Dual Role of Protein Kinase C in the Regulation of $cPLA_2$-mediated Arachidonic Acid Release by P_{2U} Receptors in MDCK-D_1 Cells: Involvement of MAP Kinase-dependent and -independent Pathways

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Abstract

Defining the mechanism for regulation of arachidonic acid (AA) release is important for understanding cellular production of AA metabolites, such as prostaglandins and leukotrienes. Here we have investigated the differential roles of protein kinase C (PKC) and mitogen-activated protein (MAP) kinase in the regulation of cytosolic phospholipase A_2 ($cPLA_2$)-mediated AA release by P_{2U}-purinergic receptors in MDCK-D_1 cells. Treatment of cells with the P_{2U} receptor agonists ATP and UTP increased PLA_2 activity in subsequently prepared cell lysates. PLA_2 activity was inhibited by the $cPLA_2$ inhibitor $AACOCF_3$, as was AA release in intact cells. Increased PLA_2 activity was recovered in anti-$cPLA_2$ immunoprecipitates of lysates derived from nucleotide-treated cells, and was lost from the immunodepleted lysates. Thus, $cPLA_2$ is responsible for AA release by P_{2U} receptors in MDCK-D_1 cells. P_{2U} receptors also activated MAP kinase. This activation was PKC-dependent since phorbol 12-myristate 13-acetate (PMA) promoted down-regulation of PKC-eliminated MAP kinase activation by ATP or UTP. Treatment of cells with the MAP kinase cascade inhibitor PD098059, the PKC inhibitor GF109203X, or down-regulation of PKC by PMA treatment, all suppressed AA release promoted by ATP or UTP, suggesting that both MAP kinase and PKC are involved in the regulation of $cPLA_2$ by P_{2U} receptors. Differential effects of GF109203X on $cPLA_2$-mediated AA release and MAP kinase activation, however, were observed: at low concentrations, GF109203X inhibited AA release promoted by ATP, UTP, or PMA without affecting MAP kinase activation. Since GF109203X is more selective for PKC_α, PKC_α may act independently of MAP kinase to regulate $cPLA_2$ in MDCK-D_1 cells. This conclusion is further supported by data showing that PMA-promoted AA release, but not MAP kinase activation, was suppressed in cells in which PKC_α expression was decreased by antisense transfection. Based on these data, we propose a model whereby both MAP kinase and PKC are required for $cPLA_2$-mediated AA release by P_{2U} receptors in MDCK-D_1 cells. PKC plays a dual role in this process through the utilization of different isoforms: PKC_α regulates $cPLA_2$-mediated AA release independently of MAP kinase, while other PKC isoforms act through MAP kinase activation. This model contrasts with our recently demonstrated mechanism (*J. Clin. Invest.* 99:1302–1310.) whereby α_1-adrenergic receptors in the same cell type regulate $cPLA_2$-mediated AA release only through sequential activation of PKC and MAP kinase. (*J. Clin. Invest.* 99:805–814.) Key words: purinergic receptors • phospholipase • eicosanoid • ATP • epithelial cells

Introduction

Arachidonic acid (AA) and its eicosanoid metabolites play critical roles in the initiation or modulation of a broad spectrum of physiological as well as pathological (e.g., inflammatory) responses in mammalian cells (1, 2). AA release, which is the limiting step in the biosynthesis of eicosanoids in response to stimulation by membrane receptors, is primarily mediated by phospholipase A_2s (PLA_2s).[1] Several types of mammalian PLA_2s have been characterized, including 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). Among these PLA_2s, $cPLA_2$ appears to be responsible for AA release mediated by many receptors (4, 5). A current model to explain activation of $cPLA_2$ (largely based on in vitro experiments using recombinant proteins) is that stimulation of receptors leads to activation of mitogen-activated protein kinase/extracellular signal-regulated kinase (MAP kinase), phosphorylating $cPLA_2$ and consequently activating the lipase (6, 7). Studies using protein kinase C (PKC) inhibitors or activators or using PKC down-regulation strategies have suggested that PKC is also involved in the regulation of $cPLA_2$ activation and AA release in numerous cell types. Because PKC can activate MAP kinase (8, 9), it has been hypothesized that sequential activation of PKC and, in turn, MAP kinase, is the principal mechanism for receptor-stimulated activation of $cPLA_2$ and AA release. Whether PKC regulates $cPLA_2$ independently of MAP kinase is an important but unresolved issue. Moreover, although a role of MAP kinase has been implicated in the regulation of endogenous $cPLA_2$ by certain receptors (10, 11), in other systems (12–14) a causal relationship between activation of endogenous $cPLA_2$ and AA release and activation of MAP kinase has not been established. Recent studies have suggested a dissociation between the activation of $cPLA_2$ and that of MAP kinase (15–

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1. *Abbreviations used in this paper:* $AACOCF_3$, arachidonyl trifluoromethyl ketone; MAP kinase, mitogen-activated protein kinase; MDCK cells, Madin-Darby canine kidney cells; PKC, protein kinase C; PLA_2, phospholipase A_2.

17). Such results suggest that one cannot yet precisely define the relationships between activation of PKC and MAP kinase in the regulation of $cPLA_2$ by cellular receptors, particularly G protein-linked receptors.

In the present study we have used Madin-Darby canine kidney cells (MDCK-D_1) cells as a model system to investigate the mechanism whereby G protein-linked P_{2U}-purinergic receptors regulate $cPLA_2$ activation and AA release. We demonstrate that in these cells P_{2U} receptors regulate AA release through activation of $cPLA_2$. We further show that both PKC and MAP kinase are required for $cPLA_2$-mediated AA release by P_{2U} receptors, and that PKC plays a dual role in this process, perhaps through different isoforms: PKC_α regulates $cPLA_2$-mediated AA release independently of MAP kinase while other PKC isoforms act through the activation of MAP kinase.

Methods

Materials. 1-stearoyl-2[1-^{14}C] arachidonyl-L-3-phosphatidylcholine ([^{14}C]PC) (specific activity, 55 mCi/mmol), horseradish peroxidase-linked donkey anti-rabbit Ig and ECL Western blotting detection reagents were bought from Amersham Intl. (Buckinghamshire, England). [^{3}H]AA (specific activity, 100 Ci/mmol) and [γ-^{32}P]ATP (specific activity, 3,000 Ci/mmol) were purchased from DuPont-NEN (Boston, MA). Okadaic acid was obtained from Gemini Bio-Products Inc. (Calabasas, CA) or from Calbiochem, (La Jolla, CA). Leupeptin, pepstatin A, PMSF, A23187, PMA, $AACOCF_3$, GF109203X, and anti-PKCα antibody were bought from Calbiochem. Potato acid phosphatase, Na_3VO_4, sodium pyrophosphate, levamisole, protein A-sepharose, benzamidine, myelin basic protein (MBP), diisopropyl fluorophosphate (DIFP), PBS tablet, and arachidonic acid were purchased from Sigma Chemical Co. (St. Louis, MO). PKI (6–22 amide), a protein kinase A inhibitor, was bought from Gibco-BRL (Gaithersburg, MD). Immobilon-P PVDF transfer membrane (0.45 μM) was purchased from Millipore Corp. (Milford, MA). P-81 phosphocellulose paper was from Whatman Inc. (Clifton, NJ). TLC silica gel plates were from Analtech Inc. (Newark, DE). Anti-$cPLA_2$ serum was obtained from Genetics Institute (Cambridge, MA) (6). Anti-p42-MAP kinase (ERK_2) serum was obtained from Dr. Y. Wang (18). The MAP kinase cascade inhibitor PD098059 was provided by Dr. A.R. Saltiel at Parke-Davis (Ann Arbor, MI) (19).

Cell culture and maintenance. MDCK-D_1 cells were cultured and passaged every 3–4 days by trypsinization using trypsin/EDTA as previously described (20). Cells were routinely used for experiments at about 70% confluence, which was usually achieved 2–3 d after the subculture.

[^{3}H]Arachidonic acid release in intact cells. Cells were labeled with [^{3}H]AA by incubation with 0.5 μCi [^{3}H]AA/ml per well for approximately 20 h in 24-well plates. Before stimulation of [^{3}H]AA release, [^{3}H]AA-labeled cells were washed four times with serum- and $NaHCO_3$-free DMEM supplemented with 5 mg/ml BSA and 20 mM Hepes, pH 7.4, and then were incubated in the same medium at 37°C for 15–20 min to equilibrate the temperature. Treatment of cells with agents of interest was initiated by replacing the incubation medium with 1 ml of 37°C medium containing the specified agents. To quantify released [^{3}H]AA after agonist treatment, the incubation medium was quickly aspirated and transferred to ice-cold tubes containing EGTA and EDTA (final concentration 5 mM each). The mixture was centrifuged to eliminate cell debris, and the radioactivity in the supernatant was determined by scintillation spectrophotometry. The resultant pellets were combined with the cells left attached to the plate, which were scraped with 0.2% Triton X-100 and counted as cell-associated radioactivity. Because the cell number and labeled radioactivity were not exactly the same in each well, release of [^{3}H]AA was normalized as percent of incorporated radioactivity (the released radioactivity plus the cell-associated radioactivity at the end of stimulation) for comparison of different treatment conditions.

In vitro assay of PLA_2 activity in cell lysates. MDCK-D_1 cells cultured in 75-cm^2 flasks were washed five times with serum- and $NaHCO_3$-free DMEM supplemented with 2 mg/ml BSA and 20 mM Hepes, (pH 7.4) and equilibrated by incubation with the same medium for 1 h at 37°C. Stimulation was started by adding the specified agonists to the cells, and after 10 min stopped by rapidly aspirating away the incubating medium, 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 DIFP). After washing four times with this ice-cold washing buffer, we scraped cells into an ice-cold PLA_2 assay buffer that was the same as the washing buffer except that sucrose was omitted, and the buffer was supplemented with 100 nM okadaic acid and 1 mM DTT. After a 20 min incubation on ice, the scraped cells were broken by sonication, followed by centrifugation at 4°C for 10 min at 500 *g* to eliminate unbroken cells. The supernatants, defined as cell lysates, were assayed for $cPLA_2$ activity using a previously described protocol with some modifications (21). In brief, the substrate [^{14}C]PC was dried under nitrogen, resuspended in DMSO, vigorously vortexed for 2 min, and resuspended in the assay buffer containing 10 mg/ml BSA and 10 mM $CaCl_2$. The reaction was started by adding 100 μl cell lysate to an equal volume of substrate in a shaking 37°C water bath. The final concentrations of the components in the assay mixture were 10 μM [^{14}C]PC, 5 mM $CaCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 50 mM Hepes (pH 7.4), 10–30 μg protein (determined using a Bradford protein assay kit from Bio-Rad, Hercules, CA), and the phosphatase and protease inhibitors as specified above. After incubating 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 (14), 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. Under these conditions, less than 3% of the substrates were typically hydrolyzed.

Immunoprecipitation of the 85-kDa $cPLA_2$ and assay of its activity in immunoprecipitates. Immunoprecipitation of $cPLA_2$ was performed as described (10). Briefly, 500 μl cell lysate prepared in DTT-free PLA_2 assay buffer (supplemented with protease and phosphatase inhibitors as described above) was incubated in the presence of 1% NP-40 with 1–2 μl of normal or anti-$cPLA_2$ serum for 1 h, followed by incubation with 24 mg BSA-precoated protein A-Sepharose for 1 h. The mixture was then centrifuged and the supernatant was saved for assay of PLA_2 activity, as described above. The resultant antigen-antibody-protein A complex pellets were washed two times with the PLA_2 assay buffer containing 0.5% NP-40, and three times with the same buffer without NP-40. The final pellet was resuspended in assay buffer supplemented with DTT (5 mM) and assessed for PLA_2 activity. All the procedures were performed at 4°C until the final PLA_2 activity assay, which was conducted at 37°C.

Phosphorylation-induced mobility shift, SDS-PAGE, and Western blotting of MAP kinase. Cells cultured in 6-well plates were washed five times with serum- and $NaHCO_3$-free DMEM, supplemented with 2 mg/ml BSA and 20 mM Hepes (pH 7.4), and incubated for 1 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 specified for PLA_2 activity assay. Cells were then scraped and lysed into SDS-PAGE loading buffer, followed by boiling for 5 min. Samples were then subjected to SDS-PAGE using 7.5% acrylamide, followed by transfer to Immobilon-P PVDF membrane. After blocking for 1 h with 5% non-fat dry milk dissolved in PBS, the membrane was incubated with 1:2,000-

3,000 diluted anti-p42 MAP kinase rabbit serum for 1.5 h and then with 1:2,000 diluted horseradish peroxidase-linked donkey anti-rabbit Ig for 1 h, both in 5% non-fat dry milk dissolved in PBS. Each antibody incubation was followed by washing four times with PBS for 5 min. The bands of MAP kinase in the membrane were visualized using the ECL Western blotting detection reagents as instructed by the manufacturer.

MAP kinase activity assay. Cells cultured in 75-cm^2 flasks were washed and equilibrated in DMEM as described above for preparation of cell lysates for PLA_2 activity assay, followed by stimulation with agonists for the indicated times. The stimulation was stopped by 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 PLA_2 activity assay. Cells were then scraped into MAP kinase buffer consisting of 30 mM β-glycerophosphate, 20 mM Hepes (pH 7.4), 4 μM PKI (6–22 amide), 2 mM EGTA, 1 mM EDTA, and the protease and phosphatase inhibitors, as described above. The scraped cells were broken by sonication, followed by centrifugation at 4°C. The resulting supernatant was used as the protein sample for the MAP kinase activity assay. To start the assay for MAP kinase activity, 10 μl of the protein sample was added to an equal volume of substrates and $MgCl_2$ in MAP kinase buffer pre-warmed 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 ml of the reaction mixture to P81 phosphocellulose membrane (2 × 1.5 cm), followed by washing 6 times for 5 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. In some experiments, immunoprecipitation of MAP kinase was performed, and MAP kinase activity associated with immunoprecipitates was similarly assayed. The procedures were the same for the immunoprecipitation of $cPLA_2$ described above, except that MAP kinase buffer and anti-MAP kinase (ERK_2) antiserum were used instead.

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

Results

Activation of $cPLA_2$ is responsible for P_{2U} receptor-mediated AA release in a phosphorylation-dependent manner in MDCK-D_1 cells. MDCK-D_1 cells are a subclone of parental MDCK cells, an epithelial cell line derived from the distal tubule and collecting duct of the canine kidney (23, 24). Recent work from this laboratory has demonstrated the co-existence of P_{2U} and P_{2Y} receptors in MDCK-D_1 cells, both of which are coupled to AA release (25). This cellular release of AA results from activation of PLA_2 by P_2-purinergic receptors, as shown by an increase in PLA_2 activity in lysates prepared from cells treated with ATP or UTP (Fig. 1). To determine which type of PLA_2 was coupled to P_2 receptors in MDCK-D_1 cells, we examined the effect of arachidonyl trifluoromethyl ketone ($AACOCF_3$) on PLA_2 activities. $AACOCF_3$ is a trifluoromethyl ketone analogue of arachidonoyl acid that can inhibit the 85-kD $cPLA_2$, but not the 14-kD low molecular weight secretory form of PLA_2s (26). As shown in Fig. 1, both basal and stimulated PLA_2 activities in MDCK-D_1 cell lysates were inhibited by $AACOCF_3$. $AACOCF_3$ has also been shown to inhibit Ca^{2+}-independent PLA_2 (27). However, PLA_2 activity in MDCK-D_1 cells is Ca^{2+}-dependent (10), and ATP- or UTP-promoted AA release in intact MDCK-D_1 cells is dependent on Ca^{2+} (25).

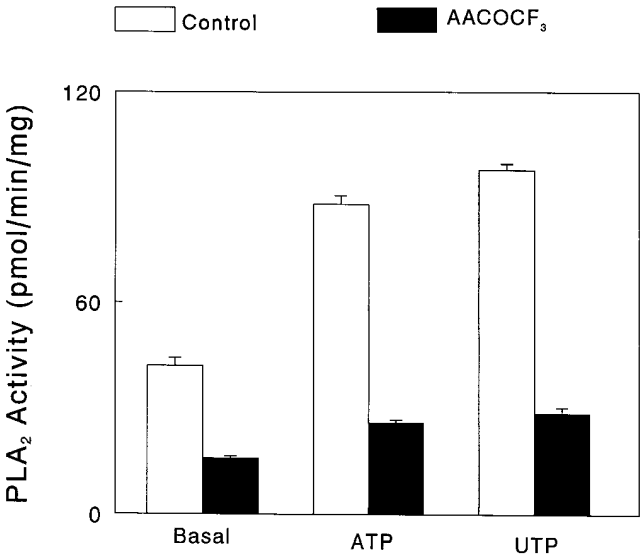

Figure 1. Effect of $AACOCF_3$ on ATP- or UTP-mediated activation of PLA_2 in MDCK-D_1 cells. Cells were treated with 100 μM ATP or UTP or the corresponding vehicle (*Basal*) for 10 min, followed by preparation of lysate protein samples. PLA_2 activities of the cell lysates were assessed in the absence (*Control*) or presence of 30 μM $AACOCF_3$. The experimental details for cell lysate preparation and PLA_2 activity assay are described in Methods.

Therefore, Ca^{2+}-independent PLA_2 appears not to contribute to P_2 receptor-promoted PLA_2 activity and AA release in these cells. Furthermore, anti-$cPLA_2$ serum immunoprecipitated increased PLA_2 activity from cells stimulated by ATP or UTP in parallel with a depletion of PLA_2 activity in the cell lysate (Fig. 2). Recovery of PLA_2 activity was associated with immunoprecipitates obtained with anti-$cPLA_2$ serum but not with normal serum. In addition, SDS-PAGE and immunoblotting studies showed that $cPLA_2$ protein was associated with immunoprecipitates obtained with anti-$cPLA_2$ serum but not with normal serum (10). Although the effect of ATP observed in the present study might be mediated through both P_{2U} and P_{2Y} receptors, the effect of UTP is mediated by P_{2U} receptors (25). Thus, these data (taken together with the finding that $AACOCF_3$ also inhibits ATP- or UTP-promoted AA release in intact MDCK-D_1 cells) (25) demonstrate that $cPLA_2$ is the PLA_2 species present in these cells that is responsible for AA release mediated by P_{2U}-purinergic receptors.

Since phosphorylation is thought to be an important mechanism for $cPLA_2$ activation (4, 5), we asked whether such a mechanism is involved in the activation of $cPLA_2$ by P_{2U} receptors in MDCK-D_1 cells by incubating lysates (prepared from MDCK-D_1 cells treated with ATP or UTP with potato acid phosphatase) and then assessing PLA_2 activity. As shown in Fig. 3, treatment of cell lysates with phosphatase decreased basal $cPLA_2$ activity, suggesting that a basal phosphorylation state maintains $cPLA_2$ activity at a higher level. Treatment of cell lysates with phosphatase also inhibited ATP- or UTP-stimulated activity of $cPLA_2$. Treatment of cell lysates with potato acid phosphatase did not affect the quantity of $cPLA_2$ protein, as determined by SDS-PAGE and immunoblotting studies. Thus, phosphorylation is involved in the activation of $cPLA_2$ by P_{2U} receptors.

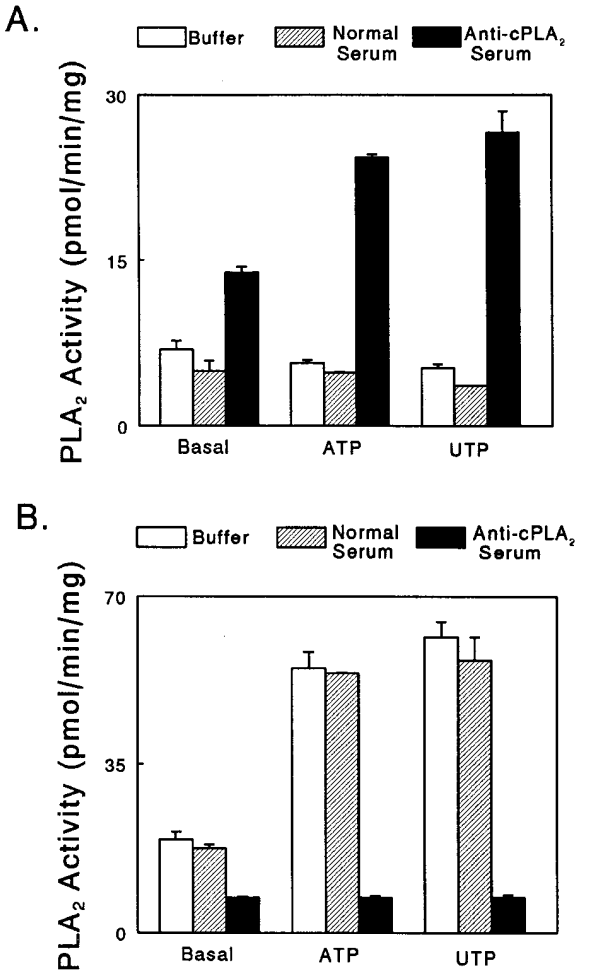

Figure 2. PLA_2 activity in immunoprecipitates and immunodepleted lysates of MDCK-D_1 cells. Lysates derived from cells treated with vehicle (*Basal*) or 100 μM ATP or UTP for 10 min were subjected to immunoprecipitation with buffer, normal serum, or anti-$cPLA_2$ serum, followed by the assay of PLA_2 activity associated with the resulting immunoprecipitates (*A*) or in the immunodepleted lysates (*B*) as described in Methods. In some experiments the normal serum partially inhibited PLA_2 activity in cell lysates (data not shown).

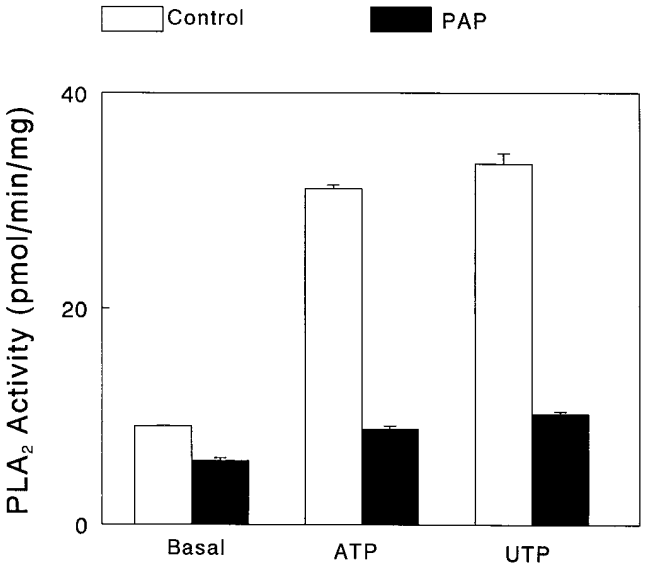

Figure 3. Effect of potato acid phosphatase on $cPLA_2$ activities in MDCK-D_1 cell lysates. Lysates were prepared from cells incubated with vehicle (*Basal*) or 100 μM ATP or UTP for 10 min and treated without (*Control*) or with 1 U/ml potato acid phosphatase (*PAP*) at 30°C (pH 6.4) for 30 min in the absence of phosphatase inhibitors and in the 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 to 7.4 and the phosphatase inhibitors were added back to the cell lysates, PLA_2 activities were assessed at 37°C, as described in Methods.

PKC-dependent activation of MAP kinase by P_{2U} receptors in MDCK-D_1 cells. Subsequent studies were focused on the nature of kinases involved in activation of $cPLA_2$. We first examined whether P_{2U} receptors are coupled to activation of MAP kinase in MDCK-D_1 cells. As shown in Fig. 4, treatment of cells with ATP or UTP increased MAP kinase activity measured in subsequently prepared cell lysates. The activation was time-dependent, and the peak response occurred 3 min after treatment with agonists. Immunoprecipitated MAP kinase, particularly ERK_2 (which is the type of MAP kinase that has been specifically shown to phosphorylate and activate recombinant $cPLA_2$ in vitro [7]) showed increased enzyme activity in response to stimulation by ATP or UTP. A molecular weight shift of MAP kinase resolved by SDS-PAGE was also observed in response to stimulation by ATP or UTP. As shown in the inset of Fig. 4, this molecular shift occurred in a time-dependent manner and, as with the increase in enzyme activity, the maximal shift occurred 3 min after stimulation with ATP. UTP induced a similar time-dependent molecular shift of MAP kinase (data not shown). The molecular shift of MAP kinase has been shown to be due to kinase phosphorylation (9), a conclusion consistent with results obtained for ATP-, UTP- or PMA-induced molecular shift of MAP kinase in MDCK-D_1 cells, and with the abolition of this shift by treatment of protein samples with potato acid phosphatase (Fig. 5 *A*). Activation of PKC by PMA increased MAP kinase activity in MDCK-D_1 cells (10), and induced a phosphorylation-dependent molecular shift of this kinase (Fig. 5 *A*). As shown in Fig. 5 *B*, the molecular weight shift of MAP kinase induced by ATP or UTP (or PMA as a positive control) was blocked by down-regulation of PKC by overnight treatment of cells with PMA. These data, therefore, suggest that coupling of P_{2U} receptors to MAP kinase is dependent on PKC in MDCK-D_1 cells.

It has been established that phosphorylation of MAP kinase is an obligatory step in the activation of this kinase (9). Indeed, the phosphorylation state of MAP kinase closely correlated with its activity state in response to agonist stimulation in MDCK-D_1 cells (Fig. 4, *inset*). The phosphorylation-based molecular weight shift of MAP kinase, which has been used by many investigators (10, 14, 28, 29) as a convenient and reliable indication of MAP kinase activation, was used in the following studies as a measure of activation of this kinase.

MAP kinase and PKC are both required for $cPLA_2$-mediated arachidonic acid release by P_{2U} receptors in MDCK-D_1 cells. To determine if MAP kinase was required for $cPLA_2$-mediated AA release by P_{2U} receptors in MDCK-D_1 cells, we tested the effect of the MAP kinase cascade inhibitor

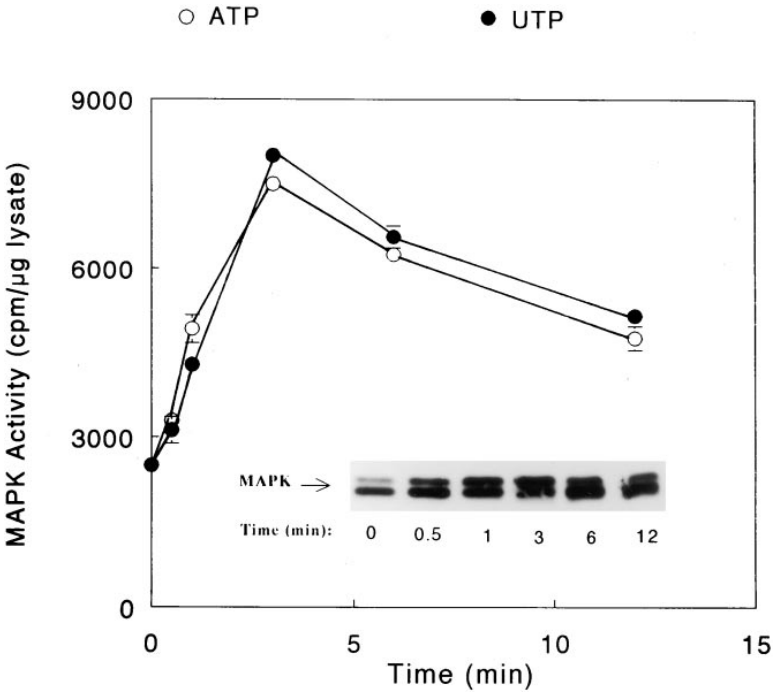

Figure 4. Enzyme activation and molecular weight shift of MAP kinase in MDCK-D_1 cells. For MAP kinase activity assay, lysates were prepared from cells incubated in the absence (*0 time point*) or presence of 100 μM ATP or UTP for the indicated times. Cell lysates were then subjected to MAP kinase activity assay, as described in Methods. For the evaluation of molecular weight shift (*Inset*), cells were similarly treated without (*0 time point*) or with 100 μM ATP for the indicated times, followed by lysis into SDS loading buffer, boiling for 5 min, SDS-PAGE, immunoblotting with anti-p42 MAP kinase serum, and detection by ECL as described in Methods. Note the double band of MAP kinase (*MAPK*). Compared with basal state (*0 time point*), the intensity of the upper band was increased by treatment of cells with ATP. This increase occurred maximally at 3 min of treatment.

PD098059 (19) on ATP- or UTP-mediated MAP kinase activation, and AA release in these cells. As shown in Fig. 6 *A*, ATP- or UTP-promoted MAP kinase activation was blocked by 30 μM PD098059. Under the same conditions, AA release promoted by ATP or UTP was suppressed by 82±11% and 68±11% (mean±SD of three experiments performed in duplicate), respectively. Fig. 6 *B* shows a representative experiment.

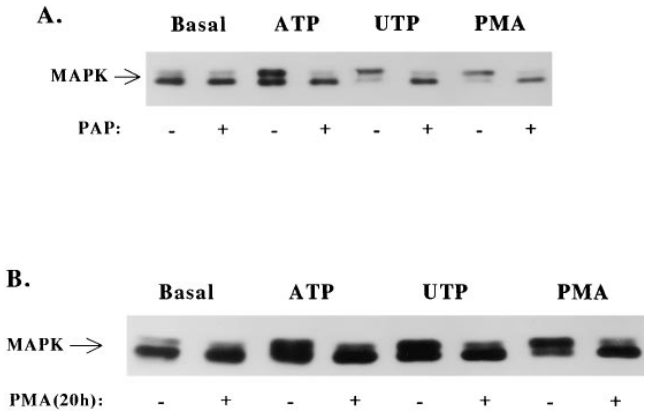

Figure 5. Effect of potato acid phosphatase (*A*) and PKC down-regulation (*B*) on molecular weight shift of MAP kinase in MDCK-D_1 cells. (*A*) Lysates derived from cells incubated with vehicle (*Basal*) or 100 μM ATP or 100 μM UTP or 100 nM PMA for 3 min were treated in the absence (−) or presence (+) of potato acid phosphatase (*PAP*) as described in the legend to Fig. 3. The lysates were then mixed with appropriately concentrated SDS loading buffer, boiled, and subjected to SDS-PAGE and immunoblotting as described in the legend to Fig. 4. (*B*) Cells were treated with vehicle (−) or 200 nM PMA (+) overnight (*20 h*) to down-regulate PKC. Cells were then incubated with the indicated agonists for 3 min, followed by preparation of protein sample in SDS loading buffer, SDS-PAGE, and immunoblotting.

These data thus suggest that MAP kinase is at least partially required for $cPLA_2$-mediated AA release promoted by P_{2U} receptors in MDCK-D_1 cells.

To determine whether PKC is required for the activation of $cPLA_2$ by P_{2U} receptors, we next examined the effects of PKC down-regulation and treatment of cells with the PKC inhibitor GF109203X (30, 31) on P_{2U} receptor-mediated AA release. Both PKC down-regulation (Fig. 7 *A*) and GF109203X (Fig. 7 *B*) suppressed ATP- or UTP-mediated AA release. AA release promoted by the Ca^{2+} ionophore A23187 was not inhibited by PKC down-regulation or treatment of cells with GF109203X (Fig. 7), suggesting that inhibition of ATP- or UTP-promoted AA release by these experimental strategies was specific for these nucleotides. Therefore, in addition to MAP kinase, PKC is also required for P_{2U} receptor regulation of $cPLA_2$-mediated AA release in MDCK-D_1 cells.

PKC is independently required for $cPLA_2$-mediated AA release through a pathway in parallel to MAP kinase activation. Since activation of MAP kinase by P_{2U} receptors was PKC-dependent (Fig. 5 *B*) and activation of $cPLA_2$ by this receptor involved MAP kinase (Fig. 6), the involvement of PKC in $cPLA_2$ regulation appears to be at least partially through activation of MAP kinase in MDCK-D_1 cells. However, the data presented above do not rule out the possibility that PKC may also play a role independent of MAP kinase in the regulation of $cPLA_2$. Indeed, we have obtained evidence for such a role for PKC in P_{2U} receptor-mediated AA release. This evidence includes the different concentration-response relationships of the PKC inhibitor GF109203X on MAP kinase activation and AA release. As shown in Fig. 8 *A*, GF109203X inhibited both ATP- and UTP-promoted AA release in a concentration-dependent manner. About 50% of ATP- or UTP-promoted response was inhibited by this inhibitor at 3 μM. However, the inhibitory effect of GF109203X on MAP kinase activation by these nucleotides was not as dramatic (Fig. 8 *B*): at 3 μM, this inhibitor

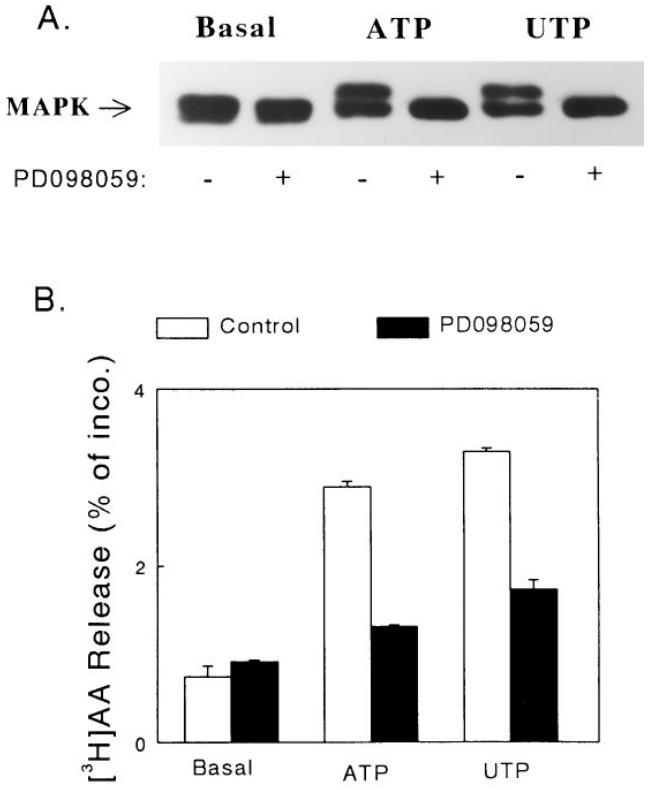

Figure 6. Effect of PD098059 on MAP kinase activation (*A*) and $cPLA_2$-mediated AA release (*B*) in MDCK-D_1 cells. (*A*) Cells were treated with vehicle (−) or 30 μM PD098059 (+) for 20 min and further treated with vehicle (*Basal*) or 100 μM ATP or UTP for 3 min, followed by preparation of protein samples in SDS loading buffer, SDS-PAGE and immunoblotting as described in the legend to Figure 4. (*B*) [^{3}H]AA-labeled cells were treated with vehicle (*Control*) or 30 uM PD098059 for 20 min, followed by assessment of [^{3}H]AA release in response to stimulation with vehicle (*Basal*) or 100 μM ATP or UTP for 10 min, as described in Methods. PD098059 had no direct inhibitory effect on immunoprecipitated $cPLA_2$ activity (*data not shown*).

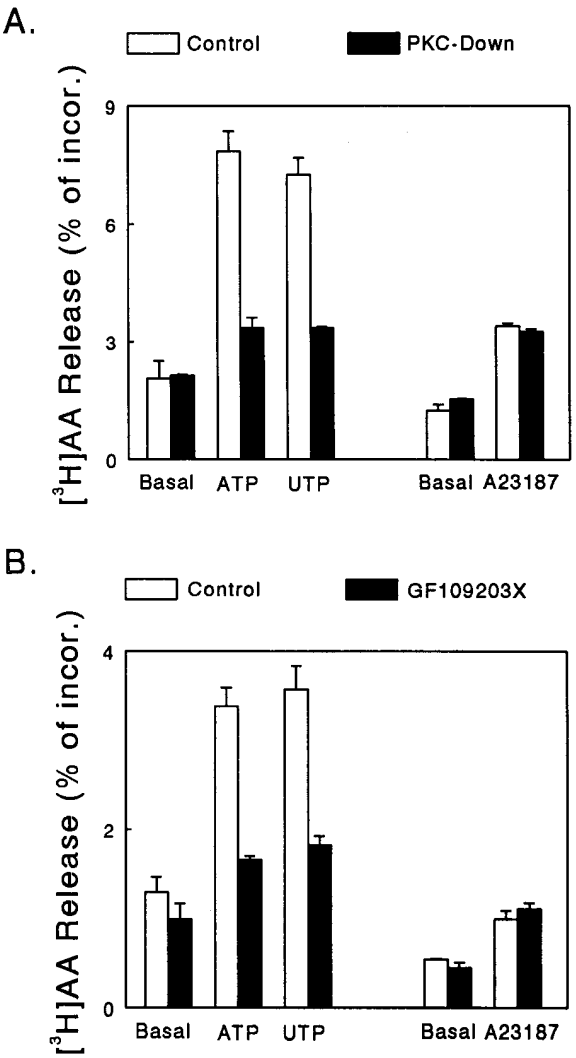

Figure 7. Effects of PKC down-regulation (*A*) and GF109203X (*B*) on $cPLA_2$-mediated AA release in MDCK-D_1 cells. (*A*) Cells without (*Control*) or with PKC down-regulation (*PKC-Down*) as described in the legend to Fig. 5 *B* were assessed for [^{3}H]AA release in response to treatment with vehicle (*Basal*), 100 μM ATP or UTP, or 4 μM A23187 for 10 min as described in Methods. (*B*) Cells were incubated with vehicle (*Control*) or 12 μM GF109203X for 30 min, followed by assessment of [^{3}H]AA release in response to treatment with the vehicle or the indicated agonists for 10 min, as described in Methods. The data with A23187 treatment and the corresponding basal conditions were obtained from separate experiments. GF109203X had no direct inhibitory effect on immunoprecipitated $cPLA_2$ activity (data not shown).

did not decrease the activation of MAP kinase mediated by ATP or UTP, although it showed a slight inhibitory effect at a higher concentration (9 μM). A similar concentration response discrepancy was observed for the effects of GF109203X on PMA-promoted AA release and MAP kinase activation (Fig. 9), although the inhibitor seemed more potent in inhibition of the response mediated by PMA than in inhibition of that mediated by nucleotides. At 0.33 μM, GF109203X inhibited greater than 50% of PMA-promoted AA release (Fig. 9 *A*), while it showed no effect on PMA-promoted MAP kinase activation at this concentration (Fig. 9 *B*). At 1 μM, this inhibitor completely blocked PMA-promoted AA release, although it had only a minimal inhibitory effect on PMA-promoted MAP kinase activation (Fig. 9 *B*). These data demonstrate that even under the conditions where MAP kinase can be activated, inhibition of PKC still suppresses $cPLA_2$-mediated AA release. Therefore, in addition to its involvement in MAP kinase activation required for $cPLA_2$ activation, PKC also may be independently required for $cPLA_2$ activation by P_{2U} receptors in MDCK-D_1 cells.

In previous work from this laboratory, stable PKC antisense transfectants of MDCK-D_1 cells were isolated that had decreased PKC_α protein, and which displayed a decrease in PMA-mediated AA release (32). As shown in Fig. 10 *A*, when we tested these transfectants in the present study, they had a 50% (as determined by densitometry) reduction of PKC_α protein compared with vector-transfected cells, but no change in MAP kinase protein level (Fig. 10 *B*). There were also no changes in the protein levels of PKCε and PKCζ (data not shown). The reduction of PKC_α protein level did not affect activation of MAP kinase by PMA (Fig. 10 *C*), although PMA-promoted AA release was inhibited by suppression of the ex-

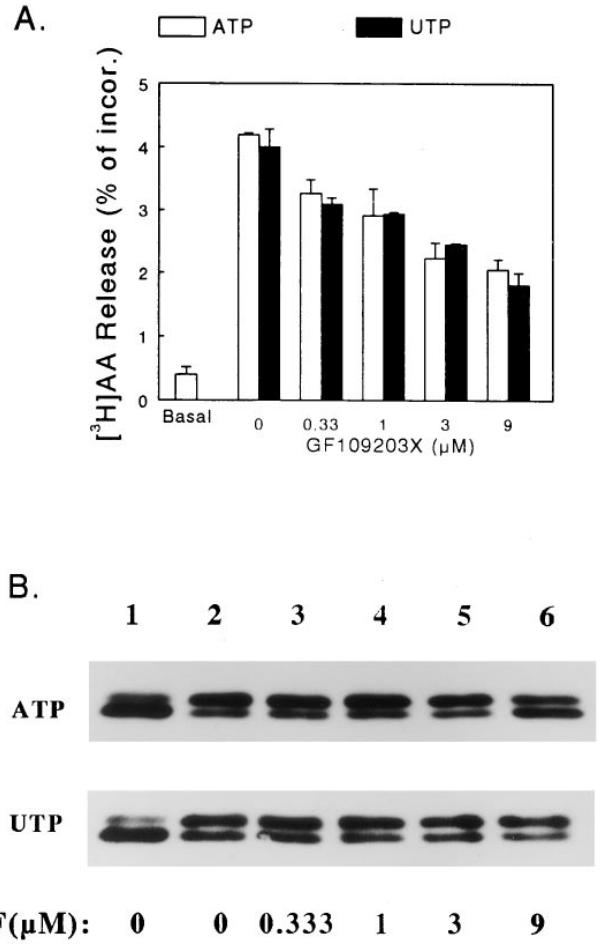

Figure 8. Concentration-response of the effects of GF109203X on P_{2U} receptor-mediated AA release (*A*) and MAP kinase activation (*B*) in MDCK-D_1 cells. (*A*) Cells were treated with GF109203X at the indicated concentrations for 30 min, followed by assessment of [^{3}H]AA release in response to stimulation with vehicle (*Basal*) or 100 μM ATP or UTP for 10 min, as described in Methods. (*B*) Cells were treated with GF109203X (*GF*) at the indicated concentrations for 30 min, followed by further treatment with vehicle (*lane 1*), or 100 μM ATP or UTP for 3 min (*lanes 2–6*) as indicated. Protein samples were then prepared in SDS loading (buffer, followed by SDS-PAGE and immunoblotting as described in the legend to Fig. 4.

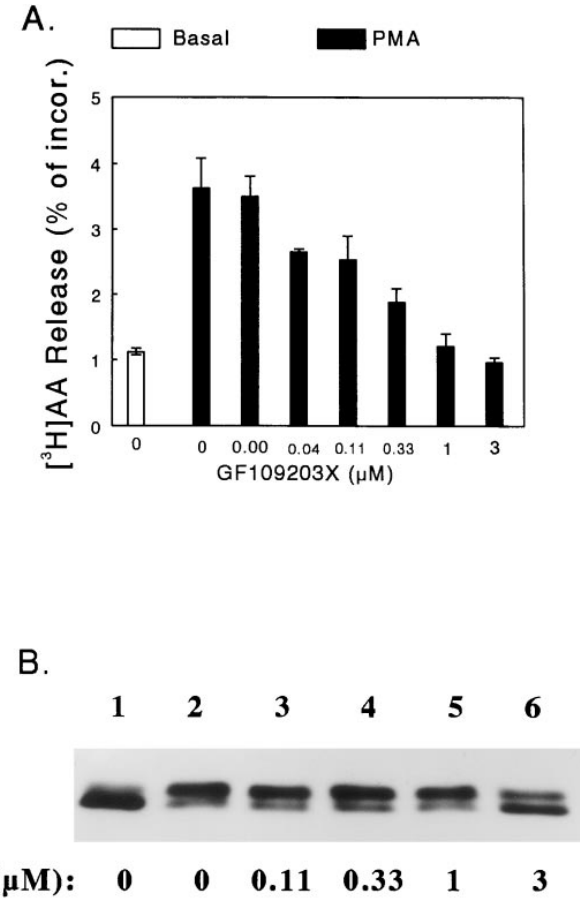

Figure 9. Concentration response of the effects of GF109203X on PMA-mediated AA release (*A*) and MAP kinase activation (*B*) in MDCK-D_1 cells. The experimental procedures were same as described in the legend to Fig. 8, except that PMA was used as agonist.

pression of PKC_α (Fig. 10 *D*). Because PMA-promoted AA release is also mediated by $cPLA_2$ in MDCK-D_1 cells (10), the present data obtained with PMA in these stable transfectants further support the idea that PKC, particularly the α isoform, acts independently of MAP kinase to regulate $cPLA_2$ in MDCK-D_1 cells. Studies of P_{2U} receptor-promoted AA release in these transfectants yielded inconsistent findings, perhaps because PKC_α may be involved in feedback regulation of P_{2U} receptor signaling as well as in the regulation of $cPLA_2$. Alternatively, PKC_α in the transfectants used in the present study was only decreased by 50% (Fig. 10 *A*). This decrease might not be enough to significantly affect the coupling of P_{2U} receptors to AA release, but enough to affect the release of AA promoted by PMA. This difference in the sensitivity of AA release to the decrease in PKC_α protein is consistent with the data that the PKC_α-selective inhibitor GF109203X was more potent in inhibiting PMA-promoted AA release than in inhibiting P_{2U} receptor-promoted AA release (Figs. 8 and 9).

Discussion

Considerable attention has been devoted to defining signal transduction by the G protein-coupled P_{2U}/P_{2Y} receptors because of their wide distribution and involvement in a variety of biological activities (33, 34). One important consequence of stimulation of these receptors is AA release, which may play a key role in many of the physiological and pathological processes mediated by these receptors. AA release by P_{2U}/P_{2Y} receptors has been described in numerous cells, including, for example, astrocytes (35), neutrophils and neutrophil-like HL60 cells (14, 36), NG108-15 neuroblastoma cells (37), FRTL-5 thyroid cells (38), airway epithelium cells (39), Chinese hamster ovary cells (40), and MDCK cells (25, 41). Although considerable effort has been directed toward defining the molecular mechanism for P_2-purinergic receptor-mediated AA release, clear-cut information has not been provided. It has been hypothesized that $cPLA_2$ is responsible for P_2-purinergic receptor-mediated AA release (25), but definitive evidence for this hypothesis is lacking. Moreover, there is evidence suggesting that other types of PLA_2 (such as the Ca^{2+}-independent

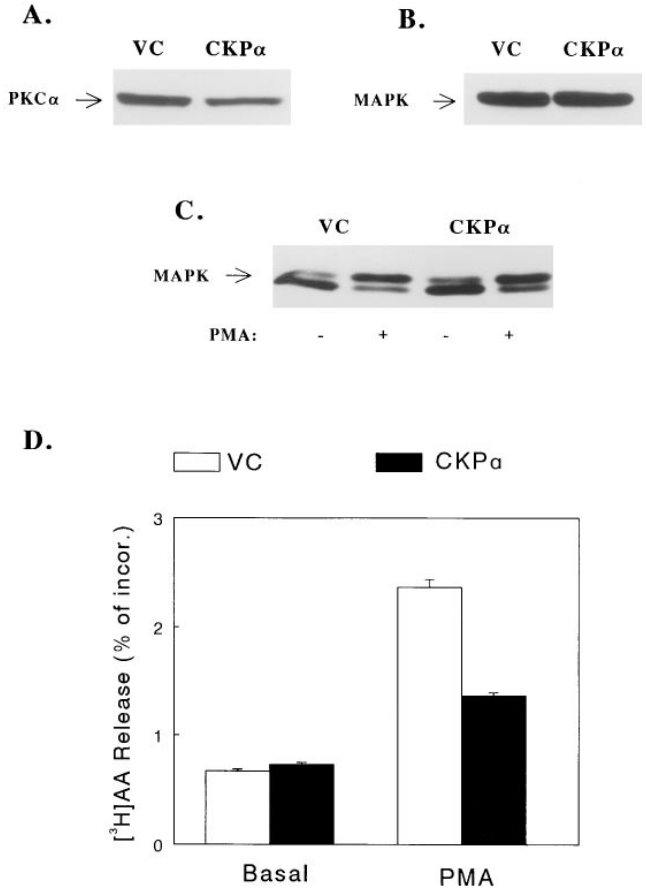

Figure 10. Effect of decreased expression of PKCα on the protein levels of PKCα (*A*) and MAP kinase (*B*), and on activation of MAP kinase (*C*) and AA release (*D*) in MDCK-D_1 cells. MDCK-D_1 transfectants carrying the vector (*VC*) or PKCα antisense cDNA (*CKPα*) were originally isolated as described (32). The protein levels of PKCα (*A*) and MAP kinase (*B*) in these transfectants were analyzed by SDS-PAGE and immunoblotting using anti-PKCα antibodies and anti-ERK_2 antibodies, respectively. To avoid the separation of the phosphorylated species from the dephosphorylated species of MAP kinase (and hence to more accurately compare the total protein of this kinase), in the experiment shown in *B*, 10%, instead of 7.5% acrylamide was used in the gel, and the gel was run for 2 h. (*C*) Cells were stimulated with vehicle (−) or 100 nM PMA (+) for 3 min, and activation of MAP kinase (in terms of molecular weight shift) was analyzed as described in the legend to Fig. 4. (*D*) Basal or 100 nM PMA-promoted [^{3}H]AA release in vector- or PKCα antisense cDNA-transfected cells were assessed as described in Methods.

PLA_2 [42] and the secretory PLA_2 [43, 44]) can be responsible for AA release mediated by certain receptors. In view of these results, one of the goals of the present study was to use MDCK-D_1 cells as a model to define whether $cPLA_2$ mediates AA release promoted by P_2-purinergic receptors, in particular P_{2U} receptors. The present data demonstrate that AA release by P_{2U} receptors in MDCK-D_1 cells is mediated by $cPLA_2$, a conclusion supported by the following findings: (*a*) PLA_2 activity in MDCK-D_1 cell lysates is Ca^{2+}-dependent and sensitive to $AACOCF_3$ (10), suggesting that the $cPLA_2$ is the type of PLA_2 preferentially expressed in these cells; (*b*) P_{2U} receptor-mediated AA release in intact cells (25) and activated PLA_2 activity in cell lysates are $AACOCF_3$-sensitive (Fig. 1); and (*c*) increased PLA_2 activity promoted by ATP or UTP is recovered in immunoprecipitates obtained using anti-$cPLA_2$ antibodies, and the PLA_2 activity is correspondingly lost in the immunodepleted cell lysates (Fig. 2).

Phosphorylation has been implicated for the activation of both in vitro recombinant $cPLA_2$ (6, 7) and in vivo endogenous $cPLA_2$ (e.g., 10–12, 45). The coupling of P_{2U} receptors to activation of $cPLA_2$ in MDCK-D_1 cells is apparently also through a phosphorylation-dependent mechanism, as suggested by the ATP- or UTP-promoted increase in $cPLA_2$ activity in lysates (Figs. 1–3) and in anti-$cPLA_2$ immunoprecipitates (Fig. 2 *A*) prepared from treated cells, and as suggested by the sensitivity of this increase of $cPLA_2$ activity to phosphatase (Fig. 3). Previous work indicated that in neutrophil-like HL60 cells P_{2U} receptors are coupled to both MAP kinase activation and AA release (14). However, a cause-effect relationship for the two responses was not established. The data in the present study show that MAP kinase is required for $cPLA_2$ activation by P_{2U} receptors since inhibition of MAP kinase by PD098059 (Fig. 6 *A*) inhibited $cPLA_2$-mediated AA release by P_{2U} receptors (Fig. 6 *B*). In addition, PKC is also required for P_{2U} receptor regulation of $cPLA_2$-mediated AA release. This PKC involvement occurs in two ways: (*a*) it is required for MAP kinase activation because down-regulation of PKC blocks P_{2U} receptor-promoted MAP kinase activation (Fig. 5 *B*), and (*b*) it is also required for $cPLA_2$-mediated AA release through a pathway that is parallel to MAP kinase activation since we were able to inhibit PKC and suppress AA release without blocking MAP kinase activation (Figs. 8–10).

The PKC inhibitor GF109203X is more selective for the α isoform than for other PKC isoforms (30, 31). Its differential effects on MAP kinase activation and $cPLA_2$-mediated AA release observed in the present study could occur if different PKC isoforms are selectively involved in activation of MAP kinase and $cPLA_2$. Thus, the PKC isoforms involved in the activation of MAP kinase by P_{2U} receptors might be other than the α type (as suggested in other systems [46, 47]), while PKC_α would be required for activation of $cPLA_2$ through a pathway independent of MAP kinase. Thus, by selectively inhibiting PKC_α, GF109203X at low concentrations would suppress $cPLA_2$-mediated AA release, but not activation of MAP kinase. This idea is also supported by the data obtained with MDCK-D_1 transfectants with decreased PKC_α, which showed inhibition of $cPLA_2$-mediated AA release, but not of MAP kinase activation (Fig. 10). It should be pointed out that our interpretation of the requirement for PKC_α and MAP kinase in the regulation of $cPLA_2$ is different from previous conclusions, which only emphasize the role of MAP kinase in the activation of $cPLA_2$ (6). We hypothesize that activation of both PKC (particularly the α isoform) and MAP kinase (mediated by PKC isoforms other than the α form) is required for maximal activation of $cPLA_2$, because inhibition of either impaired $cPLA_2$-mediated AA release (Figs. 6–10). This hypothesis of dual regulation of $cPLA_2$ by MAP kinase and PKC_α for maximal activation is consistent with other observations, such as the demonstration that $cPLA_2$ possesses distinct phosphorylation sites for MAP kinase and PKC (6), and that in Chinese hamster ovary cells, transfection of a mutant $G_{\alpha i2}$ inhibits $cPLA_2$-mediated AA release by P_2-purinergic receptors without affecting MAP kinase activation and $cPLA_2$ phosphorylation by MAP kinase (40). In the latter studies, activation of

PKC by PMA was able to eliminate this inhibition and restore the coupling of P_2-purinergic receptors to $cPLA_2$, thus suggesting that this $G_{\alpha i2}$ mutant inhibited P_2-purinergic receptor-coupled activation of PKC, and blocked activation of $cPLA_2$ by these receptors.

Direct phosphorylation and activation of $cPLA_2$ by MAP kinase has been consistently demonstrated by in vitro studies (6, 7). By contrast, in vitro studies are inconsistent with regard to the importance of PKC for the activation of $cPLA_2$. One study showed a moderate increase in $cPLA_2$ activity after in vitro phosphorylation of this lipase by PKC (7), but two other studies failed to show activation of lipase activity after such phosphorylation (6, 48). Although there is no clear explanation for this inconsistency, the role of different PKC isoforms was not evaluated in these studies, nor was the MAP kinase-mediated phosphorylation level of $cPLA_2$ that existed prior to assessment of the function of phosphorylation by PKC. This could explain the inconsistency of the results in these studies, since we hypothesize that phosphorylation of $cPLA_2$ by both MAP kinase and PKC is required for maximal activation of this lipase.

In MDCK-D_1 cells, both $alpha_1$-adrenergic receptors (10) and P_{2U} receptors (present study) utilize MAP kinase to regulate $cPLA_2$. However, unlike the regulation of $cPLA_2$ by P_{2U} receptors (which utilize different PKC isoforms through MAP kinase-dependent and -independent pathways to achieve maximal activation of $cPLA_2$), regulation of $cPLA_2$ by α_1-adrenergic receptors occurs only through sequential activation of PKC and MAP kinase. Interestingly, in the same cell type, bradykinin receptors regulate $cPLA_2$ through a phosphorylation-dependent mechanism that does not involve MAP kinase and PKC, but may involve a tyrosine kinase (49). Several recent studies also show that certain receptors can promote phosphorylation-dependent activation of $cPLA_2$ without involvement of MAP kinase and PKC, but the kinases involved remain unidentified (15, 16). It appears, therefore, that the mechanisms and the protein kinases involved in the regulation of $cPLA_2$ vary with receptor types, even in the same cell type.

In summary, in the present study we demonstrate that in MDCK-D_1 cells' P_{2U}-purinergic receptors mediate AA release via activation of $cPLA_2$. This $cPLA_2$-mediated AA release by P_{2U} receptors is through a phosphorylation mechanism which appears to require activation of both MAP kinase and PKC. In this process PKC plays a dual role: it is required for MAP kinase activation, and it also acts through a pathway independent of MAP kinase to regulate $cPLA_2$-mediated AA release. Involvement of different PKC isoforms may explain this dual role of PKC. We speculate that dual phosphorylation of $cPLA_2$ by PKC_α and MAP kinase (perhaps activated by PKC isoforms other than PKC_α) is required for the maximal activation of $cPLA_2$ by P_{2U} receptors in MDCK-D_1 cells. This dual requirement for PKC and MAP kinase in the activation of $cPLA_2$ is not consistent with previous ideas that have only emphasized the role of the sequential activation of PKC and MAP kinase in the regulation of $cPLA_2$.

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