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Cytosolic phospholipase A_2 is coupled to hormonally regulated release of arachidonic acid

(Ca^{2+}/phosphorylation)

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ABSTRACT **Cytosolic phospholipase A_2 ($cPLA_2$) binds to natural membrane vesicles in a Ca^{2+}-dependent fashion, resulting in the selective release of arachidonic acid, thus implicating $cPLA_2$ in the hormonally regulated production of eicosanoids. Here we report that the treatment of Chinese hamster ovary (CHO) cells overexpressing $cPLA_2$ with ATP or thrombin resulted in an increased release of arachidonic acid as compared with parental CHO cells, demonstrating the hormonal coupling of $cPLA_2$. In contrast, CHO cells overexpressing a secreted form of mammalian PLA_2 ($sPLA_2$-II) failed to show any increased hormonal responsiveness. Interestingly, we have noted that the activation of $cPLA_2$ with a wide variety of agents stimulates the phosphorylation of $cPLA_2$ on serine residues. Pretreatment of cells with staurosporin blocked the ATP-mediated phosphorylation of $cPLA_2$ and strongly inhibited the activation of the enzyme. Increased $cPLA_2$ activity was also observed in lysates prepared from ATP-treated cells and was sensitive to phosphatase treatment. These results suggest that in addition to Ca^{2+}, the phosphorylation of $cPLA_2$ plays an important role in the agonist-induced activation of $cPLA_2$.**

Arachidonic acid is the rate-limiting precursor in the biosynthesis of two potent classes of inflammatory mediators, prostaglandins and leukotrienes (1, 2). The production of these two inflammatory mediators is initiated primarily by phospholipase A_2 (PLA_2), which hydrolyzes arachidonic acid from the *sn*-2 position of membrane phospholipids. Lysophospholipid, the other product of the reaction, can serve as a precursor for platelet-activating factor, another potent inflammatory mediator (3). Therefore, the activation of an arachidonic acid-selective PLA_2 serves as a key step in initiating an inflammatory response.

To date, much of the work on PLA_2 has focused on structural and mechanistic studies on a group of closely related secreted forms of PLA_2 ($sPLA_2$) (4–8). However, these enzymes fail to selectively hydrolyze arachidonyl-containing phospholipids, suggesting that their primary function is not to initiate the biosynthesis of prostaglandins and leukotrienes (9, 10). It is possible, however, that the secreted form $sPLA_2$-II may, in combination with secreted proteases, participate in tissue destruction at a site of inflammation.

Recently, several groups have reported the purification of an arachidonic acid-selective cytosolic PLA_2 ($cPLA_2$) (11–14) that translocates to natural membrane vesicles in response to submicromolar Ca^{2+} concentrations (15). This observation is of particular significance because many of the ligands that cause the rapid production of eicosanoids are Ca^{2+}-mobilizing agents. Several of these ligands such as bradykinin, histamine, ATP, and thrombin mediate their effects through guanine nucleotide-binding proteins (G proteins) (16–18). At present, however, it is unclear if G proteins directly interact with PLA_2 or if they modulate the function of an intermediary regulator of PLA_2.

PLA_2 can also be activated by growth factors that stimulate receptor tyrosine kinases, such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF). In response to the treatment of kidney mesangial cells with EGF, an increase in PLA_2 activity has been detected in both intact cells and lysates (19–21). These data suggested that the activity of a cytosolic PLA_2 might be affected by a stable modification such as phosphorylation.

In this report, we demonstrate that $cPLA_2$ mediates the ATP- and thrombin-stimulated release of arachidonic acid and show that the hormonal activation of $cPLA_2$ requires the synergistic actions of increased intracellular Ca^{2+} and $cPLA_2$ phosphorylation.

MATERIALS AND METHODS

Cell Culture. Chinese hamster ovary (CHO) cells were maintained in alpha medium (GIBCO) containing 50 units of penicillin per ml, 50 μg of streptomycin per ml, and 10% (vol/vol) fetal calf serum and supplemented with either 10 μg each of adenosine, deoxyadenosine, and thymidine per ml for the parental cells or 0.1 μM methotrexate for E5-CHO cells (22). Rat-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% fetal calf serum.

Release of Arachidonic Acid and Its Metabolites. Cells were plated into a 12-well cluster plate (Costar) at 1.5×10^5 cells per well in growth medium. After a 20-hr incubation (about 50–70% confluency), the medium was removed and replaced with 0.5 ml of alpha medium containing 0.5 μCi (18.5 kBq) of [^{3}H]arachidonic acid (NEN; 100 Ci/mmol) and incubated for another 20 hr at 37°C. Cells were then washed three times with medium containing 0.1% bovine serum albumin and incubated with various agents for the indicated time. Medium was removed, and radioactivity was determined by scintillation counting.

Isolation of Synovial Fluid $sPLA_2$ cDNA and an Overexpressing CHO Cell Line. Synovial fluid $sPLA_2$ cDNA was cloned from a cDNA library prepared from HEL cells, an erythroleukemia cell line, by the PCR method using the oligonucleotides complementary to the 5′ and 3′ ends of the published sequence (8). This DNA contained the identical sequence as reported and was transfected into CHO cells as described (15, 22). The medium collected from one of the transfectants, R1-CHO, contained 100-fold more PLA_2 activity than that of the parental CHO cells—i.e., 16 and 0.16 nmol/min per ml for R1-CHO and CHO, respectively, assayed essentially as described (11) except that 1-palmitoyl-

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Abbreviations: PLA_2, phospholipase A_2; $cPLA_2$, cytosolic PLA_2; $sPLA_2$, secretory PLA_2; PAP, potato acid phosphatase; TPA, phorbol 12-tetradecanoate 13-acetate; PDGF, platelet-derived growth factor; EGF, epidermal growth factor.

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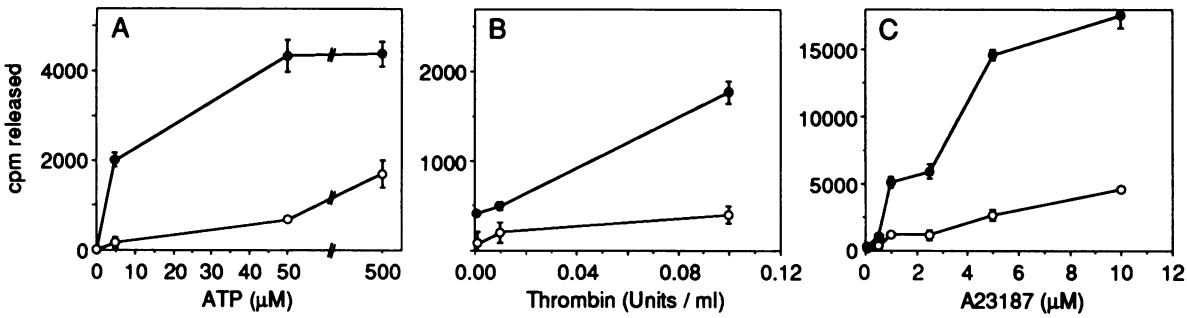

FIG. 1. E5-CHO cells (●) stimulated with ATP (*A*), thrombin (*B*), or A23187 (*C*) show enhanced release of [^{3}H]arachidonic acid as compared with CHO cells (○). Cells were seeded, labeled with [^{3}H]arachidonic acid, washed, and then treated with the indicated concentrations of ATP, thrombin, or A23187 for 15 min. Radioactivity released into the medium was determined; error bars indicate ± SEM. Background released from untreated cells (about 500 cpm) has been subtracted from the data.

2-[^{14}C]arachidonyl phosphatidylethanolamine instead of phosphatidylcholine was used as a substrate (11).

Phosphorylation of cPLA$_2$ and Immunoprecipitation. E5-CHO cells were grown in six-well plates to 50–70% confluency and starved in medium without serum for 20 hr. The medium was removed, replaced with 1 ml of phosphate-free DMEM containing 0.1% bovine serum albumin and 0.1 mCi of [^{32}P]orthophosphate, and incubated for 3–4 hr. Cells were then stimulated with various agents for 10 min at 37°C, lysed in 1 ml of buffer A (20 mM Tris, pH 8.0/100 mM NaCl/1 mM EDTA/0.5% Nonidet P-40/50 mM NaF/30 mM sodium pyrophosphate/0.2 mM sodium orthovanadate), and centrifuged. The supernatant was then immunoprecipitated with 10 μl of a cPLA$_2$ polyclonal antibody (15). For labeling Rat-2 cells, cells were seeded onto 10-cm plates, and 1 mCi of [^{32}P]orthophosphate per plate was used.

RESULTS AND DISCUSSION

ATP, Thrombin, and Ca^{2+} Ionophore Activate cPLA$_2$. ATP and thrombin have been shown to activate PLA$_2$ and to stimulate the release of arachidonic acid in CHO cells (18). We were interested in determining if this hormone-induced arachidonic acid release was mediated by cPLA$_2$. We reasoned that if the amount of cPLA$_2$ in CHO cells was a limiting factor in the ligand-induced arachidonic acid release, increased expression of cPLA$_2$ should result in increased arachidonic acid release. To test this hypothesis, E5-CHO cells, which overexpress cPLA$_2$ about 20- to 40-fold (15), were labeled with [^{3}H]arachidonic acid, and cPLA$_2$ activation was monitored by measuring the release of [^{3}H]arachidonic acid. The stimulation of either parental or E5-CHO cells with increasing concentrations of ATP or thrombin resulted in an increased release of [^{3}H]arachidonic acid in the E5-CHO cells as compared with the parental cells (Fig. 1 *A* and *B*). In response to 50 μM ATP, 6-fold more [^{3}H]arachidonic acid was released from the E5-CHO cells as compared with the parental cells. Similar results were obtained for E5-CHO cells treated with thrombin, where a 4-fold increase in [^{3}H]arachidonic acid release was noted. The increased responsiveness of the E5-CHO cells indicates that cPLA$_2$ mediates the hormone-induced arachidonic acid release. Many of the agents that stimulate the production of arachidonic acid are Ca^{2+}-mobilizing agents, including ATP and thrombin. Therefore, in an effort to mimic the effects of a Ca^{2+}-mobilizing ligand, cells were stimulated with the Ca^{2+} ionophore A23187. The incubation of cells with increasing concentrations of A23187 resulted in about a 5-fold increase in the production of [^{3}H]arachidonic acid in the E5-CHO cells as compared with the parental CHO cells (Fig. 1*C*), indicating that cPLA$_2$ mediates the Ca^{2+}-stimulated release of arachidonic acid.

Overexpression of sPLA$_2$ Fails to Increase Hormonal Responsiveness. To determine if a secreted form of PLA$_2$, which was originally isolated from the synovial fluid of rheumatoid arthritis patients (sPLA$_2$-II) (8), was also capable of mediating the hormonally stimulated release of arachidonic acid, we developed a CHO cell line, R1-CHO, that overexpressed this enzyme 100-fold (see *Materials and Methods*). Stimulation of R1-CHO cells with ATP, thrombin, or A23187 failed to result in an increased release of arachidonic acid relative to the parental CHO cells (Fig. 2), indicating that in these cells, sPLA$_2$-II is not coupled to ATP-, thrombin-, or A23187-stimulated release of arachidonic acid.

Agonist-Induced Phosphorylation of cPLA$_2$. In addition to the well-documented role of increased intracellular Ca^{2+} in activating PLA$_2$, several investigators have speculated that the activity of a cPLA$_2$ may be increased by phosphorylation (19–21). To determine if cPLA$_2$ is phosphorylated in response to various stimuli, E5-CHO cells were labeled with [^{32}P]orthophosphate and stimulated with ATP or phorbol 12-tetradecanoate 13-acetate (TPA) for 10 min. TPA and ATP caused about a 2-fold increase in the incorporation of ^{32}P into cPLA$_2$ (Fig. 3 *Upper*). Phosphoamino acid analysis indicated that both TPA and ATP treatment caused an increase in the phosphorylation of serine residues of cPLA$_2$ but no detectable phosphorylation of tyrosine or threonine residues (Fig. 3 *Lower*). In a separate experiment, thrombin treatment of E5-CHO cells also stimulated the phosphorylation of cPLA$_2$, exclusively on serine residues (Fig. 3 *Lower*).

PDGF and EGF, which activate receptor tyrosine kinases, can also stimulate PLA$_2$ and cause the release of arachidonic acid. Therefore, we were interested in determining if these agents could stimulate phosphorylation of tyrosine residues

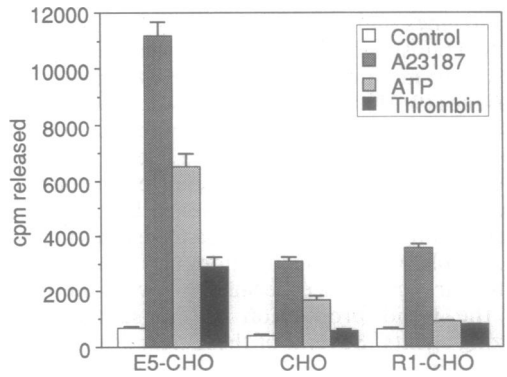

FIG. 2. CHO cells overexpressing synovial fluid sPLA$_2$ (R1-CHO) fail to show enhanced release of [^{3}H]arachidonic acid as compared with CHO cells. Cells labeled with [^{3}H]arachidonic acid were treated with medium only (control), 1 μM A23187, 50 μM ATP, or 0.1 unit of thrombin per ml for 15 min. Radioactivity released was then determined.

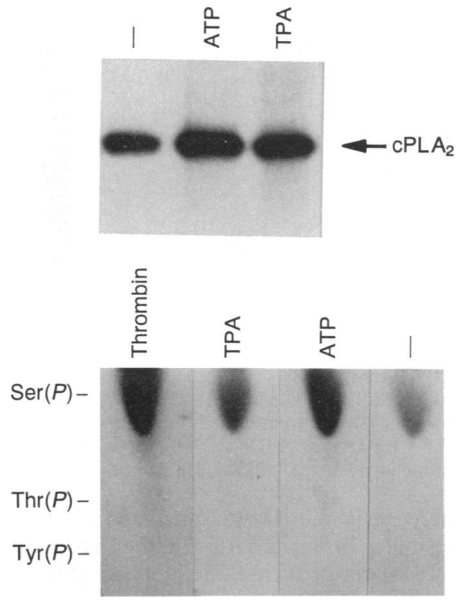

FIG. 3. ATP and TPA stimulate the phosphorylation of $cPLA_2$ on serine residues. (*Upper*) E5-CHO cells were labeled with [^{32}P]orthophosphate and then incubated with medium only (first lane), 1 μM TPA, or 50 μM ATP for 10 min. Cells were lysed and immunoprecipitated with a $cPLA_2$ antiserum. Immunoprecipitated material was electrophoresed on a SDS/7.5% polyacrylamide gel, which was exposed to XAR film at −70°C for 16 hr. (*Lower*) Phosphoamino acid analysis was done on the $cPLA_2$ band excised from the gel in *Upper* as described (23, 24) and then exposed to XAR film with an intensifying screen at −70°C for 7 days. Ser(*P*), Thr(*P*), and Tyr(*P*), phosphoserine, phosphothreonine, and phosphotyrosine.

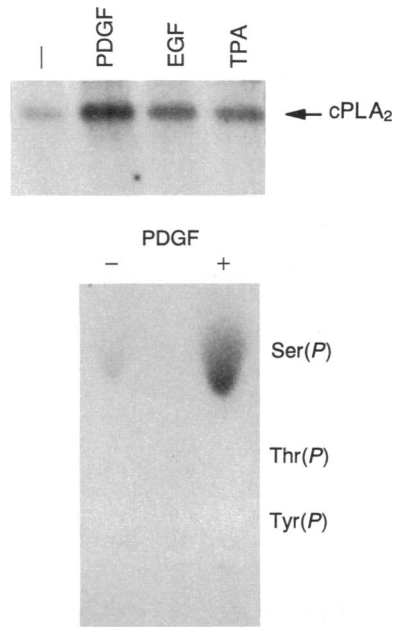

FIG. 4. PDGF-, EGF-, and TPA-stimulated phosphorylation of serine residues of $cPLA_2$ in Rat-2 cells. (*Upper*) Rat-2 cells labeled with [^{32}P]orthophosphate were treated with 50 ng of PDGF per ml, 150 ng of EGF per ml, or 1 μM TPA for 10 min. Cells were then lysed, and contents were immunoprecipitated with a $cPLA_2$ specific antiserum. The immunoprecipitates were separated on a SDS/7.5% polyacrylamide gel and exposed to XAR film at −70°C for 48 hr. (*Lower*) The ^{32}P-labeled $cPLA_2$ band in *Upper* was transferred to an Immobilon membrane, and a phosphoamino acid analysis was performed. Abbreviations are as in Fig. 3 *Lower*.

of $cPLA_2$. To test for this, Rat-2 cells, which release arachidonic acid in response to EGF or PDGF treatment, were labeled with ^{32}P and stimulated with PDGF, EGF, or (as a control) TPA. The ^{32}P content of $cPLA_2$ from the treated cells increased 2- to 4-fold as compared with the untreated cells (Fig. 4 *Upper*). Interestingly, when phosphoamino acid analysis of the ^{32}P-labeled $cPLA_2$ from PDGF-treated cells was performed, only phosphoserine was detected (Fig. 4 *Lower*). Similar results were obtained with $cPLA_2$ derived from EGF- or TPA-treated cells (data not shown). Therefore, $cPLA_2$ is not a direct substrate for the PDGF or EGF receptor tyrosine kinase but must be phosphorylated by a growth factor-activated serine/threonine kinase(s). At present, the identity of the kinase(s) that directly phosphorylates $cPLA_2$ is unknown. Interestingly however, $cPLA_2$ contains consensus phosphorylation sites for the mitogen-activated protein kinase 2 (MAP-2)-related kinase and protein kinase C (15, 25).

Immunoblot analysis of $cPLA_2$ derived from resting E5-CHO cells revealed that $cPLA_2$ migrated as a doublet, with about 20% of the total $cPLA_2$ being present in the upper band (Fig. 5 *Upper*). Interestingly, when cells were treated with ATP, 100% of the $cPLA_2$ comigrated with the upper band. To determine if the phosphorylation of $cPLA_2$ caused the decreased mobility, the lysate derived from ATP-treated cells was incubated with PAP, which resulted in the loss of the more slowly migrating species and the regeneration of the more rapidly migrating species, indicating that the generation of this more slowly migrating species was due to the phosphorylation of $cPLA_2$. Using this gel shift as a measure of phosphorylation, we show in Fig. 5 *Lower* that ATP treatment resulted in nearly 100% of the $cPLA_2$ becoming phosphorylated within 2 min, consistent with the rapid release of arachidonic acid in response to ATP (data not shown). Interestingly, in addition to ATP treatment, thrombin or TPA also caused 100% of the $cPLA_2$ to comigrate with the upper band observed in resting cells (Fig. 5 *Upper*). Treatment of E5-CHO cells with A23187 also caused the appearance of 50% $cPLA_2$ as the more slowly migrating species, indicating that A23187 treatment activates a kinase that phosphorylates $cPLA_2$. Therefore, four of the agents that have shown to activate $cPLA_2$ all cause the phosphorylation of $cPLA_2$, suggesting that the phosphorylation of $cPLA_2$ may be associated with its activation.

Increased Activity of Phosphorylated $cPLA_2$. In view of our observations that agents that stimulate the activity of $cPLA_2$ also cause increased phosphorylation of $cPLA_2$, we were interested in determining if the phosphorylation of $cPLA_2$ alters its activity in an *in vitro* liposome assay. In Table 1, we show that the PLA_2 activity present in the lysate derived from ATP-treated E5-CHO cells was increased 2-fold as compared with untreated cells. Importantly, when the lysates were treated with PAP, the PLA_2 activities in the lysates derived from both the stimulated and unstimulated cells were nearly equal, suggesting that the phosphorylation of $cPLA_2$ increases its specific activity by about 2-fold.

TPA Synergizes with A23187 to Activate $cPLA_2$. Treatment of cells with TPA, a direct activator of protein kinase C, results in the stoichiometric phosphorylation of $cPLA_2$ (Fig. 5 *Upper*) but only a slight stimulation of arachidonate release. However, it has been shown in a variety of cells that TPA treatment followed by the addition of a Ca^{2+} ionophore results in a greater-than-additive effect of the two agents (26, 27). This synergistic effect of TPA can be referred to as "priming." To determine if TPA primes $cPLA_2$ to respond to A23187, we analyzed the abilities of both parental and E5-CHO cells to release arachidonate in response to A23187 with or without prior exposure to TPA (Fig. 6). With the parental cell line, treatment with TPA or a suboptimal dose of A23187 resulted in <2-fold and about a 3-fold increase in production of arachidonate, respectively. However, pre-

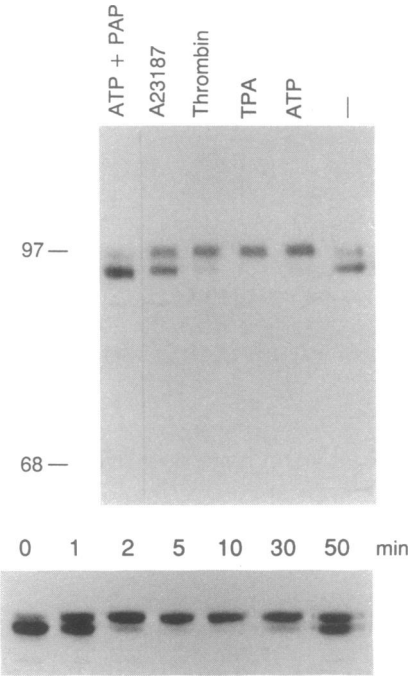

FIG. 5. Agonist-mediated mobility shift of $cPLA_2$. (*Upper*) E5-CHO cells were treated with 50 μM ATP, 1 μM TPA, 0.1 unit of thrombin per ml, or 1 μM A23187 for 10 min. Ten micrograms of protein from cell lysate were subjected to SDS/7.5% PAGE, transferred to nitrocellulose, immunoblotted with a polyclonal $cPLA_2$ antibody, and developed by using the Amersham ECL system. To observe the phosphorylation-induced gel shift, the electrophoresis was run for an additional 2 hr at 250 V after the bromophenol blue dye reached the bottom of the gel. The "ATP+PAP" sample was prepared by treating the cell lysate from ATP-treated cells with potato acid phosphatase (PAP). Molecular weight is shown $\times 10^{-3}$. (*Lower*) E5-CHO cells were treated with 50 μM ATP for the indicated time, and the lysates were immunoblotted as described in *Upper*.

treatment with TPA for 5 min followed by A23187 resulted in a 10-fold increase in arachidonate release. Similar synergies were observed for the E5-CHO cells, except that the overall arachidonate release in all cases was amplified. The enhanced responsiveness of these cells to TPA/A23187 suggests that TPA treatment primes $cPLA_2$ to respond to A23187, causing

Table 1. Comparison of PLA_2 activity from control and ATP-treated cells using liposome assay

	PLA_2 activity pmol/min per mg
Cell lysate	
Control	46.8 ± 1.9
ATP	104.5 ± 2.8
Cell lysate + PAP	
Control	56.6 ± 4.4
ATP	61.0 ± 0.2

E5-CHO cells were incubated with or without 50 μM ATP at 37°C for 10 min and lysed by sonication. Sixty micrograms of protein from total lysate was assayed as described (20) with some modifications. Substrate, 1-stearoyl-2-[^{14}C]arachidonyl phosphatidylcholine (Amersham) was dried down under N_2 and resuspended in dimethyl sulfoxide by vigorous mixing for 2 min. Substrate was then diluted to the final concentration of 1 μM in 10 mM Hepes buffer (pH 7.4), and Ca^{2+} was added to 5 mM. The reaction mixtures were incubated at 37°C for 30 min. The lysate from untreated cells liberated about 5300 cpm, while the lysate derived from ATP-treated cells liberated ≈14,000 cpm (blank was about 100 cpm). +PAP, cell lysate was treated with 2 μg of PAP at 30°C for 30 min. These data consist of duplicate assays from three separate experiments. Error bars indicate ± SEM.

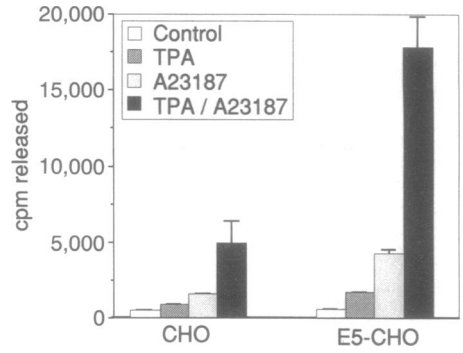

FIG. 6. Synergistic effect of TPA on A23187-induced release of [^{3}H]arachidonate from E5-CHO and CHO cells. Cells labeled with [^{3}H]arachidonic acid were treated with medium only (control) for 15 min, 1 μM TPA for 15 min, 1 μM A23187 for 10 min, or 1 μM TPA for 5 min followed by 1 μM A23187 for 10 min (TPA/A23187).

a 3-fold increase in the A23187-mediated response. These results are similar to our *in vitro* finding that the phosphorylation of $cPLA_2$ results in a 2-fold increase in the activity of the enzyme. We suggest that the phosphorylation of $cPLA_2$ causes a 2- to 3-fold increase in the intrinsic activity of the enzyme that can be realized only when the enzyme associates with its substrate, which we have previously shown to be a Ca^{2+}-dependent process (15).

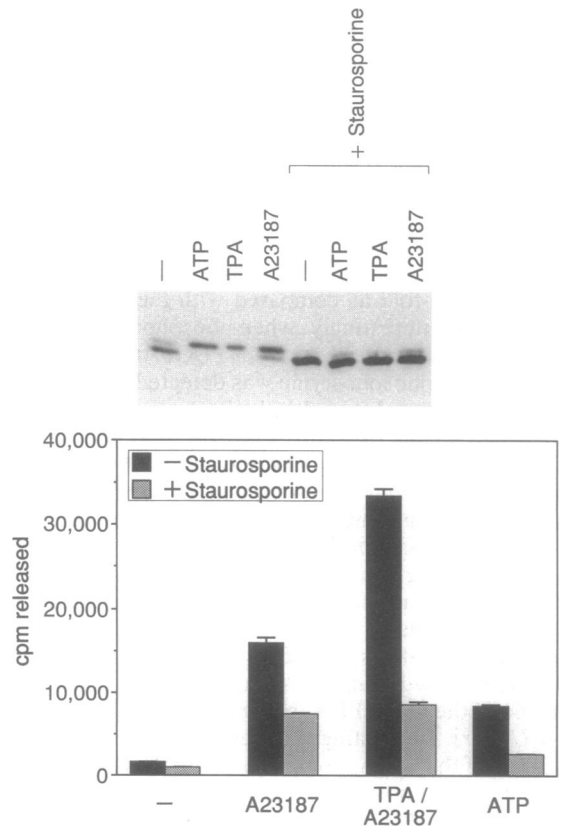

FIG. 7. Staurosporin inhibition of $cPLA_2$ phosphorylation and arachidonic acid release. (*Upper*) E5-CHO cells were treated with 50 μM ATP, 1 μM TPA, or 1 μM A23187 for 15 min, or were preincubated first with 1 μM staurosporin for 20 min. Cell lysates were immunoblotted with $cPLA_2$ specific antisera as described in Fig. 5 *Upper*. (*Lower*) E5-CHO cells labeled with [^{3}H]arachidonic acid were either preincubated with 1 μM staurosporin (stippled bar), or control medium (solid bar) for 20 min and then treated with 1 μM A23187 for 10 min, 1 μM TPA for 5 min followed by 1 μM A23187 for 10 min (TPA/A23187), or 50 μM ATP for 15 min, and radioactivity released into the medium was determined.

Staurosporin Blocks Agonist-Induced $cPLA_2$ Phosphorylation and Activation. To determine the importance of the hormone-mediated phosphorylation of $cPLA_2$ in the activation of $cPLA_2$, the effect of a protein kinase inhibitor, staurosporin (28), was examined. Pretreatment of E5-CHO cells with staurosporin inhibited the ATP-, TPA-, and A23187-induced phosphorylation of $cPLA_2$ (Fig. 7 *Upper*). Importantly, the staurosporin pretreatment also completely blocked the priming effect of TPA on A23187-induced arachidonate release and strongly inhibited the ATP- and A23187-induced arachidonate release (80% and 50% inhibition, respectively), further supporting the suggestion that the phosphorylation of $cPLA_2$ is an important component of the agonist-mediated stimulation of $cPLA_2$ (Fig. 7 *Lower*).

Previously we have suggested that the Ca^{2+}-dependent association of $cPLA_2$ with membrane provided a simple mechanism for the regulation of $cPLA_2$ activity by Ca^{2+}-mobilizing agents. Here we have extented this model by demonstrating the importance of both a rise in intracellular Ca^{2+} and $cPLA_2$ phosphorylation as synergistic mechanisms for regulating the hormonal activation of $cPLA_2$. Moreover, the coupling of $cPLA_2$ to hormonally regulated release of arachidonic acid suggests that the activation of $cPLA_2$ is likely to be an essential step in the initiation of the inflammatory cascade. Therefore, $cPLA_2$ is an important target for the development of antiinflammatory drugs that act at a very early step in the biosynthesis of a number of potent inflammatory mediators.

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