtained from venous blood anticoagulated with heparin. [9,10-3H]Myristic acid (53 Ci/mmol), [1-14C]oleate (35, 9 mCi/mmol), and [1-14C]arachidonic acid (100 Ci/mmol) were from Amersham International, Bucks, United Kingdom. Essentially fatty acid-free BSA was from Miles Laboratories, Kankakee, IL. Reagent for the measurement of proteins according to the method of Bradford (33) was purchased from Bio-Rad. Heparin-agarose type I, 2%-D-mannopyranoside-BSA (mannose-BSA), and porcine pancreatic PLA₂ were from Sigma. A C127 mouse fibroblast line stably transfected with the coding sequence of type IIA PLA₂ from human placenta (34) was purchased from Bio-Rad. E. coli LPS was from Escherichia coli ATCC 25015 ( Obtained from the American Type Culture Collection, Rockville, MD). Human recombinant type IIA PLA₂ was from Sigma. A C127 mouse fibroblast line stably transfected with the coding sequence of type IIA PLA₂ from human placenta (34) was used as a source of human recombinant type IIA PLA₂. Rabbit polyclonal anti-cPLA₂ 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 E. coli Sodizer and 75 column (Pharmacia LKB, Uppsala, Sweden). Fractions containing PLA₂, after this step were made in 0.1% Tris buffered, and applied into a C₁₅₃ reverse-phase FPLC column (ProRPC HR 5/2, Pharmacia LKB). Fractions showing PLA₂ activity were pooled and concentrated and loaded into a 10% SDS-PAGE gel, and transferred to polyvinylidene difluoride membrane (Immobilon P, Millipore Corp., Bedford, MA) using a semidry transfer system was used and the membrane was incubated 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-p85 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-phosphotyrosin antibody.

**Assay of c-Jun 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 (IPTG) in cell cultures at about 10⁶ cells/ml and lysed using a French press and the fusion protein purified with glutathione-agarose beads. The cytosolic extracts for the kinase assay were obtained from the lysis of 5 × 10⁶ 1321N1 cells in 200 μl of a medium containing 25 mM Hepes, 0.3 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 100 mM orthovanadate, 20 μM β-glycerophosphate, 10 μg/ml leupeptin, and 10 μg/ml aprotonin, pH 7.4. After centrifugation (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 in 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 μM ATP and 5 μCi of [γ-32P]ATP in a volume of 30 μl. The reaction was diluted in buffer and centrifuged to discard supernatant 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**

**sPLA₂ Produces [3H]AA Release and Mitogenesis in 1321N1 Astrocytoma Cells, but Does Not Release [1-14C]Oleic Acid**

Incubation of 1321N1 cells with sPLA₂ at concentrations of 10 to 0.4 μg induced the release of [3H]AA into the medium (Table I, Fig. 1A). This release was similar to that produced by thrombin on monolayer of nontreated rat astrocyte cells. The cells were plated at 3000 cells/cm², named carbachol (25), thrombin (26), and LPA (Fig. 1B). Astrocytes labeled with [1-14C]Oleic acid were treated with sPLA₂ under the same conditions used for the assay of [3H]AA release. As shown in Table I, no significant release of [1-14C]Oleic acid was observed. Since sPLA₂ produces mitogenesis in astrocytes (9), we addressed whether this response was also elicited in quies-
1321N1 cells were incubated in the presence of 0.1 μM agonist somewhat more potent than thrombin.

% Results represent mean ± S.E. of three independent experiments in triplicate.

| sPLA2 | [3H]AA | [1-14C]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 [3H]AA in response to sPLA2 and LPA. 1321N1 cells were incubated in the presence of 0.1 μg/ml sPLA2 (A) or 0.2 μM LPA (B) for the times indicated, and then the release of [3H]AA assayed in the culture medium. Closed circles indicate cells incubated in the presence of agonist. Open circles indicate cells incubated in the absence 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 indicates cells incubated in the presence of vehicle. Data represent mean ± S.E. of three independent experiments in triplicate.

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, sPLA2 behaved as a mitogenic agonist somewhat more potent than thrombin.

sPLA2 Induces the Phosphorylation of Both cPLA2 and MAP Kinases—Since cPLA2 is the most specific enzyme that releases arachidonate from phospholipids, and sPLA2 does not display selectivity for any unsaturated fatty acid on the sn-2 position of phospholipids (43, 44), sPLA2 responses might a priori reflect either a direct consequence of its catalytic activity or recruitment of the arachidonate-selective enzyme cPLA2. Considering that 1321N1 cells contain cPLA2 as the main PLA2 activity detected in cell-free homogenates and the implication of this activity in the mobilization of [3H]AA produced by receptor stimulation (25, 26), we hypothesized that activation cPLA2 could explain the release of [3H]AA triggered by sPLA2. The increase in catalytic activity of the 85-kDa PLA2 has been linked to phosphorylation of the enzyme, which results in reduced mobility upon electrophoresis. As shown in Fig. 2A, 0.1 μg/ml sPLA2 purified from the plasma of patients with sepsisemia or 0.2 μM LPA, and at the times indicated washed and then lysed in Laemmlli’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-cPLA2; P-p42-MAP) from the nonphosphorylated forms (cPLA2, p42-MAP) of cPLA2 and p42 MAP. The proteins were electrophoretically transferred to polyvinylidifluoride membranes, and immunoblotting was carried out with anti-cPLA2 antibody or with anti-MAP/ERK antibody.

activity in lysates from cells stimulated with sPLA2 showed an increase in catalytic activity of the 85-kDa PLA2 has been linked to phosphorylation of the enzyme, which results in reduced mobility upon electrophoresis. As shown in Fig. 2A, 0.1 μg/ml sPLA2 purified from the plasma of patients with sepsisemia or 0.2 μM LPA, and at the times indicated washed and then lysed in Laemmlli’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-cPLA2; P-p42-MAP) from the nonphosphorylated forms (cPLA2, p42-MAP) of cPLA2 and p42 MAP. The proteins were electrophoretically transferred to polyvinylidifluoride membranes, and immunoblotting was carried out with anti-cPLA2 antibody or with anti-MAP/ERK antibody.

FIG. 2. Activation of cPLA2 and p42 MAP kinase by sPLA2 (A) and LPA (B). Cells were stimulated with 0.1 μg/ml sPLA2 purified from patients with sepsisemia or 0.2 μM LPA, and at the times indicated washed and then lysed in Laemmlli’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-cPLA2; P-p42-MAP) from the nonphosphorylated forms (cPLA2, p42-MAP) of cPLA2 and p42 MAP. The proteins were electrophoretically transferred to polyvinylidifluoride membranes, and immunoblotting was carried out with anti-cPLA2 antibody or with anti-MAP/ERK antibody.
cytoma cells also increased the amount of P-cPLA2 detected upon electrophoresis (Fig. 4). To confirm that the observed increase of the cPLA2 phosphorylation was due to sPLA2 rather than linked to a possible lipopolysaccharide contamination in the sPLA2 preparation from septic patients, we treated our cells with 10 μg/ml lipopolysaccharide. SDS-PAGE revealed that lipopolysaccharide is not able to induce cPLA2 phosphorylation (data not shown), thus ruling out the view that the observed activation of cPLA2 could be due to contamination by lipopolysaccharide. Having established that addition of either of the two secreted forms of PLA2 induced phosphorylation of cPLA2, we hypothesized two possible mechanisms either a direct action of sPLA2 on its receptor or an indirect effect through lipid mediators generated as a consequence of its catalytic activity.

sPLA2 Elicits Its Effect Independently of Lysophosphatidate Formation—Since LPA is a mitogenic agonist (reviewed in Ref. 45) and a product of sPLA2 (14), we put forward the hypothesis that sPLA2 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. 1B, a concentration of LPA as low as 0.2 μM induced [3H]AA release. To determine the time course of LPA-induced phosphorylation of cPLA2, astrocytes were exposed to 0.2 μM LPA for 0–60 min. As shown in Fig. 2B, the response is already evident by 5 min and is fully developed by 10 min. cPLA2 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. 2B). Fig. 4 shows the dose-dependent effect of LPA. Since both sPLA2 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 sPLA2 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 protein. On this basis, if sPLA2 were acting through LPA generation, cPLA2 and MAP kinase activation in response to sPLA2 should show identical sensitivity to PTX. Then, in astrocytes preincubated with or without 100 nM PTX, we looked at the effect of sPLA2 on the phosphorylation of p42 MAP kinase and cPLA2. Whereas overnight incubation of 1321N1 cells with PTX inhibited the LPA-induced shift in electrophoretic mobility of both cPLA2 and p42 MAP kinase, this treatment did not affect the ability of sPLA2 to phosphorylate cPLA2 or p42 MAP kinase (Fig. 5). This suggests not only that LPA is not involved in the cellular response to sPLA2, but also that sPLA2 acts through a pathway independent of G proteins. Attempts to demonstrate formation of LPA by sPLA2 were carried out by labeling the phospholipid pools with [3H]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 sPLA2 treatment, [3H]LPA accumulation was not detected, even though a high concentration of lipid-free BSA was added to the medium to trap LPA.
because of its strong binding to albumin (48).

Inactivation of sPLA2 Catalytic Activity Does Not Block the Ability to Induce Phosphorylation of cPLA2—As we failed to find accumulation of LPA or any other fatty acid but [3H]AA in the cell culture medium, we addressed the possibility of regarding sPLA2 as the direct responsible for cPLA2 phosphorylation. To determine whether blockade of sPLA2 catalytic activity may affect its ability to induce cPLA2 activation, the actions of known sPLA2 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 sPLA2 for 30 min with different doses of the inhibitor resulted in a dose-dependent loss of its catalytic activity on the E. coli membrane system, reaching a complete blockage at 100 μM. However, even in the presence of these doses of BPB, the cPLA2 band-shift induced by sPLA2 was not affected (Fig. 6). It should be noted that 1 mM BPB (but not the other doses) alters agonist-induced cPLA2 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 sPLA2 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 sPLA2 to phosphorylate cPLA2 upon addition to 1321N1 cells (Fig. 6). Taken together, the above results show that both cPLA2 phosphorylation and AA mobilization induced by sPLA2 are events independent of the catalytic activity of the enzyme.

Compounds Blocking Binding of sPLA2 to Cell Membrane Surface Inhibit the Ability to Phosphorylate cPLA2—In contrast to the aforementioned data, previous treatment of 1321N1 cells with mannose-BSA prior to sPLA2 addition blocked cPLA2 band-shift (Fig. 7) with an EC50 similar to that described for the inhibition of binding of sPLA2 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 sPLA2 may trigger mast cell activation through binding of its heparin-binding domain to the cell surface (52), the effect of exogenous heparin on sPLA2-induced cPLA2 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 cPLA2 band-shift, without affecting significantly sPLA2 catalytic activity on [1-14C]oleate-labeled autoclaved E. coli (data not shown). All these findings suggesting that both cPLA2 and p42 MAP kinase activation can be explained by interaction of sPLA2 with a binding structure on 1321N1 cell surface.

Blockade of MAP/ERK Kinase Activation Inhibits cPLA2 Phosphorylation and [3H]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 cPLA2 and p42 MAP kinase activation over the same range of concentrations (Fig. 8), as well as the release of [3H]AA (Fig. 1A), thus suggesting the involvement of the MAP/ERK subgroup of MAP kinases in the phosphorylation of cPLA2 elicited by sPLA2. Pretreatment of the cells with the p38 inhibitor SB 203580 at the concentration of 25 μM also caused inhibition of [3H]AA release (Fig. 1A), thus suggesting that this subfamily of MAP kinases could be involved in the pathway for cPLA2 activation elicited by sPLA2.

**DISCUSSION**

We have selected 1321N1 astrocytoma cells to study the effect of sPLA2 because these cells do not express this enzyme, but do contain high amounts of cPLA2, which is the form of enzyme most usually involved in the release of [3H]AA coupled to receptor stimulation. Analysis of the physiological effects of sPLA2 indicates several possible mechanisms through which they might be exerted. One of them takes into account the lysosphospholipids 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_o, 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 adenyl 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 μM (45–47), and the effect of sPLA_2 on platelets incubated with lipid microspheres has been related to the production of LPA (14). Some of our findings agree with this mechanism of signalizing 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 different features, 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 [1-14C]oleate, but did show [3H]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 ~1 μg/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 [3H]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 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.

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