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Protein kinase C-dependent activation of cytosolic phospholipase A2 and mitogen-activated protein kinase by alpha 1-adrenergic receptors in Madin-Darby canine kidney cells.

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Research Article

We have characterized the mechanism whereby a G protein-coupled receptor, the alpha 1-adrenergic receptor, promotes cellular AA release via the activation of phospholipase A2 (PLA2) in Madin-Darby canine kidney (MDCK-D1) cells. Stimulation of cells with the receptor agonist epinephrine or with the protein kinase C (PKC) activator PMA increased AA release in intact cells and the activity of PLA2 in subsequently prepared cell lysates. The effects of epinephrine were mediated by alpha 1-adrenergic receptors since they were blocked by the alpha 1-adrenergic antagonist prazosin. Epinephrine- and PMA-promoted AA release and activation of the PLA2 were inhibited by AACOCF3, an inhibitor of the 85-kD cPLA2. The 85-kD cPLA2 could be immunoprecipitated from the cell lysate using a specific anti-cPLA2 serum. Enhanced cPLA2 activity in cells treated with epinephrine or PMA could be recovered in such immunoprecipitates, thus directly demonstrating that alpha 1-adrenergic receptors activate the 85-kD cPLA2. Activation of cPLA2 in cell lysates by PMA or epinephrine could be reversed by treatment of lysates with exogenous phosphatase. In addition, both PMA and epinephrine induced a molecular weight shift, consistent with phosphorylation, as well as an increase in activity of mitogen-activated protein (MAP) kinase. The time course of epinephrine-promoted activation of MAP kinase preceded that of the accumulation of released AA and correlated with the time course of cPLA2 activation. Down-regulation of […]

Protein Kinase C–dependent Activation of Cytosolic Phospholipase A_2 and Mitogen-activated Protein Kinase by Alpha$_1$-Adrenergic Receptors in Madin-Darby Canine Kidney Cells

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Abstract

We have characterized the mechanism whereby a G protein–coupled receptor, the α_1-adrenergic receptor, promotes cellular AA release via the activation of phospholipase A_2 (PLA_2) in Madin-Darby canine kidney (MDCK-D1) cells. Stimulation of cells with the receptor agonist epinephrine or with the protein kinase C (PKC) activator PMA increased AA release in intact cells and the activity of PLA_2 in subsequently prepared cell lysates. The effects of epinephrine were mediated by α_1-adrenergic receptors since they were blocked by the α_1-adrenergic antagonist prazosin. Epinephrine- and PMA-promoted AA release and activation of the PLA_2 were inhibited by $AACOCF_3$, an inhibitor of the 85-kD $cPLA_2$. The 85-kD $cPLA_2$ could be immunoprecipitated from the cell lysate using a specific anti-$cPLA_2$ serum. Enhanced $cPLA_2$ activity in cells treated with epinephrine or PMA could be recovered in such immunoprecipitates, thus directly demonstrating that α_1-adrenergic receptors activate the 85-kD $cPLA_2$. Activation of $cPLA_2$ in cell lysates by PMA or epinephrine could be reversed by treatment of lysates with exogenous phosphatase. In addition, both PMA and epinephrine induced a molecular weight shift, consistent with phosphorylation, as well as an increase in activity of mitogen-activated protein (MAP) kinase. The time course of epinephrine-promoted activation of MAP kinase preceded that of the accumulation of released AA and correlated with the time course of $cPLA_2$ activation. Down-regulation of PKC by overnight incubation of cells with PMA or inhibition of PKC with the PKC inhibitor sphingosine blocked the stimulation of MAP kinase by epinephrine and, correspondingly, epinephrine-promoted AA release was inhibited under these conditions. Similarly, blockade of MAP kinase stimulation by the MAP kinase cascade inhibitor PD098059 inhibited epinephrine-promoted AA release. The sensitivity to Ca^{2+} was similar, although the maximal activity of $cPLA_2$ was enhanced by treatment of cells with epinephrine or PMA. The data thus demonstrate that in MDCK-D1 cells α_1-adrenergic receptors regulate AA release through phosphorylation-dependent activation of the 85-kD $cPLA_2$ by MAP kinase subsequent to activation of PKC. This may represent a general mechanism by which G protein–coupled receptors stimulate AA release and formation of products of AA metabolism. (*J. Clin. Invest.* 1996. 97:1302–1310.) Key words: arachidonic acid • G protein–coupled receptor • phosphorylation • renal epithelium • calcium

Introduction

AA and its eicosanoid metabolites (e.g., prostaglandins and leukotrienes) play critical roles in the initiation or modulation of a broad spectrum of physiological responses and certain abnormal (e.g., inflammatory) processes in mammalian cells (1, 2). This fatty acid is not freely stored in cells but is esterified to cellular phospholipids, mainly at the *sn*-2 position. Its release can be catalyzed by phospholipase A_2 (PLA_2)[1] and is believed to be the limiting step in the biosynthesis of eicosanoids in response to stimulation by receptors such as G protein–coupled receptors. Three groups of mammalian PLA_2s have been characterized, namely, the 14-kD Ca^{2+}-dependent secreted PLA_2s, the 85-kD Ca^{2+}-dependent and *sn*-2 arachidonyl-specific cytosolic PLA_2 ($cPLA_2$), and the Ca^{2+}-independent PLA_2s (3, 4). Overexpression of Chinese hamster ovary cells with recombinant $cPLA_2$ enhanced AA release stimulated by ATP or thrombin receptors (5). However, little definitive evidence is available for the coupling of the native $cPLA_2$ to receptors, particularly G protein–coupled receptors, although it has been proposed, largely based on indirect evidence, that the $cPLA_2$ is responsible for G protein–coupled receptor-mediated AA release (3, 6). Furthermore, more recent studies have suggested that the 14-kD secreted group II PLA_2 (7, 8), the calcium-independent PLA_2 (9), and a 29-kD cytosolic PLA_2 (10) could each be responsible for AA release mediated by receptors.

Numerous studies have implicated the involvement of protein kinase C (PKC) in the regulation of receptor-mediated AA release in a variety of cells (3, 6, 11). Nevertheless, in vitro studies have failed to consistently show direct phosphorylation-dependent activation of $cPLA_2$ by PKC (12–14). Because mitogen-activated protein (MAP) kinase, which has been shown in vitro to phosphorylate and activate the recombinant $cPLA_2$ (12, 13), can be stimulated in cells through both PKC-dependent and independent pathways, it has been proposed that the PKC-dependent activation of $cPLA_2$ is via the activation of MAP kinase (12). However, incomplete information is available regarding the relationship of the activation of PKC and MAP kinase with that of the endogenous $cPLA_2$ by G protein–coupled receptors in native cells, although activation of

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1. *Abbreviations used in this paper:* $cPLA_2$, cytosolic PLA_2; MAP, mitogen-activated protein; MBP, myelin basic protein; MDCK, Madin-Darby canine kidney; PKC, protein kinase C; PLA_2, phospholipase A_2.

each of these enzymes has been separately studied in many reports. In fact, MAP kinase stimulation and Ca^{2+} mobilization promoted by G protein–coupled P_{2U} receptors fail to stimulate $cPLA_2$-mediated AA release in undifferentiated HL60 cells (15). Furthermore, in Chinese hamster ovary cells a G_{i2} α mutant inhibits G protein–coupled P_2-purinergic receptor- or thrombin receptor–promoted AA release by $cPLA_2$ while not altering Ca^{2+} mobilization, MAP kinase activation, and phosphorylation of $cPLA_2$ (16). Thus, the role of MAP kinase, and its relationship with PKC, in the regulation of the endogenous $cPLA_2$ by G protein–coupled receptors need to be further defined in native cells.

$Alpha_1$-adrenergic receptors are an important class of the G protein–coupled receptors. They play fundamental roles in the regulation of a wide variety of cardiovascular, renal, and metabolic functions (17). These receptors are also coupled to release of AA and eicosanoids in many cells. Although some evidence, such as assessment of lysophospholipid formation, has suggested that PLA_2 is involved (18), no data has directly defined which type of PLA_2, if any, mediates α_1-adrenergic receptor-promoted AA release in cells. Moreover, the specific mechanism(s) regulating this receptor-promoted activation of PLA_2, especially in terms of involvement of protein kinases, has not been defined. In the present study with Madin-Darby canine kidney (MDCK)-D1 cells, we investigated the molecular mechanism for the regulation of AA release by α_1-adrenergic receptors. We demonstrate that α_1-adrenergic receptors stimulate AA release in MDCK-D1 cells by phosphorylation-dependent activation of the 85-kD $cPLA_2$, which involves activation of PKC and MAP kinase.

Methods

Materials. Leupeptin, pepstatin A, A23187, PMA, $AACOCF_3$, and PMSF were purchased from Calbiochem Corp. (La Jolla, CA). DTT was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). [5,6,8,9,11,12,14,15-3H(N)]-arachidonic acid ([^{3}H]AA) (sp act, 100 Ci/mmol) and [γ-^{32}P]ATP (sp act, 3,000 Ci/mmol) were obtained from DuPont NEN (Boston, MA). 1-stearoyl-2-[1-^{14}C]arachidonyl-L-3-phosphatidylcholine ([^{14}C]PC) (sp act, 55 mCi/mmol), horseradish peroxidase–linked donkey anti–rabbit Ig and ECL Western blotting detection reagents were bought from Amersham Corp. (Arlington Heights, IL). Potato acid phosphatase, Na_3VO_4, sodium pyrophosphate, levamisole, protein A-Sepharose, benzamidine, myelin basic protein (MBP), diisopropyl fluorophosphate, PBS, arachidonic acid, and (-) epinephrine were purchased from Sigma Chemical Co. (St. Louis, MO). Okadaic acid was obtained from Gemini Bio-Products, Inc. PKI (6-22 amide), a protein kinase A inhibitor, was bought from Gibco-BRL (Gaithersburg, MD). Prazosin hydrochloride was bought from Pfizer. P-81 phosphocellulose paper was from Whatman Inc. (Clifton, NJ). Immobilon-P PVDF transfer membrane (0.45 μM) was purchased from Millipore Corp. (Bedford, MA). TLC silica gel plates were bought from Analtech. Rabbit anti-p42-MAP kinase serum was originally generated and obtained from the laboratory of Dr. Michael J. Dunn (19). Standard 85-kD $cPLA_2$ protein and its specific antiserum were from Dr. Lih-ling Lin (Genetics Institute, Cambridge, MA) (5). PD098059 was from Dr. Alan R. Saltiel (Parke-Davis, Ann Arbor, MI) (20).

Cell culture. MDCK-D1 cells were cultured as previously described (21). Subconfluent cells were subcultured every 3–4 d by trypsinization using trypsin/EDTA. Cells at 60–80% confluence usually achieved 3 d after the subculture were normally used for experiments.

[^{3}H]AA release in intact cells. After labeling with 0.5 μCi [^{3}H]AA/ml per well for 20 h in a 24-well plate, cells were washed four times with serum- and $NaHCO_3$-free DME supplemented with 5 mg/ml BSA and 20 mM Hepes, pH 7.4, and incubated in the same medium at 37°C for 15 min to equilibrate the temperature. Stimulation of cells was then started by replacing the medium with 1 ml of 37°C medium containing the specified agonists. After a 10-min incubation in a 37°C water bath with constant agitation, the stimulation was stopped by aspirating the incubation medium and transferring it to ice-cold tubes containing 100 μl of 55 mM EGTA and EDTA (final concentration, 5 mM each). The medium was then subjected to centrifugation to eliminate cell debris, and the radioactivity in the supernatant was determined by scintillation spectrophotometry. Cells left attached to the plate were scraped with 0.2% Triton-X100 and also counted for radioactivity. The release of [^{3}H]AA was normalized as percentage of the total prestimulation incorporated radioactivity (the total released radioactivity plus the total cell-associated radioactivity at the end of stimulation) for the comparison of different treatment conditions.

In vitro $cPLA_2$ activity assay using cell lysates. Cells cultured in 75-cm^2 flasks were washed four times with serum- and $NaHCO_3$-free DME supplemented with 2 mg/ml BSA and 20 mM Hepes, pH 7.4, followed by incubation with the same medium for 2 h at 37°C. Stimulation was started by adding the specified agonists to the cells and, after 5–10 min, stopped by rapidly aspirating away the incubating medium and replacing it with an ice-cold washing buffer containing 250 mM sucrose, 50 mM Hepes, pH 7.4, 1 mM EGTA, 1 mM EDTA, phosphatase inhibitors (200 μM Na_3VO_4, 1 mM levamisole), and protease inhibitors (500 μM PMSF, 8 μM pepstatin A, 16 μM leupeptin, and 1 mM diisopropyl fluorophosphate). Cells were washed four times with ice-cold washing buffer and then were scraped into an ice-cold assay buffer that was the same as the washing buffer except that sucrose was omitted, but the buffer was supplemented with 100 nM okadaic acid. The scraped cells were then homogenized by sonication, followed by centrifugation at 4°C for 10 min at 500 *g* to eliminate the unbroken cells. The supernatants, defined as cell lysates, were used for $cPLA_2$ activity assay, using a previously described protocol with some modifications (5). Briefly, the substrate [^{14}C]PC was dried under nitrogen, resuspended in DMSO, vigorously shaken (vortex) for 2 min, and resuspended in the assay buffer containing 10 mM $CaCl_2$. The reaction was started by adding 100 μl cell lysate to an equal volume of 37°C substrate in an agitating water bath. The final concentrations of the components in the assay were 10 μM [^{14}C]PC, 5 mM $CaCl_2$, 1 mM EGTA, 1 mM EDTA, 50 mM Hepes, pH 7.4, and 10–30 μg protein (measured with the Bradford assay kit; Bio-Rad Laboratories, Richmond, CA). Unless otherwise specified, 5 mg/ml BSA and 1 mM DTT were included in the final assay. After incubation for 30–40 min, the reaction was stopped by adding 750 μl of 1:2 (vol:vol) chloroform/methanol. The total lipids were then extracted following the method of Bligh and Dyer (22) and subjected to TLC, as previously described (15), using as running solvent the upper phase of the mixture of ethyl acetate/isooctane/water/acetic acid (33:45:60:6, vol/vol). The TLC plates were stained with iodine and the bands containing [^{14}C]AA that comigrated with AA standards were scraped and counted. The activity of PLA_2 was normalized as picomoles of hydrolyzed substrate/min per milligram cell lysate protein. Under these conditions, less than 3-5% of the substrates were normally hydrolyzed.

Immunoprecipitation of the 85-kD $cPLA_2$. Cell lysates were prepared as described for the in vitro $cPLA_2$ activity assay. After the protein concentrations were matched for different samples, 400–700 μg cell lysate in 0.5 ml assay buffer (the same buffer as described above for $cPLA_2$ activity assay) was supplemented with 1–2 μl of normal rabbit serum or anti–85-kD $cPLA_2$ rabbit serum and 1% NP-40, followed by incubation at 4°C with agitation for 1 h. The mixtures were then transferred to a microcentrifuge tube containing 24 mg protein A–Sepharose, which was precoated with 3% BSA for 2–3 h at 4°C. After a 1-h incubation at 4°C with agitation, the antigen-antibody-protein A complex was precipitated by centrifugation in an Eppendorf microcentrifuge. The resultant pellets were washed three times by repeated centrifugation and resuspending in new assay buffer con-

taining 1% N-P40, followed by two more washings in NP-40-free buffer. The pellets were finally resuspended either in SDS-loading buffer for SDS-PAGE and Western blotting or in the assay buffer supplemented with 5 mM DTT for the $cPLA_2$ activity assay.

Phosphorylation-induced mobility shift, SDS-PAGE, and Western blotting of MAP kinase. Cells cultured in a 6-well plate were washed four times with serum- and $NaHCO_3$-free DME supplemented with 2 mg/ml BSA and 20 mM Hepes, pH 7.4, and incubated for 2 h at 37°C in the same medium, followed by stimulation with specified agonists for indicated times. The stimulation was stopped by quickly aspirating the medium and washing the cells four times with an ice-cold solution consisting of 10% glycerol, 62.5 mM Tris-HCl, pH 6.8, and protease and phosphatase inhibitors as described above. Cells were then scraped and lysed into SDS-PAGE loading buffer, followed by heating for 5 min at 100°C. Samples were then subjected to SDS-PAGE using either 7.5 or 10% acrylamide, with the former concentration of acrylamide requiring shorter time to run the gel and the latter requiring longer time, followed by transfer to Imobilon-P PVDF membrane. After being blocked for 1 h with 5% nonfat dry milk dissolved in PBS, the membrane carrying the proteins was sequentially incubated with 1:2,000–3,000 diluted anti-p42 MAP kinase rabbit serum for 1.5 h and with 1:2,000 diluted horseradish peroxidase–linked donkey anti–rabbit Ig for 1 h, both in 5% nonfat dry milk dissolved in PBS. Each antibody incubation was followed by washing three to four times with PBS for 5–10 min. The bands of MAP kinase in the membrane, including the mobility-shifted species due to phosphorylation, were visualized using the ECL Western blotting detection reagents following the manufacturer's instructions.

Immunoprecipitation and activity assay of MAP kinase. Immunoprecipitation and activity assay of MAP kinase were performed using a modified version of several previously published protocols (16, 23, 24). Cells cultured in 75-cm^2 flasks were washed four times with serum- and $NaHCO_3$-free DME supplemented with 2 mg/ml BSA and 20 mM Hepes, pH 7.4, followed by a 2-h incubation at 37°C in the same medium. Cells were then stimulated for 3 min with indicated agonists. The stimulation was stopped by quickly aspirating away the medium and washing the cells four times with ice-cold PBS supplemented with 2 mM EGTA, 1 mM EDTA, 1 mM benzamidine, 5 mM sodium pyrophosphate, and other protease and phosphatase inhibitors as specified above for $cPLA_2$ assay. Cells were then scraped into MAP kinase buffer consisting of 30 mM β-glycerophosphate, 20 mM Hepes, pH 7.4, 2 mM EGTA, 1 mM EDTA, and the protease and phosphatase inhibitors as described above for the washing solution. The scraped cells were disrupted by sonication and adjusted to the same protein concentrations for different samples before immunoprecipitation. 400 μg cell lysate protein in 500 μl MAP kinase buffer was supplemented with 5 μl NP-40 (1% final) and 1 μl anti-p42 MAP kinase serum and incubated at 4°C with constant agitation for 1 h. The antigen-antibody mixture thus formed was then transferred to tubes containing 24 mg protein A–Sepharose (precoated at 4°C with 3% BSA for 2–3 h before use) and incubated at 4°C with agitation for 1 h, followed by centrifugation in a microcentrifuge to precipitate the antigen-antibody-protein A complex. The pellet was sequentially washed three times with MAP kinase buffer supplemented with 1% NP-40, and three times with the same buffer without detergent. The immunoprecipitates were resuspended in 40 μl MAP kinase buffer supplemented with 2 mM DTT and 4 μM PKI (6–22 amide). To start the assay for MAP kinase activity, 10 μl of the resuspended immunoprecipitate was added to an equal volume of substrates and $MgCl_2$ in MAP kinase buffer prewarmed at 30°C, generating (final concentrations) 10 mM $MgCl_2$, 2 μM PKI (6-22 amide), 40 μM ATP, 2 μCi [γ-^{32}P]ATP and 10 μg MBP. After a 20-min incubation at 30°C with constant agitation, the reaction was stopped by spotting 10 μl of the reaction mixture to P81 phosphocellulose membrane (2 × 1.5 cm), followed by washing six times for 10 min each in 125 mM phosphoric acid. The radioactivity associated with the membrane, which represented the phosphorylation of MBP by MAP kinase, was determined by scintillation spectrophotometry. Alternatively, the MAP kinase reaction was terminated by adding equal volume of twofold concentrated Laemmli's buffer and heating the mixture for 5 min, followed by SDS-PAGE and autoradiography. The major band corresponding to MBP on the exposed film was recognized, and its intensity represented MAP kinase activity.

Preparation of calcium-EGTA buffer. Desired concentration of free calcium ions in the PLA_2 assay buffer was obtained by adding appropriate amount of $CaCl_2$ to the buffer containing 1 mM EGTA and 1 mM EDTA, based on the calculation using the FREECA computer program (25).

Data presentation. Unless otherwise specified, the data shown in the figures are mean±SD of triplicate or duplicate measurements and are representative of results obtained in two to five experiments.

Results

α_1-adrenergic receptors mediate AA release through the 85-kD $cPLA_2$. MDCK-D1 cells are a subclone derived from parental MDCK cells, an epithelial cell line derived from distal tubule/collecting duct of the canine kidney (26). These cells possess a single population of α_1-adrenergic receptors, namely the α_{1b} type, and these receptors are coupled to AA release (Fig. 1 *A* and references. 21, 27, 28). To determine whether this AA release is secondary to activation of $cPLA_2$, we initially established conditions to assay the activation of this enzyme in cell lysates prepared from agonist-stimulated cells. Treatment of the cells with epinephrine increased the PLA_2 activity in the subsequently prepared cell lysates (Fig. 1 *B*). The α_1-adrenergic antagonist prazosin not only inhibited epinephrine-triggered AA release in intact cells (Fig. 1 *A*) but also inhibited epinephrine-induced activation of PLA_2 activity in the cell lysates (Fig. 1 *B*). Stimulation of AA release in intact cells or activation of PLA_2 in cell lysates by the PKC activator PMA, which is cell membrane receptor independent, was not affected by prazosin. These results indicate that agonist occu-

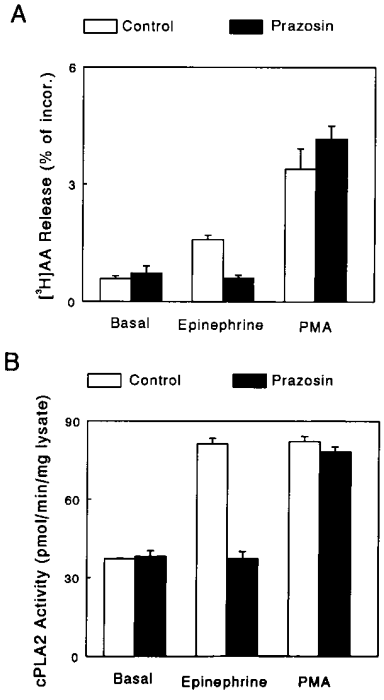

Figure 1. Effects of prazosin on AA release in intact MDCK-D1 cells (*A*) and on activation of PLA_2 activity in cell lysates (*B*). (*A*) [^{3}H]AA release in intact cells in response to the stimulation by 100 μM epinephrine or 100 nM PMA (plus 5 μM A23187) was measured as described in Methods. Before stimulation, cells were treated with or without 0.5 μM prazosin for 20 min. Prazosin was also included when cells were stimulated with agonists. (*B*) Cells were treated with prazosin and stimulated with agonists as described for panel A (except for the omission of A23187 from the PMA treatment) before cell lysates were made. Activity of PLA_2 in cell lysates was assessed as described in Methods.

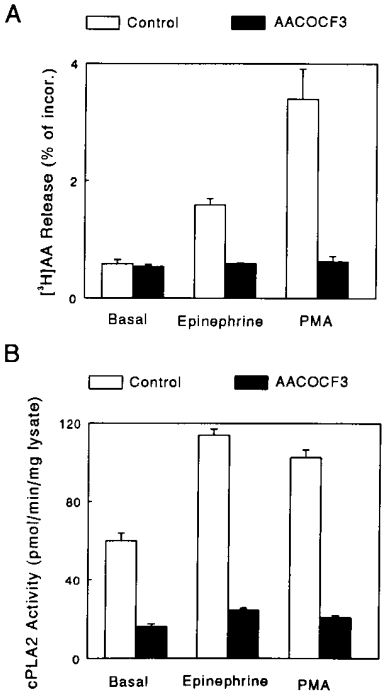

Figure 2. Effects of $AACOCF_3$ on AA release in intact MDCK-D1 cells (*A*) and on the activity of PLA_2 in cell lysates (*B*). (*A*) [^{3}H]AA release in intact cells was assessed as described in Methods. Before stimulation, cells were treated with or without 150 μM $AACOCF_3$ for 12 min. $AACOCF_3$ was also included with the corresponding cells during the stimulation with the indicated agonists as specified in the legend to Fig. 1 *A*. (*B*) Cells were stimulated with the agonists as indicated in the figure before cell lysates were made. PLA_2 activity in the cell lysates was assessed as described in Methods, except for the inclusion of 20 μM $AACOCF_3$ in some assay conditions as indicated in the figure. DTT was omitted in this experiment.

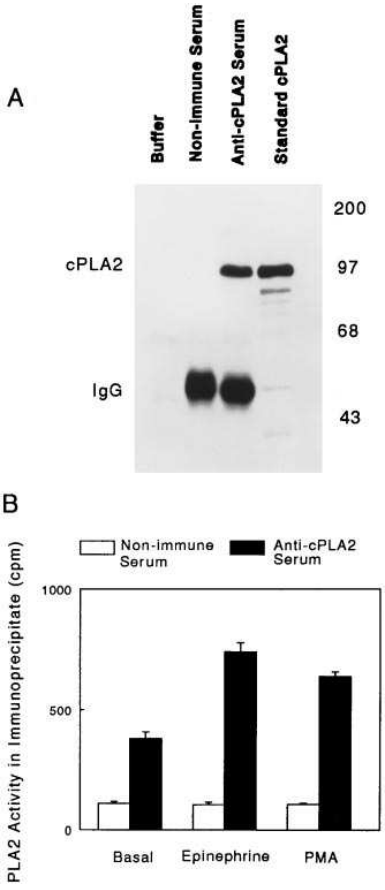

Figure 3. Immunoprecipitation of the 85-kD $cPLA_2$ and the recovery of PLA_2 activity in the immunoprecipitates. (*A*) Cell lysates were immunoprecipitated either without serum (buffer only), or with nonimmune serum, or with anti–85-kD $cPLA_2$ serum, as described in Methods. The immunoprecipitates were resuspended in SDS loading buffer, boiled for 5 min, and subjected to SDS-PAGE. Standard 85-kD $cPLA_2$ was also loaded in parallel to the immunoprecipitate samples in order to define the position of this protein on the gel. The proteins were then transferred to PVDF membranes, immunoblotted with anti–85-kD $cPLA_2$ serum and detected by ECL as described for Western blotting of MAP kinase in Methods. (*B*) Cell lysates were prepared from cells stimulated with or without 100 μM epinephrine or 100 nM PMA and subjected to immunoprecipitation with nonimmune serum or with anti-85-kD $cPLA_2$ serum as described for *A*. The immunoprecipitates were then assessed for PLA_2 activity as described in Methods.

pancy of α_1-adrenergic receptors in MDCK-D1 cells promotes activation of a PLA_2. No substantial release of AA in intact MDCK-D1 cells was observed during the initial period (< 10–15 min) of treatment with PMA, unless the Ca^{2+} ionophore A23187 was also included in the medium to increase the intracellular Ca^{2+} (data not shown), suggesting the involvement of a Ca^{2+}-dependent PLA_2 in PMA-mediated AA release as in α_1-adrenergic receptor-promoted AA release in these cells (21).

We hypothesized that this α_1-adrenergic receptor-coupled PLA_2 was the 85-kD cytosolic form, since the PLA_2 activity in cell lysates was insensitive to the reducing agent DTT (included in all the in vitro PLA_2 assays except for the experiment shown in Fig. 2 *B*) and micromolar Ca^{2+} was sufficient for the activation of the cell lysate PLA_2 activity (see below), as expected for the 85-kD $cPLA_2$. To test this hypothesis, we examined the effect of $AACOCF_3$, a trifluoromethyl ketone analogue of arachidonyl acid that can inhibit the 85-kD $cPLA_2$ but not the 14-kD low molecular weight form of PLA_2 (29). As shown in Fig. 2, epinephrine- and PMA-promoted AA release were inhibited by $AACOCF_3$ both in intact cells (Fig. 2 *A*) and when assessed as PLA_2 activity in cell lysates (Fig. 2 *B*). These data suggest that, in MDCK-D1 cells, α_1-adrenergic receptors, as well as PMA, induce AA release through the activation of the 85-kD $cPLA_2$.

To more definitively demonstrate the coupling of α_1-adrenergic receptors to the 85-kD $cPLA_2$, we examined $cPLA_2$ activity in immunoprecipitates obtained using anti-$cPLA_2$ antibody. As shown in Fig. 3 *A*, an 85-kD $cPLA_2$ (its apparent molecular weight on SDS-PAGE gel is ~ 100 kD) could be immunoprecipitated from MDCK-D1 cell lysate with a rabbit antiserum directed against the 85-kD $cPLA_2$ protein, but not with the nonimmune serum, indicating the specificity of the anti-$cPLA_2$ antibody. We found substantial PLA_2 activity in the immunoprecipitates obtained with the anti–85-kD $cPLA_2$ serum, but not with the nonimmune serum, especially when assays were conducted in the presence of DTT (data not shown). DTT presumably helps to release bound $cPLA_2$ from the antibody by impairing the binding affinity of the antibody for its antigen as a result of the reduction of the disulfide bonds in the antibody molecule. With this experimental strategy, we found increased $cPLA_2$ activity in the immunoprecipitates obtained with anti–85-kD $cPLA_2$ serum and cell lysates derived from cells prestimulated with epinephrine or PMA (Fig. 3 *B*).

Activation of the $cPLA_2$ by α_1-adrenergic receptors is mediated through protein phosphorylation. The stable increase in the activity of the $cPLA_2$ detected in cell-free systems derived from MDCK-D1 cells pretreated with agonists (Figs. 1 *B*, 2 *B*, and 3 *B*) suggested that covalent modification of the lipase was the mechanism for the change in its enzyme activity. To test whether such modification of $cPLA_2$ by agonists in MDCK-D1 cells was the result of phosphorylation, cell lysates were treated with potato acid phosphatase before the assay for $cPLA_2$ activity. This treatment abolished the increase of the $cPLA_2$ activity produced by incubation of cells with epinephrine or with PMA (Fig. 4 *A*). Treatment with potato acid phosphatase did not lead to proteolysis of $cPLA_2$ under our experimental conditions, as judged by Western blotting studies (data not shown). Thus, both α_1-adrenergic receptors and PMA ap-

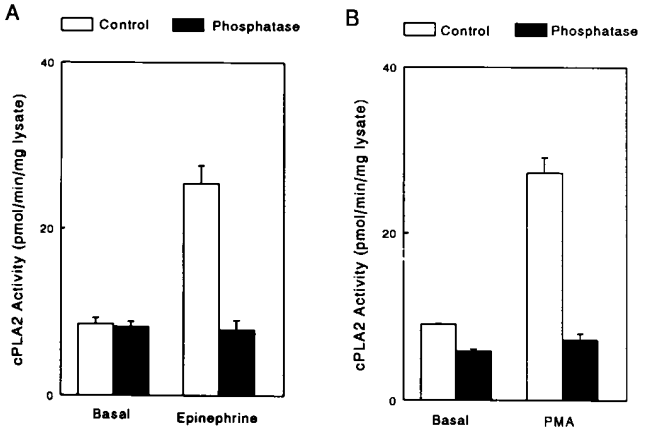

Figure 4. Effect of potato acid phosphatase on the stimulated activity of PLA_2 in MDCK-D1 cell lysates. Cell lysates were prepared from cells stimulated with 100 μM epinephrine (*A*) or 100 nM PMA (*B*) and treated with 1 U/ml potato acid phosphatase at 30°C and pH 6.4 for 30 min in the absence of phosphatase inhibitors but presence of protease inhibitors as defined in Methods. The control samples were treated identically except for the omission of phosphatase. After the pH of the lysates was brought up to 7.4 and the phosphatase inhibitors were added back to the cell lysates, PLA_2 activity was assessed at 37°C as described in Methods.

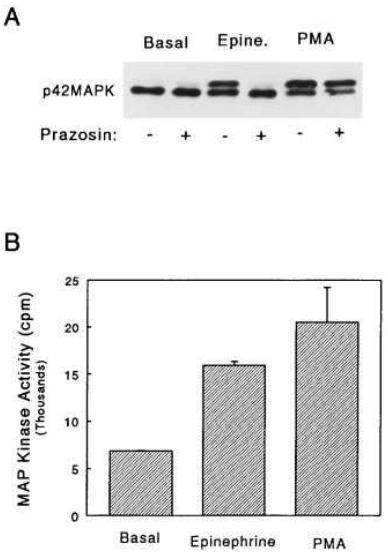

Figure 5. Phosphorylation-induced molecular weight shift and activation of MAP kinase by α_1-adrenergic receptors in MDCK-D1 cells. (*A*) Cells were incubated with or without 0.5 μM prazosin for 20 min, followed by incubation with 100 μM epinephrine (*Epine.*) or 100 nM PMA for 3 min. Cells were then lysed into SDS loading buffer and the samples were boiled, subjected to SDS-PAGE, immunoblotted with anti-p42 MAP kinase serum and detected by ECL as described in Methods. (*B*) Cell lysates were prepared from cells stimulated with or without epinephrine or PMA and subjected to immunoprecipitation with anti-p42 MAP kinase serum. The immunoprecipitates were then assessed for MAP kinase activity as described in Methods.

pear to mediate the activation of $cPLA_2$ in MDCK-D1 cells through protein phosphorylation.

α_1-adrenergic receptor-promoted phosphorylation and activation of MAP kinase correlates with the activation of $cPLA_2$. We next sought to investigate whether α_1-adrenergic receptor-promoted phosphorylation-dependent activation of $cPLA_2$ occurred in MDCK-D1 cells secondary to activation of MAP kinase, as has been observed in in vitro experiments using recombinant enzyme (12, 13). We first examined whether this receptor could cause phosphorylation and activation of MAP kinase in MDCK-D1 cells in response to the stimulation by epinephrine. Activation of MAP kinase requires tyrosine and threonine phosphorylation of the enzyme (30). A unique feature of this activation is the molecular weight shift of the phosphorylated species of MAP kinase as assessed by SDS-PAGE, which has been used as a measure of the stimulation of MAP kinase (15, 23, 31). We observed such a mobility shift of MAP kinase in MDCK-D1 cells in response to stimulation by a variety of agonists (Fig. 5 *A* and data not shown); this mobility shift is attributable to phosphorylation because it can be reversed by treatment of the protein sample with potato acid phosphatase (data not shown). As shown in Fig. 5 *A*, stimulation of MDCK-D1 cells with either epinephrine or PMA caused phosphorylation of MAP kinase, as suggested by the appearance of a band with decreased molecular mobility. Epinephrine-, but not PMA-, induced phosphorylation of MAP kinase could be blocked by prazosin, suggesting that the same α_1-adrenergic receptor that is coupled to activation of $cPLA_2$ is also coupled to MAP kinase. In addition to the enhanced phosphorylation of MAP kinase in response to stimulation of cells by epinephrine or PMA, both agents increased MAP kinase activity (Fig. 5 *B*).

To further investigate the relationship between the activation of $cPLA_2$ and that of MAP kinase by α_1-adrenergic receptors, we compared the time courses of the two α_1-adrenergic receptor-mediated events. As shown in Fig. 6 *A*, stimulation of MAP kinase by epinephrine occurred in a time-dependent manner: it became appreciable at 0.5 min, was most prominent at 3 min after treatment of cells with epinephrine, and gradually declined thereafter. Accumulation of released AA in the medium occurred somewhat more slowly but was prominent by 3 min of cell stimulation with epinephrine (Fig. 6 *B*). The stimulation of $cPLA_2$ activity more closely followed the stimulation of MAP kinase and was faster than AA accumulation (Fig. 6 *C*). This type of temporal relationship suggests a cause-and-effect relationship between the activation of MAP kinase and that of $cPLA_2$. Further support for this conclusion was obtained by the inhibition of epinephrine-promoted AA release by PD098059 (Fig. 7), which prevents MAP kinase activation by inhibiting MAP kinase kinase (20).

α_1-adrenergic receptor-induced activation of MAP kinase and $cPLA_2$ is mediated by PKC. The similar effects of epinephrine with those of PMA on the activation of $cPLA_2$ and MAP kinase (Figs. 1–5) suggested the possible involvement of PKC in the regulation of $cPLA_2$ and MAP kinase by α_1-adrenergic receptors. Previous studies from this laboratory have suggested that PKC is involved in α_1-adrenergic receptor-mediated AA release in intact MDCK-D1 cells (18, 32). To further investigate the mechanism for this PKC involvement, we examined the effect of down-regulation of PKC on α_1-adrenergic receptor-mediated stimulation of MAP kinase and $cPLA_2$. Down-regulation of PKC was achieved by incubation of cells overnight (20 h) with 200 nM PMA. Stimulation of MAP kinase by PMA and by epinephrine was completely blocked by such down-regulation of PKC (Fig. 8 *A*). Correspondingly, down-regulation of PKC completely blocked the stimulation of AA release by epinephrine and the potentiating effect of PMA on the Ca^{2+} ionophore A23187-stimulated AA release (Fig. 8 *B*). Down-regulation of PKC did not affect AA release by A23187, which has been shown to stimulate AA release in a PKC-independent manner (24). Interpretation of the results obtained with PKC down-regulation by overnight treatment of cells with PMA, as shown in Fig. 8, could be complicated by

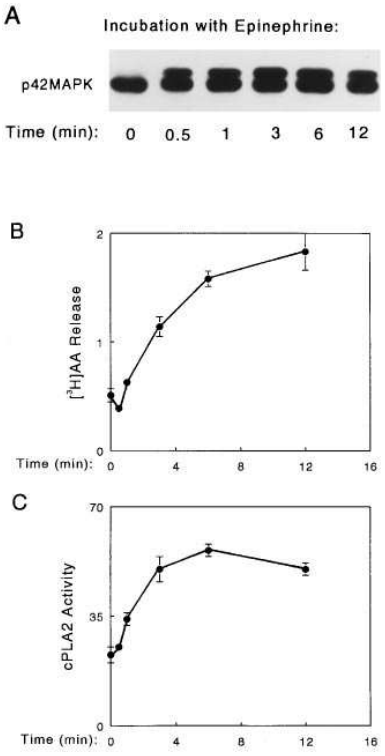

Figure 6. Time courses of α_1-adrenergic stimulation of MAP kinase (*A*) , AA release (*B*) and PLA_2 activity (*C*) in MDCK-D1 cells. (*A*) Cells were incubated with 100 μM epinephrine for the indicated times and the proteins samples derived from the cells were detected for p42 MAP kinase (*MAPk*) molecular weight shift by Western blotting. (*B*) Accumulated free [^{3}H]AA (expressed as percentage of incorporation) released in the medium was measured after the [^{3}H]AA-labeled intact cells were stimulated with 100 μM epinephrine for the indicated times. (*C*) Cells were incubated with 100 μM epinephrine for the indicated times, followed by preparation of cell lysates and assay for PLA_2 activity (expressed as picomoles per min per milligram lysates). The experimental details are described in Methods.

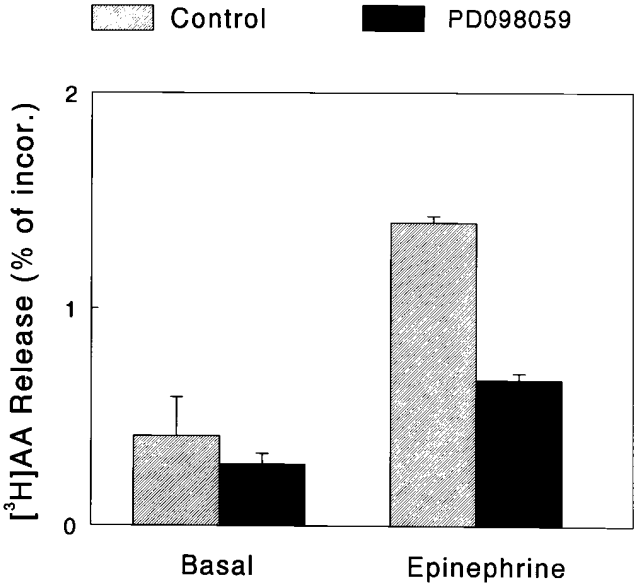

Figure 7. Effect of PD098059 on AA release in MDCK-D1 cells. [^{3}H]AA release in intact cells at basal state or in response to the stimulation by 100 μM epinephrine was measured as described in Methods. Before stimulation, cells were treated with or without 30 μM PD098059 for 30 min. PD098059 was also included when cells were stimulated with the agonist.

the fact that PKC is also involved in the desensitization of α_{1b} receptors (33). To circumvent this potential problem, we also tested the effect of the PKC inhibitor sphingosine on agonist stimulation of MAP kinase and AA release. As shown in Fig. 9 *A*, stimulation of the molecular weight shift of MAP kinase by epinephrine or PMA was blocked by sphingosine. Sphingosine treatment also blocked epinephrine-stimulated AA release (Fig. 9 *B*). In addition, sphingosine treatment or PKC down-regulation blocked epinephrine-promoted activation of MAP kinase activity (Fig. 10). Taken together, these data demonstrate the mandatory involvement of PKC in the activation of both MAP kinase and $cPLA_2$ by α_1-adrenergic receptors in MDCK-D1 cells.

Alpha$_1$-adrenergic receptor and protein kinase C activation increase the maximal activity of cPLA$_2$. Since calcium plays a critical role in the regulation of $cPLA_2$, we were interested to determine whether the ability of Ca^{2+} to activate $cPLA_2$ was altered by α_1-adrenergic receptor or phorbol ester stimulation. Compared with control cells, treatment of cells with either epinephrine or PMA increased the maximal activity of PLA_2, as assayed in subsequently prepared cell lysates (Fig. 11). However, we observed a similar sensitivity of the $cPLA_2$ to Ca^{2+} in cells treated with or without epinephrine or PMA. Fig. 11 also illustrates that the agonist-stimulated PLA_2 activity is sensitive to Ca^{2+} in the micromolar range, characteristic of the involvement of $cPLA_2$. Taken together with the evidence presented above, these data suggest that a mechanism whereby α_1-adrenergic receptor and PMA stimulate $cPLA_2$ activity is to increase the maximal activity of the enzyme through phosphorylation by protein kinases in MDCK cells.

Discussion

Stimulation of AA release by α_1-adrenergic receptors has been demonstrated in a variety of cells, including FRTL5 cells (34), spinal cord neurons (35), MDCK cells (11), vascular smooth muscle cells (36), transfected COS-1 cells (37), and striatal astrocytes (38). Although some efforts have been made in these studies to define the molecular mechanism(s) for α_1-adrenergic regulation of AA release, no clear-cut information regarding this issue has been provided. It has been hypothesized that a PLA_2 is involved in this receptor-mediated release of AA, but definitive evidence for this hypothesis has been lacking.

The results shown here provide substantial evidence in support of the conclusion that in MDCK-D1 cells the 85-kD $cPLA_2$ is coupled to α_1-adrenergic receptors and is responsible for this receptor-mediated AA release. The evidence for this conclusion is several-fold: (*a*) The only PLA_2 that is known to be activated by membrane receptors through phosphorylation is the 85-kD form, and α_1-adrenergic receptor-stimulated activation of the PLA_2 in MDCK-D1 cells was mediated through phosphorylation (Fig. 4); (*b*) To date, the only PLA_2 whose activation has been suggested to involve PKC is the 85-kD form, and PKC mediates the activation of the PLA_2 by α_1-adrenergic receptors in MDCK-D1 cells (Figs. 8 and 9); (*c*) Activation of the 85-kD $cPLA_2$ requires micromolar Ca^{2+}. PLA_2 activities in MDCK-D1 cell lysates are Ca^{2+}-dependent and micromolar Ca^{2+} provides substantial activation of the enzyme (Fig. 11), consistent with the previous observation that omission of extracellular Ca^{2+} blocks epinephrine-stimulated AA release in MDCK-D1 cells (21); (*d*) Unlike the 14-kD PLA_2s, the α_1-adrenergic receptor-coupled PLA_2 activity is insensitive to the reducing agent DTT, which was included in the assays of PLA_2 activity in the present study (except for the experiment shown

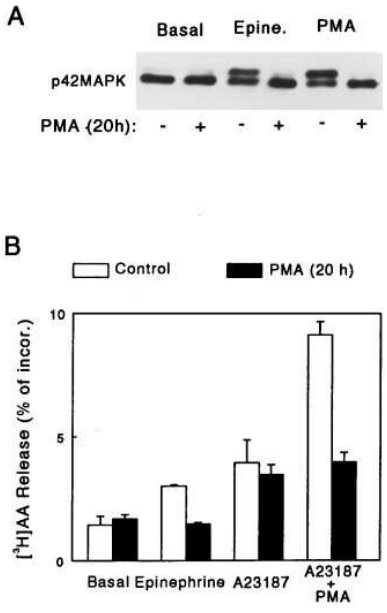

Figure 8. Effect of PKC down-regulation on agonist stimulation of MAP kinase and AA release in MDCK-D1 cells. (*A*) After incubation overnight (20 h) with or without 200 nM PMA, cells were incubated with 100 μM epinephrine or 100 nM PMA for 3 min. Protein samples derived from the cells were then detected for p42 MAP kinase by Western blotting as described in Methods. (*B*) After incubation of cells with 200 nM PMA overnight (20 h), [^{3}H]AA release in intact cells in response to the stimulation by 100 μM epinephrine or 5 μM A23187 or 5 μM A23187 plus 100 nM PMA was assessed as described in Methods.

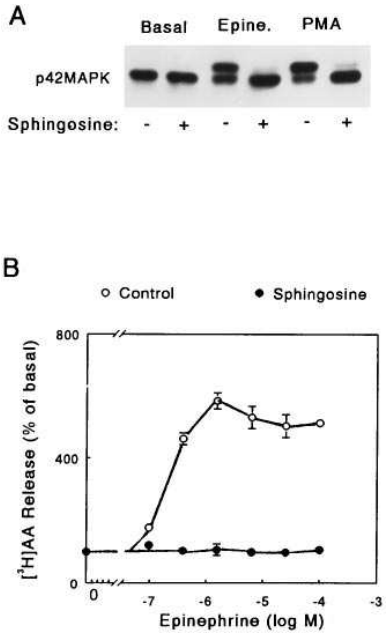

Figure 9. Effect of sphingosine on agonist stimulation of MAP kinase and AA release in MDCK-D1 cells. (*A*) Cells were incubated with 15 μM sphingosine for 20 min, followed by a 3-min stimulation with 100 μM epinephrine (*Epine.*) or 100 nM PMA. Protein samples derived from the cells were then detected by Western blotting as described in Methods. (*B*) Cells were incubated with 15 μM sphingosine for 20 min and [^{3}H]AA release was then assessed in response to the stimulation by epinephrine at the indicated concentrations as described in Methods.

in Fig. 2 *B*); (*e*) The α_1-adrenergic receptor-coupled PLA_2 is sensitive to the recently characterized $cPLA_2$ inhibitor $AACOCF_3$ (Fig. 2); and (*f*) α_1-adrenergic activation of $cPLA_2$ was recovered in the immunoprecipitates obtained with anti–85-kD $cPLA_2$ serum (Fig. 3). $AACOCF_3$ has recently also been shown to inhibit a calcium-independent PLA_2 purified from P388D1 cells (39). However, calcium-independent PLA_2 is apparently not the type activated by α_1-adrenergic receptors in MDCK-D1 because receptor-promoted AA release in intact cells (21) and activation of PLA_2 activity measured in cell lysates (Fig. 11) are both Ca^{2+} dependent.

Another major effort of the present study was to define the regulatory mechanism(s) by which the 85-kD $cPLA_2$ is activated by α_1-adrenergic receptors in MDCK-D1 cells. In particular, we sought to define the role of PKC and MAP kinase. Based on in vitro studies of phosphorylation and activation of the recombinant 85-kD $cPLA_2$ by MAP kinase, it has been proposed that phosphorylation of the 85-kD $cPLA_2$ by MAP kinase, in coordination with an increase in the concentration of intracellular Ca^{2+}, is the mechanism whereby membrane receptors fully activate the enzyme (12, 13). Other evidence in favor of this mechanism is the correlation of activation of MAP kinase with that of PLA_2 activity in macrophages stimulated with zymosan particles (24) or colony-stimulating factor 1 (40) and in endothelial cells stimulated with basic fibroblast growth factor (41). In contrast, data have not previously been provided for parallel activation of endogenous MAP kinase and $cPLA_2$ in native cells by G protein–coupled receptors, although separate reports showing G protein receptor-coupled phosphorylation of $cPLA_2$ or activation of MAP kinase in different cells are available. In fact, a more complex situation regarding the role of MAP kinase in the regulation of the endogenous $cPLA_2$ by G protein–coupled receptors has been suggested by the findings that in Chinese hamster ovary cells or undifferentiated HL60 cells certain G protein–coupled receptors promote normal Ca^{2+} mobilization and MAP kinase activation without inducing $cPLA_2$-mediated AA release (15, 16). These results could suggest either that a factor separate from Ca^{2+} and MAP kinase is also required to modify the $cPLA_2$ molecule for its activation or that MAP kinase is not involved in the activation of the endogenous $cPLA_2$ by these G protein–coupled receptors. Data reported in the present study that activation of $cPLA_2$ temporally follows the activation of MAP kinase by α_1-adrenergic receptors (Fig. 6) and that PD098059, a MAP kinase cascade inhibitor (20), blocked α_1-adrenergic release of AA (Fig. 7) strongly support the idea that MAP kinase is required for the regulation of $cPLA_2$ by α_1-adrenergic receptors in MDCK-D1 cells. This conclusion is further supported by the fact that blockade of MAP kinase stimulation by PKC down-regulation or by PKC inhibitor also blocks AA release (Figs. 8–10).

Although MAP kinase alone (12) or both MAP kinase and PKC (13) have been reported to phosphorylate and activate recombinant $cPLA_2$, most in vitro studies have shown no direct activation of $cPLA_2$ by PKC (12, 14). Since MAP kinase can be activated by PKC (present study and reference 30), our data support the idea that sequential activation of PKC and MAP kinase is an important mechanism in α_1-adrenergic receptor-mediated activation of the endogenous $cPLA_2$ in MDCK-D1 cells. Agonist-promoted phosphorylation of $cPLA_2$ that involved PKC has been observed in macrophages and smooth muscle cells (42–44) although the involvement of MAP kinase in this cellular event was not addressed in these studies. The PKC-dependent activation of MAP kinase and $cPLA_2$ by α_1-adrenergic receptors is consistent with the kinetics of production of the native PKC activator diacylglycerol and the activation of PKC by α_1-adrenergic receptors in MDCK-D1 cells (18, 45). Our results indicate that the effect of phosphorylation on $cPLA_2$ is to increase its maximal activity rather than its sensitivity to Ca^{2+} (Fig. 11), as also found for the activation of PLA_2 by thrombin in human platelets (46).

In summary, we have used MDCK-D1 cells to demonstrate that the 85-kD $cPLA_2$ is activated by α_1-adrenergic receptors and is responsible for this receptor-promoted AA release. In

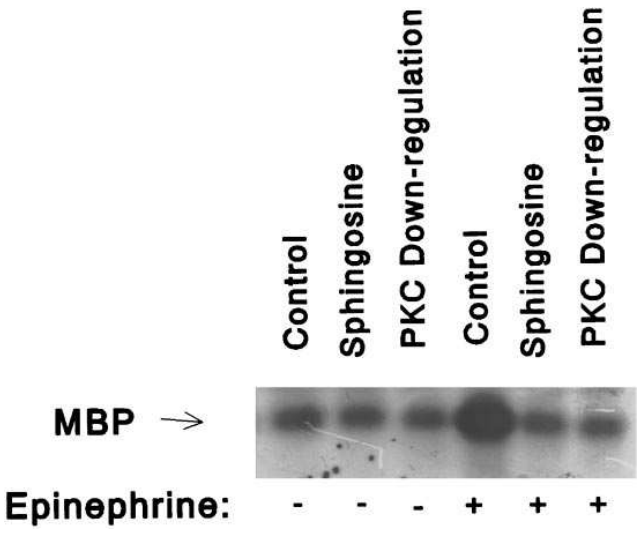

Figure 10. Effects of sphingosine and PKC down-regulation on epinephrine stimulation of MAP kinase activity. MDCK-D1 cells were treated with vehicle (*Control*), 15 μM sphingosine (20 min), or 200 nM PMA (20 h, to down-regulate PKC) (*PKC Down-regulation*), followed by incubation with or without 100 μM epinephrine for 3 min. Cell lysates were then prepared and immunoprecipitated for MAP kinase as described in Methods. The immunoprecipitated MAP kinase was assayed for kinase activity using MBP and [γ-^{32}P]ATP as substrates. The kinase assay reaction was terminated by adding equal volume of twofold concentrated Laemmli's buffer and heating for 5 min, followed by SDS-PAGE. After drying, the gel was exposed to film, and the major band corresponding to MBP was recognized as shown in the figure.

addition, results in the present study strongly suggest the involvement of MAP kinase activation, secondary to the activation of PKC, in the stimulation of $cPLA_2$ activity by α_1-adrenergic receptors in these cells. Our present data, together with the previous work from this laboratory, lead us to propose a model whereby α_1-adrenergic receptors in MDCK-D1 cells activate the 85-kD $cPLA_2$ by the sequential activation of one or more forms of phospholipase C, PKC, and MAP kinase and thereby the phosphorylation of $cPLA_2$. Such phosphorylation appears to increase the maximal activity of $cPLA_2$ rather than to change the sensitivity to intracellular Ca^{2+}. We speculate that this may be a general mechanism whereby G protein–linked receptors stimulate AA release and formation of products of AA metabolism.

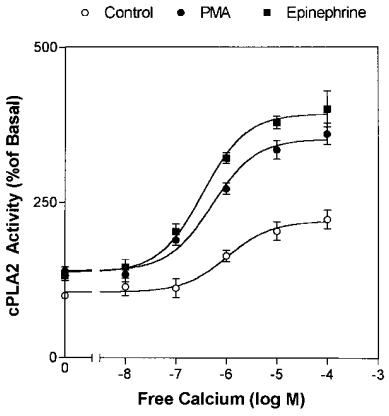

Figure 11. Calcium concentration-response of $cPLA_2$ activity from MDCK-D1 cells treated with epinephrine or PMA. Cells were incubated with vehicle (*Control*) or 100 μM epinephrine or 100 nM PMA, followed by preparation of cell lysates. PLA_2 activity in the cell lysates was assessed in the presence of increasing concentrations of free Ca^{2+} prepared as described in Methods. Each point in the figure represents the mean±SEM of data obtained from four independent experiments except for the point of 10^{-4} M which is the average of three experiments.

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References

1. Irvine, R.F. 1982. How is the level of arachidonic acid controlled in mammalian cells? *Biochem. J.* 204:3–16.

2. Smith, W.L. 1989. The eicosanoids and their biochemical mechanisms of action. *Biochem. J.* 259:315–324.

3. Mukherjee, A.B., L. Miele, and N. Pattabiraman. 1994. Phospholipase A_2 enzymes: regulation and physiological role. *Biochem. Pharmacol.* 48:1–10.

4. Dennis, E.A. 1994. Diversity of group types, regulation, and function of phospholipase A_2. *J. Biol. Chem.* 269:13057–13060.

5. Lin, L.-L., A.Y. Lin, and J.L. Knopf. 1992. Cytosolic phospholipase A_2 is coupled to hormonally regulated release of arachidonic acid. *Proc. Natl. Acad. Sci. USA.* 89:6147–6151.

6. Exton, J. 1994. Phosphatidylcholine breakdown and signal transduction. *Biochim. Biophys. Acta.* 1212:26–42.

7. Barbour, S.E., and E.A. Dennis. 1993. Antisense inhibition of group II phospholipase A_2 expression blocks the production of prostaglandin E_2 by P388D1 cells. *J. Biol. Chem.* 826:21875–21882.

8. Murakami, M., I. Kudo, and K. Inoue. 1993. Molecular nature of phospholipase A_2 involved in prostaglandin I_2 synthesis in human umbilical vein endothelial cells. Possible participation of cytosolic and extracellular type II phospholipase A_2. *J. Biol. Chem.* 268:839–844.

9. Lehman, J.J., K.A. Brown, S. Ramanadham, J. Turk, and R.W. Gross. 1993. Arachidonic acid release from aortic smooth muscle cells induced by [Arg8]vasopressin is largely mediated by calcium-independent phospholipase A_2. *J. Biol. Chem.* 268:20713–20716.

10. Du, X, S.J. Harris, T.J. Tetaz, M.H. Ginsberg, and M.C. Berndt. 1994. Association of a phospholipase A_2 (14-3-3 protein) with the platelet glycoprotein Ib-IX complex. *J. Biol. Chem.* 269:18287–18290.

11. Insel, P.A., B.A. Weiss, S.R. Slivka, M.J. Howard, J.J. Waite, and C.A. Godson. 1991. Regulation of phospholipase A_2 by receptors in MDCK-D1 cells. *Biochem. Soc. Trans.* 19:329–333.

12. Lin, L.-L., M. Wartmann, A.Y. Lin, J.L. Knopf, A. Seth, and R.J. Davis. 1993. $cPLA_2$ is phosphorylated and activated by MAP kinase. *Cell.* 72:269–278.

13. Nemenoff, R.A., S. Winitz, N.X. Qian, V. Van Putten, G.L. Johnson, and L.E. Heasley. 1993. Phosphorylation and activation of a high molecular weight form of phospholipase A_2 by p42 microtubule-associated protein 2 kinase and protein kinase C. *J. Biol. Chem.* 268:1960–1964.

14. Wijkander, J., and R. Sundler. 1991. An 100-kDa arachidonate-mobilizing phospholipase A_2 in mouse spleen and the macrophage cell line J774. Purification, substrate interaction and phosphorylation by protein kinase C. *Eur. J. Biochem.* 202:873–880.

15. Xing, M., P.L. Wilkins, B.K. McConnel, and R. Mattera. 1994. Regulation of phospholipase A_2 activity in undifferentiated and neutrophil-like HL60 cells. Linkage between impaired responses to agonists and absence of protein kinase C-dependent phosphorylation of cytosolic phospholipase A_2. *J. Biol. Chem.* 269:3117–3124.

16. Winitz, S., S.K. Gupta, N.-X. Qian, L.E. Heasley, R.A. Nemenoff, and G.L. Johnson. 1994. Expression of a mutant Gi2 alpha subunit inhibits ATP and thrombin stimulation of cytoplasmic phospholipase A_2-mediated arachidonic acid release independent of Ca^{2+} and mitogen-activated protein kinase regulation. *J. Biol. Chem.* 269:1889–1895.

17. Minneman, K.P., and T.S. Esbenshade. 1994. Alpha 1-adrenergic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.* 34:117–133.

18. Weiss, B.A., and P.A. Insel. 1991. Intracellular Ca^{2+} and protein kinase C interact to regulate alpha 1-adrenergic- and bradykinin receptor-stimulated phospholipase A_2 activation in Madin-Darby canine kidney cells. *J. Biol. Chem.* 266:2126–2133.

19. Wang, Y., M.S. Simonson, J. Pouyssegur, and M.J. Dunn. 1992. Endo-

thelin rapidly stimulates mitogen-activated protein kinase activity in rat mesangial cells. *Biochem. J.* 287:589–594.

20. Dudley, D.T., L. Pang, S.J. Decker, A.J. Bridges, and A.R. Saltiel. 1995. A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci. USA.* 92:7686–7689.

21. Slivka, S.R., and P.A. Insel. 1987. Alpha 1-adrenergic receptor-mediated phosphoinositide hydrolysis and prostaglandin E_2 formation in Madin-Darby canine kidney cells. Possible parallel activation of phospholipase C and phospholipase A_2. *J. Biol. Chem.* 262:4200–4207.

22. Bligh, E.G., and W.J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37:911–917.

23. de Vries-Smits, A.M.M., B.M.T. Burgering, S.J. Leevers, C.J. Marshall, and J.L. Bos. 1992. Involvement of p21ras in activation of extracellular signal-regulated kinase 2. *Nature (Lond.).* 357:602–604.

24. Qiu, Z.-H., and C.C. Leslie. 1994. Protein kinase C-dependent and -independent pathways of mitogen-activated protein kinase activation in macrophages by stimuli that activate phospholipase A_2. *J. Biol. Chem.* 269:19480–19487.

25. Fabiato, A. 1979. A rapid method of total lipid extraction *J. Physiol. (Paris).* 75:463–505.

26. Gstraunthaler, G.J.A. 1988. Epithelial cells in tissue culture. *Renal Physiol. Biochem.* 11:1–42.

27. Klijn, K., S.R. Slivka, K. Bell, and P.A. Insel. 1991. Renal alpha 1-adrenergic receptor subtypes: MDCK-D1 cells, but not rat cortical membranes possess a single population of receptors. *Mol. Pharmacol.* 39:407–413.

28. Blue, D.R., Jr., D.A. Craig, J.T. Ransom, J.A. Camacho, P.A. Insel, and D.E. Clarke. 1994. Characterization of the alpha-1 adrenoceptor subtype mediating [^{3}H]-arachidonic acid release and calcium mobilization in Madin-Darby canine kidney cells. *J. Pharmacol. Exp. Ther.* 268:1588–1596.

29. Street, I.P., H.-K. Lin, F. Laliberte, F. Ghomashchi, Z. Wang, H. Perrier, N.M. Tremblay, Z. Huang, P.K. Weech, and M.H. Gelb. 1993. Slow- and tight-binding inhibitors of the 85-kDa human phospholipase A_2. *Biochemistry.* 32:5935–5940.

30. Seger, R., and E.G. Krebs. 1995. The MAPK signaling cascade. *FASEB J.* 9:726–735.

31. Alblas, J., E.J. Van Corven, P.L. Hordijk, G. Milligan, and W.H. Moolenaar. 1993. Gi-mediated activation of the p21ras-mitogen-activated protein kinase pathway by alpha 2-adrenergic receptors expressed in fibroblasts. *J. Biol. Chem.* 268:22235–22238.

32. Weiss, B.A., S.R. Slivka, and P.A. Insel. 1989. Defining the role of protein kinase C in epinephrine- and bradykinin-stimulated arachidonic acid metabolism in Madin-Darby canine kidney cells. *Mol. Pharmacol.* 36:317–326.

33. Lattion, A.L., D. Diviani, and S. Cotecchia. 1994. Truncation of the receptor carboxyl terminus impairs agonist-dependent phosphorylation and desensitization of the alpha 1B-adrenergic receptor. *J. Biol. Chem.* 269:22887–22893.

34. Burch, R.M., A. Luini, D.E. Mais, D. Corda, J.Y. Vanderhoek, L.D. Kohn, and J. Axelrod. 1986. Alpha 1-adrenergic stimulation of arachidonic acid release and metabolism in a rat thyroid cell line. Mediation of cell replication by prostaglandin E_2. *J. Biol. Chem.* 261:11236-11241.

35. Kanterman, R.Y., C.C. Felder, D.E. Brenneman, A.L. Ma, S. Fitzgerald, and J. Axelrod. 1990. Alpha 1-adrenergic receptor mediates arachidonic acid release in spinal cord neurons independent of inositol phospholipid turnover. *J. Neurochem.* 54:1225–1232.

36. Nebigil, C., and K.U. Malik. 1992. Prostaglandin synthesis elicited by adrenergic stimuli is mediated via alpha-2C and alpha-1A adrenergic receptors in cultured smooth muscle cells of rabbit aorta. *J. Pharmacol. Exp. Ther.* 260: 849–858.

37. Perez, D.M., M.B. DeYoung, and R.M. Graham. 1993. Coupling of exressed alpha 1B- and alpha 1D-adrenergic receptor to multiple signaling pathways is both G protein and cell type specific. *Mol. Pharnacol.* 44:784–795.

38. Marin, P., N. Stella, J. Cordier, J. Glowinski, and J. Premont. 1993. Role of arachidonic acid and glutamate in the formation of inositol phosphates induced by noradrenalin in striatal astrocytes. *Mol. Pharmacol.* 44:1176–1184.

39. Ackermann, E.J., K. Conde-Frieboes, and E.A. Dennis. 1995. Inhibition of macrophage Ca^{2+}-independent phospholipase A_2 by bromoenol lactone and trifluoromethyl ketones. *J. Biol. Chem.* 270:445–450.

40. Xu, X.-X., C.O. Rock, Z.-H. Qiu, C.C. Leslie, and S. Jackowski. 1994. Regulation of cytosolic phospholipase A_2 phosphorylation and eicosanoid production by colony-stimulating factor 1. *J. Biol. Chem.* 269:31693–31700.

41. Sa, G., G. Murugesan, M. Jaye, Y. Ivashchenko, and P.L. Fox. 1995. Activation of cytosolic phospholipase A_2 by basic fibroblast growth factor via a p42 mitogen-activated protein kinase-dependent phosphorylation pathway in endothelial cells. *J. Biol. Chem.* 270:2360–2366.

42. Wijkander, J., and R. Sundler. 1992. Regulation of arachidonate-mobilizing phospholipase A_2 by phosphorylation via protein kinase C in macrophages. *FEBS Lett.* 311:299–301.

43. Qiu, Z.-H., M.S. de Carvalho, and C.C. Leslie. 1993. Regulation of phospholipase A_2 activation by phosphorylation in mouse peritoneal macrophages. *J. Biol. Chem.* 268:24506–24513.

44. Rao, G.N., B. Lassegue, R.W. Alexander, and K.K. Griendling. 1994. Angiotensin II stimulates phosphorylation of high-molecular-mass cytosolic phospholipase A_2 in vascular smooth-muscle cells. *Biochem. J.* 299:197–201.

45. Slivka, S.R., K.E. Meier, and P.A. Insel. 1988. Alpha 1-adrenergic receptors promote phosphatidylcholine hydrolysis in MDCK-D1 cells. A mechanism for rapid activation of protein kinase C. *J. Biol. Chem.* 263:12242–12246.

46. Kramer, R.M., E.F. Roberts, J.V. Mametta, P.A. Hyslop, and J.A. Jakubowski. 1993. Thrombin-induced phosphorylation and activation of Ca^{2+}-sensitive cytosolic phospholipase A_2 in human platelets. *J. Biol. Chem.* 268: 26796–26804.