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Involvement of Mitogen-Activated Protein Kinase and Translocation of Cytosolic Phospholipase A_2 to the Nuclear Envelope in Acetylcholine-Induced Prostacyclin Synthesis in Rabbit Coronary Endothelial Cells

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SUMMARY

We previously showed that acetylcholine (ACh) stimulates production of prostacyclin, measured as immunoreactive 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), in coronary endothelial cells (CEC) of rabbit heart by increasing influx of extracellular Ca^{2+} through a receptor-operated Ca^{2+} channel and by activating a pertussis toxin-insensitive G protein. The purposes of this study were to elucidate the type of phospholipase A_2 (PLA_2) involved in 6-keto-$PGF_{1\alpha}$ production and the mechanism(s) by which ACh activates PLA_2 in cultured CEC. In CEC transiently transfected with cytosolic PLA_2 but not secretory PLA_2 antisense oligonucleotide, ACh failed to increase 6-keto-$PGF_{1\alpha}$; this was prevented by cotransfection with $cPLA_2$ sense oligonucleotide. ACh increased production of prostacyclin and increased protein kinase C (PKC) activity. The PKC inhibitor calphostin C attenuated the ACh-induced increase in PKC activity but not 6-keto-$PGF_{1\alpha}$ production. Phorbol-12-myristate-13-acetate and phorbol-12,13-dibutyrate increased PKC activity but failed to alter 6-keto-$PGF_{1\alpha}$ production. ACh enhanced the activity of $cPLA_2$ and p42 mitogen-activated protein kinase (MAPK) in cell lysate prepared from CEC. ACh also caused phosphorylation of p42 MAPK and $cPLA_2$, which was inhibited by AG126 {[α-cyano-(3-hydroxy-4-nitro)cinnamonitrile]}, a tyrosine kinase inhibitor known to decrease MAPK activity. In addition, ACh stimulated translocation of $cPLA_2$ from cytosol to nuclear envelope; the translocation of $cPLA_2$ was prevented by removal of extracellular calcium but not by AG126 treatment. Okadaic acid, a protein phosphatase inhibitor, increased $cPLA_2$ activity in cell lysate prepared from CEC but did not alter basal 6-keto-$PGF_{1\alpha}$ production in intact CEC; however, ACh-induced 6-keto-$PGF_{1\alpha}$ was enhanced by okadaic acid. These data suggest that ACh stimulates prostacyclin synthesis by activation of $cPLA_2$ in a PKC-independent mechanism and that both $cPLA_2$ translocation to nuclear envelope and phosphorylation by MAPK are required for ACh-induced 6-keto-$PGF_{1\alpha}$ synthesis in CEC.

Cholinergic nerve stimulation or ACh stimulates prostaglandin synthesis in the heart (1, 2). PGI_2, the major prostanoid formed in the heart by ACh, may contribute to its cardioprotective effects by producing coronary vasodilation (3), inhibiting platelet aggregation (4), inhibiting release of norepinephrine (5, 6), and decreasing free radical generation (7). ACh stimulates PGI_2 synthesis in the rabbit heart via activation of M_2 and M_3 muscarinic receptors in ventricular myocytes and M_3 muscarinic receptor in CEC (8). PGI_2 is derived from AA, which has been reported to be released consequent to activation of one or more lipase, mainly PLA_2 (9–11).

In mammalian cells, two types of Ca^{2+}-dependent PLA_2, $cPLA_2$ and $sPLA_2$, have been implicated in the release of AA, the precursor for PGI_2 synthesis, in response to various stimuli (9–11). $sPLA_2$ requires millimolar levels of Ca^{2+}, can hydrolyze a variety of unsaturated fatty acids from the 2′ position of glycerophospholipids, and is sensitive to reducing conditions (12). $cPLA_2$ can be activated at micromolar Ca^{2+} concentrations (μM) and preferentially hydrolyzes AA in the *sn*-2 position of phospholipids, and its activity is not affected by disulfide-reducing agents (12). Recent studies have shown

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ABBREVIATIONS: ACh, acetylcholine; PGI_2, prostacyclin; CEC, coronary endothelial cells; $cPLA_2$, cytosolic phospholipase A_2; $sPLA_2$, secretory phospholipase A_2; PKC, protein kinase C; PDBu, phorbol-12,13-dibutyrate; PMA, phorbol-12-myristate-13-acetate; MAPK, mitogen-activated protein kinase; AA, arachidonic acid; mAChR, muscarinic acetylcholine receptor; PLC, phospholipase C; BSS, balanced salt solution; HBSS, Hanks' balanced salt solution; FBS, fetal bovine serum; DTT, dithiothreitol; PBS, phosphate-buffered saline; $PGF_{1\alpha}$, prostaglandin $F_{1\alpha}$; AS, antisense; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; TRITC, tetrarhodamine isothiocyanate.

that $cPLA_2$ activity is regulated by phosphorylation via p42 MAPK activation; this process is protein kinase C dependent or independent with different stimuli and cell types (13, 14). However, in human platelets, it has been reported that a proline-directed kinase other than MAPK might phosphorylate and activate $cPLA_2$ in response to the thrombin agonist SFLLRN (15). On the other hand, in rabbit vascular smooth muscle cells, norepinephrine-induced release of AA has been shown to be due to stimulation of $cPLA_2$ by MAPK via calmodulin-dependent kinase activation (16). $cPLA_2$ has a Ca^{2+}-dependent phospholipid-binding domain in the amino-terminal region, and in the presence of submicromolar concentrations of Ca^{2+}, it translocates from cytosol to cell membranes (17, 18). In rat nonstimulated basophilic leukemia cells, $cPLA_2$ is distributed throughout the cytosol and is excluded from the nucleoplasm; once cells are stimulated with calcium ionophore or IgE/antigen, most of the enzyme is found around the nuclear envelope, which contains the substrate for PLA_2 and the enzymes that oxygenate AA (19). The type of PLA_2 involved in ACh-induced PGI_2 synthesis is not known. Also, it is not known whether ACh stimulates PLA_2 via phosphorylation by MAPK and/or calmodulin-dependent kinase and causes its translocation to nuclear or plasma membrane.

In a previous study, we demonstrated that ACh-stimulated PGI_2 production in rabbit CEC occurs via activation of mAChRs (8), which results in increased influx of extracellular Ca^{2+} through a receptor-operated Ca^{2+} channel and does not involve PLC or nitric oxide (20). The purpose of this study was to characterize the subtype of PLA_2 involved and to determine the possible contribution of MAPK and calmodulin-dependent kinase in its activation for PGI_2 production in the CEC of rabbit heart. Furthermore, the role of $cPLA_2$ phosphorylation and its possible translocation from cytosol to nuclear envelope or plasma membrane in response to ACh were investigated.

Experimental Procedures

Materials

ACh and calphostin C were purchased from Research Biochemicals (Natick, MA). AG126 [[α-cyano-(3-hydroxy-4-nitro)cinnamonitrile]], okadaic acid, and KN62 were from Calbiochem, (La Jolla, CA). Carrier-free [^{32}P]orthophosphoric acid, L-3-phosphatidyl-choline-1-stearoyl-2-(1-^{14}C)-arachidonyl, and L-3 phosphatidylcholine-1-palmitoyl-2-(1-^{14}C)-oleyl were from Amersham Life Science (Clearbrook, IL). 1,2-Dioleoylglycerol was from Avanti Polar Lipids (Birmingham, AL). PMA, PDBu, phosphate-free Dulbecco's modified Eagle's medium, M-199, FBS, *Naja Naja* PLA_2, DTT, and TRITC conjugate anti-mouse IgG (Fc specific) were from Sigma Chemical (St. Louis, MO). Anti-MAP_2 kinase monoclonal antibody was purchased from GIBCO BRL (Gaithersburg, MD).

Preparation of CEC

Endothelial cells were isolated from rabbit coronary vessels through modification of the method of Nees *et al.* (21). Briefly, the isolated heart of rabbit was initially perfused with BSS via the aorta for 15 min; the perfusion solution was then changed to BSS without Ca^{2+}. When the heart stopped beating, it was immersed in 20 ml of HBSS containing 20% sucrose in a 50-ml conical tube. Dispersion of the endothelial lining of the coronary vessels was initiated by infusion of 5 ml of HBSS containing 0.15% of collagenase (Boehringer-Mannheim Biochemicals, Indianapolis, IN), soybean trypsin inhibitor (type I-2; Sigma), and BSA and maintenance for 10 min exposure of the coronary vascular system to solution retained in the vascular space. CEC were then flushed by perfusion of 40 ml of BSS. The cell suspension that overlaid the sucrose medium was aspirated and centrifuged. The sedimentated cells were resuspended in 10 ml of M-199 supplemented with 20% FBS, plated onto 100-mm Falcon tissue culture dishes, and incubated at 37° in an atmosphere of 5% CO_2/95% humidified air for 1.5 hr. The incubation medium was then replaced with fresh culture medium and thereafter was changed every 2–3 days. Confluent cells were passed to 48-well plates for experimental purposes. The cells were divided into three batches from each heart, and three or four hearts were used for each group of experiments. Nine to 12 wells of cells from different hearts were used for each experiment.

Experimental Protocols

Protocol 1. To determined the type or types of PLA_2 involved in 6-keto-$PGF_{1\alpha}$ production in response to ACh, CEC were transfected with $cPLA_2$ (85 kDa) AS (5′-TACAGTAAATATCTAGGAATG-3′) and group II $sPLA_2$ (14 kDa) AS (5′-AGCCAGGACAAGGAATTTCAT-3′) or their complementary sense oligomer (all thio-oligonucleotides were synthesized at the Molecular Resource Center, University of Tennessee, Memphis, TN). $cPLA_2$ AS and $sPLA_2$ AS sequences are directed against the translation initiation sites of PLA_2 (22, 23). Subconfluent CEC cultured in 12-well plates were incubated with various sense or AS oligonucleotides (300 nM) in the presence of 5 μg/ml lipofectin (Life Technologies, Grand Island, NY) in serum-free culture medium (M-199) for 5 hr and then cultured in the same medium containing 20% FBS for an additional 16 hr before any experimental intervention. Before the experiment, the cells were washed with 1 ml of HBSS for three times and then incubated for 10 min with BSS containing ACh or its vehicle. The content of 6-keto-$PGF_{1\alpha}$ in the medium was determined by radioimmunoassay.

The activity of PLA_2 in CEC transfected with $cPLA_2$ and $sPLA_2$ sense and AS oligonucleotides was measured according to the method of Leslie (9). Briefly, subconfluent CEC transfected with various sense or AS oligonucleotides and cultured in 100-mm diameter dishes were scraped and sonicated in HEPES buffer, pH 7.4, containing 340 mM sucrose, 1 mM EGTA, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 20 μg/ml soybean trypsin inhibitor. The concentration of protein in the lysate was determined by Lowry's assay and adjusted to 1 mg/ml. The PLA_2 activity of cell lysate was measured by hydrolysis of L-3-phosphatidylcholine-1-stearoyl-2-(1-^{14}C)arachidonyl. The substrate was cosonicated with 9 μM dioleoylglycerol, 5 mM $CaCl_2$, 150 mM NaCl, 1 mg/ml BSA, and 25 mM HEPES, pH 7.4, and incubated (50,000 cpm) with cell lysate (25 μg of protein) in a final volume of 50 μl. The reaction mixture in a 5-ml glass tube was incubated at 37° for 30 min and extracted by the addition of 2.5 ml of Dole solution [2-propanol/heptane/0.5 M H_2SO_4 (20:5:1)], 1.5 ml of heptane, and 1 ml of water containing 20 μg of nonradiolabeled AA. The heptane phase containing radiolabeled fatty acid was passed through a silicic acid chromatography column (SepPak Cartridges, Waters Division of Millipore, Milford, MA), and the effluent was collected in scintillation vials and dried; then, radioactivity was determined by liquid scintillation counting using high flash-point LSC cocktail (Packard Instrument, Meriden, CT).

The effect of $cPLA_2$ and $sPLA_2$ sense and AS oligonucleotides on the PLA_2 protein synthesis in CEC was also determined by immunoblotting. Subconfluent CEC transfected with various sense or AS oligonucleotides and cultured in 100-mm-diameter dishes were washed three times with ice-cold PBS, and the dishes were cooled in a dry ice/ethanol slurry. The cells were scraped and lysed by sonication in HEPES buffer, pH 7.4, containing various protease inhibitors as mentioned above. For detection of $cPLA_2$, the samples were treated with 2× Laemmli loading buffer (50 mM Tris · Cl, pH 6.8, 2% β-merocaptoethanol, 2% sodium dodecyl sulfate, 0.1% bromphenol blue, 10% glycerol), boiled for 5 min, and subjected to 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After samples

were transferred to nitrocellulose, the blots were probed with a 1:2000 dilution of mouse monoclonal anti-cPLA$_2$ (85 kDa) antibody (24). The PLA$_2$ was detected using the Amersham ECL system.

To further characterize the type of enzyme, cell lysates prepared from CEC were incubated with different phospholipid substrates or the reducing agent DTT for 30 min on ice. The activity of PLA$_2$ was measured using the method described above.

Protocol 2. To determine the contribution of PKC to ACh-induced 6-keto-PGF$_{1\alpha}$ production in CEC, the cells cultured in 48-well plates were preincubated with the PKC inhibitor calphostin C (25) (1 μM) for 30 min and then stimulated with ACh for an additional 10 min. Alternatively, the cultured cells were stimulated with 0.1 μM PMA or 0.1 μM PDBu alone, and accumulation of 6-keto-PGF$_{1\alpha}$ in the medium was measured by radioimmunoassay. The activity of PKC in response to ACh, PMA, and PDBu was measured by using a PKC enzyme assay kit (RPN 77) (Amersham). Cell lysate was prepared as described above.

Protocol 3. To determine the effect of AG126, a tyrosine kinase inhibitor known to decrease MAPK activity (26), and KN62 [1-[*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine], a calcium/calmodulin-dependent kinase inhibitor (27), on 6-keto-PGF$_{1\alpha}$ synthesis elicited by ACh, the CEC cultured in 48-well plates were preincubated with AG126 (1–100 μM) or KN62 (1–100 μM) for 30 min and then washed three times with BSS. Then, the cells were exposed to ACh for an additional 10 min in the presence of AG126 or KN62; accumulation of 6-keto-PGF$_{1\alpha}$ in the medium was measured by radioimmunoassay. PLA$_2$ activity in cell lysates was measured as described above, and the effect of AG126 on MAPK activity in CEC in response to ACh stimulation was determined in cell lysates with the use of a BIOTRAK kit (Amersham) using a synthetic peptide substrate (KRELVEPLTPAGEAPNQALLR). The transfer of γ-^{32}P from ATP to the threonine on the substrate was measured.

Protocol 4. cPLA$_2$ is activated through phosphorylation by one or more kinase, including MAPK (10, 15, 16). Because activation of muscarinic receptors has been reported to increase MAPK activity in some cells, we examined the possible contribution of phosphorylation in response to ACh. CEC cultured in 100-mm-diameter dishes were washed three times with phosphate-free Dulbecco's modified Eagle's medium and then prelabeled for 4 hr with 300 μCi/ml [^{32}P]orthophosphate and treated with 3 μM ACh in the presence or absence of AG126 for the last 30 min of the labeling. The cells were quickly washed three times with ice-cold PBS and immersed in a slurry of ice and ethanol. The cells were scraped and sonicated in buffer, pH 7.5, containing 10 mM HEPES, 250 mM sucrose, 5 mM EGTA, protease inhibitors (100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 20 μg/ml soybean trypsin inhibitor, and phosphatase inhibitors (5 μM concentrations of phosphoserine, phosphothreonine, phosphotyrosine, β-glycerophosphate, *p*-nitrophenyl phosphate, and sodium vanadate). The amount of protein of each sample was adjusted to 1 mg/ml, and 500 μl was incubated with rat monoclonal anti-cPLA$_2$ or anti-p42 MAPK antibody for 4 hr at 4° and then with protein A/Sepharose beads for 1 hr. The immunoprecipitate was centrifuged at 10,000 × *g* for 2 min at 4°, and the pellets were washed with ice-cold PBS containing phosphatase inhibitors. The pellets were suspended in 2× Laemmli buffer and boiled for 5 min. The samples were centrifuged at 10,000 × *g* for 10 min, and the supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8% gel) and autoradiography.

Protocol 5. To determine whether ACh promotes translocation of cPLA$_2$ to the plasma membrane or nuclear envelope in CEC, the cells were grown to ~50% confluency in multiwell plastic slides (Nunc, Naperville, IL). After the cells were stimulated with ACh in the presence or absence of extracellular Ca^{2+} or in the presence of AG126, they were washed three times with cold PBS and fixed by cold methanol for 10 min at room temperature. The cells were then washed with PBS containing 3% BSA (two quick washes; last wash for 30 min). Fixed cells were incubated with rat monoclonal anti-cPLA$_2$ (1:200 diluted in PBS containing 3% BSA) for 1 hr at room temperature; then, the cells were washed three times with PBS (10-min wash each time). The nonspecific binding was blocked by incubation for 1 hr of cells with PBS containing 3% BSA. TRITC-conjugated goat anti-mouse IgG (1:200 diluted in PBS containing 3% BSA) was applied to each well. After a 45-min incubation in the dark, the cells were washed three times with PBS (two quick washes; last wash for 30 min), and one drop of galvetol was applied to the cell surface and covered with a coverslip. Slides were immediately viewed with the use of confocal fluorescence microscopy or stored at 4° in the dark before microscopy.

Radioimmunoassay of 6-Keto-PGF$_{1\alpha}$

6-Keto-PGF$_{1\alpha}$ synthesis and release were determined in the incubation medium by radioimmunoassay as described by Shaffer and Malik (28). Briefly, 100 μl of sample was mixed with 3000–4000 cpm of 6-keto-PGF$_{1\alpha}$ tracer (0.925 MBq/0.25 mCi) and the appropriate amount of antibody. The tracer and antibody were prepared in a buffer consisting of 1.0 g/liter NaN_3, 9.0 g/liter NaCl, 6.8 g/liter KH_2PO_4, 26.1 g/liter K_2HPO_4, and 2.0 g/liter gelatin. The tubes were vortexed and incubated overnight at 4°. Bound and free tracer was separated by the addition of 1.0 ml of dextran-coated charcoal to each tube, and radioactivity was determined by liquid scintillation spectroscopy. The antibody for 6-keto-PGF$_{1\alpha}$ was provided by Dr. C. Leffler (Department of Physiology, University of Tennessee, Memphis, TN).

Data Analysis

Results are expressed as mean ± standard error. The data were analyzed by one-way analysis of variance and the unpaired Student's

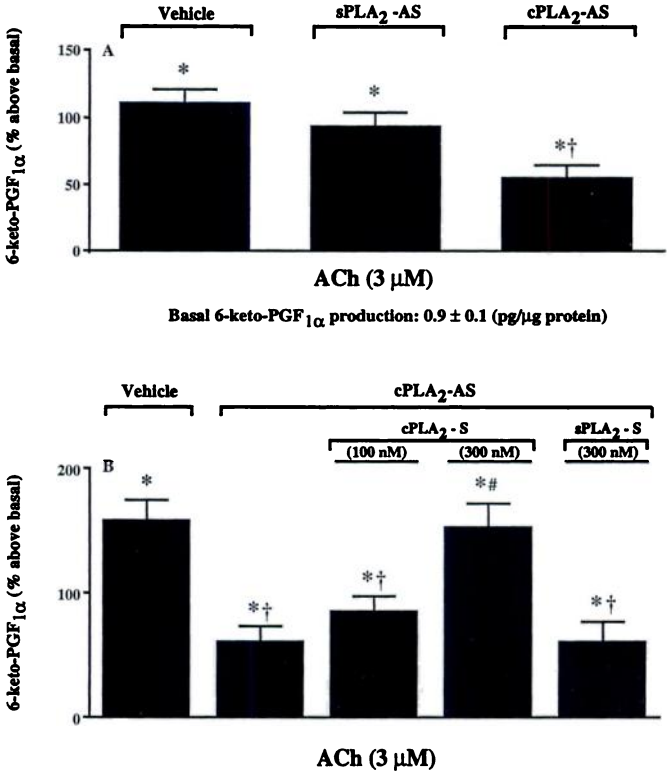

Fig. 1. Effects of (A) cPLA$_2$ and sPLA$_2$ AS (300 nM) and (B) the complementary sense (*S*) oligonuclotides on 6-keto-PGF$_{1\alpha}$ production induced by ACh in transiently transfected CEC. Data are mean ± standard error of 12 wells of cells prepared from four different hearts (three batches of cells from each). *, Value significantly different from basal. †, Value significantly different from the value (ACh) that obtained in the presence of the vehicle of cPLA$_2$ AS, lipofectin. #, Value significantly different from that obtained in the presence of cPLA$_2$ AS.

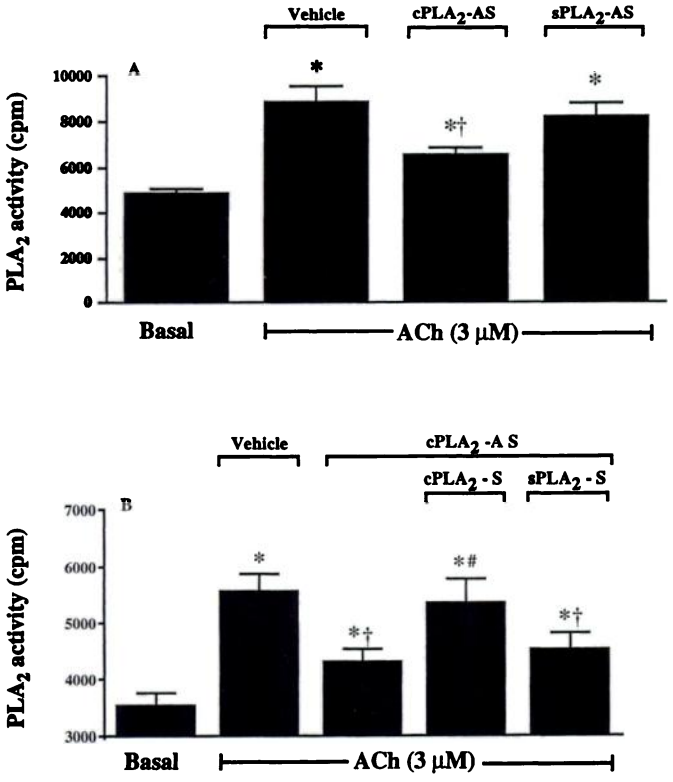

Fig. 2. Effects of ACh on PLA_2 activity in the lysates prepared from the CEC cotransfected with (A) $cPLA_2$ and $sPLA_2$ AS (300 nM) and (B) $cPLA_2$ and $sPLA_2$ sense (*S*) (300 nM). Data are mean ± standard error of six samples of CEC from three different experiments. *, Value significantly different from basal. †, Value significantly different from vehicle of $cPLA_2$ AS, lipofectin. #, Value significantly different from that obtained in the presence of $cPLA_2$ AS.

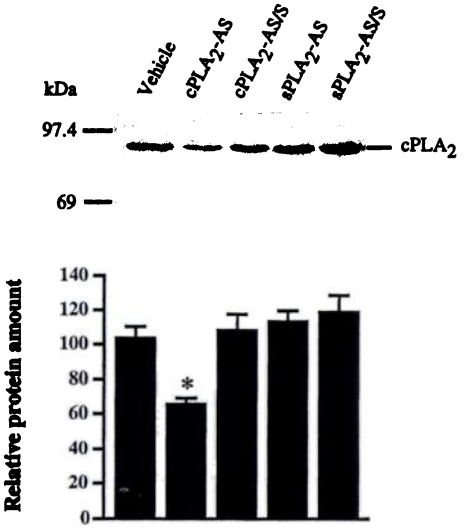

Fig. 3. $cPLA_2$ immunoreactive protein in CEC treated with $cPLA_2$ and $sPLA_2$ AS oligonucleotides in the absence and presence of $cPLA_2$ and $sPLA_2$ sense () oligonucleotides. Subconfluent CEC transfected with various sense or AS oligonucleotides (300 nM) were cultured in 100-mm-diameter dishes. The samples were prepared as described in Experimental Procedures, and 30 μg of protein from each sample was assayed by Western blot analysis using mouse monoclonal anti-$cPLA_2$ antibody. Three experiments were performed that had similar results. The relative amount of $cPLA_2$ protein synthesis was quantified by densitometric analysis. *, Value significantly different from that obtained in the presence of vehicle.

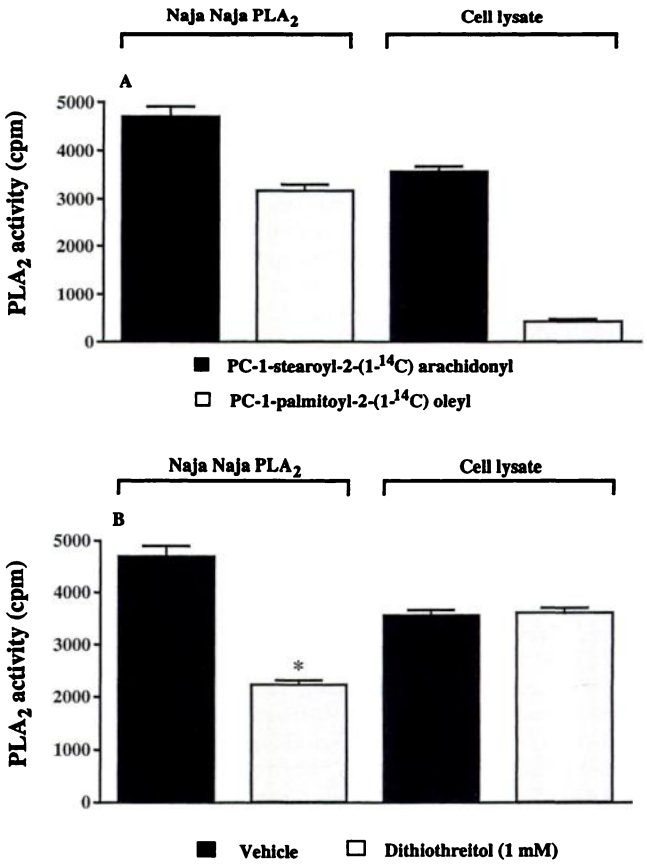

Fig. 4. Effects of (A) *N. Naja* PLA_2 (1 ng) and cell lysate (20 μg) prepared from ACh-stimulated CEC on the hydrolysis of phospholipids containing different fatty acids at *sn*-2 position and of (B) DTT on *N. Naja* PLA_2 (1 ng) and cell lysates (20 μg) prepared from ACh-stimulated CEC on the hydrolysis of L-3-phosphatidylcholine-1-stearoyl-2-(1-^{14}C)-arachidonyl. Data are mean ± standard error of nine samples from three separate experiments. *, Value significantly different from those obtained in the absence of DTT ($p < 0.05$).

t test for difference between groups. The null hypothesis was rejected at $p < 0.05$. Basal 6-keto-$PGF_{1\alpha}$ is expressed in pg of immunoreactive 6-keto-$PGF_{1\alpha}$/mg of protein. Because basal 6-keto-$PGF_{1\alpha}$ production was variable in different batches of cells and the increase in basal 6-keto-$PGF_{1\alpha}$ production elicited by ACh was consistent compared with its vehicle control within the same batch of cells and independent of basal levels, the changes in ACh-induced 6-keto-$PGF_{1\alpha}$ synthesis produced by various experimental interventions are expressed as percentage above basal.

Results

Effects of $cPLA_2$ and $sPLA_2$ AS and sense oligonucleotides on ACh-induced 6-keto-$PGF_{1\alpha}$ production, PLA_2 activity, and protein level in CEC. Transient transfection of CEC with $cPLA_2$ AS but not $sPLA_2$ AS oligonucleotides (300 nM) inhibited 6-keto-$PGF_{1\alpha}$ synthesis elevated by ACh (3 μM) (Fig. 1A). The inhibitory effect of $cPLA_2$ AS was minimized by transient cotransfection with $cPLA_2$ sense but not by $sPLA_2$ sense oligonucleotides (Fig. 1B). Transfection of CEC with $cPLA_2$ but not $sPLA_2$ AS also reduced PLA_2 activity in the lysate of CEC exposed to ACh for 10 min (Fig. 2A). The decrease in PLA_2 activity elicited by ACh was reversed in the lysate of CEC cotransfected with $cPLA_2$ sense (Fig. 2B). The basal PLA_2 activity was variable in different batches of cells, but it was constant within the same batch,

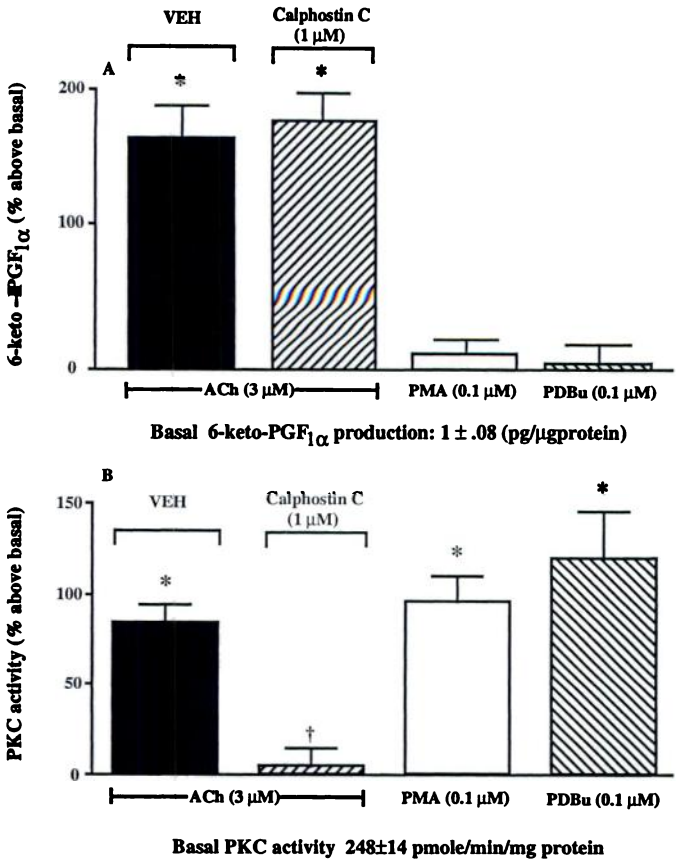

Fig. 5. Effects of calphostin C on ACh-induced 6-keto-PGF$_{1\alpha}$ (A) production and (B) PKC activity and of PMA and PDBu on (A) 6-keto-PGF$_{1\alpha}$ and (B) PKC activity in CEC. Data are mean ± standard error of nine samples from three separate experiments. *, Value significantly different from basal; †, value significantly different from the corresponding value obtained in the absence of calphostin C ($p < 0.05$).

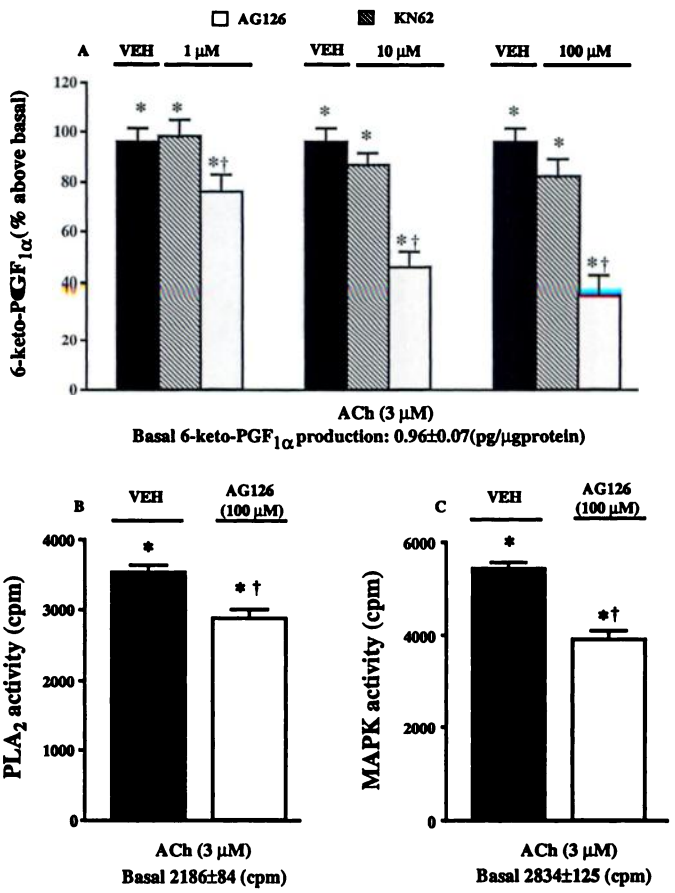

Fig. 6. Effects (A) of AG126 and KN62 on ACh-induced 6-keto-PGF$_{1\alpha}$ production and of AG126 on (B) ACh-stimulated cPLA$_2$ and (C) MAPK activity. Data are ± mean ± standard error of nine samples from three separate experiments. *, Value significantly different from basal; †, value significantly different from the corresponding value obtained in the presence of vehicle (*VEH*) of AG126 ($p < 0.05$).

and ACh produced a consistent increase in PLA$_2$ activity in all batches of cells. The basal PLA$_2$ activity in CEC transfected with 300 nM of cPLA$_2$ AS was decreased slightly but not significantly. A higher concentration of cPLA$_2$ (500 nM) reduced significantly the basal PLA$_2$ activity (3659 ± 378 cpm with vehicle versus 2173 ± 201 cpm with cPLA$_2$ AS, six samples from three experiments; $p < 0.05$). In CEC transfected with cPLA$_2$ but not sPLA$_2$ AS oligonucleotides (300 nM), PLA$_2$ protein level detected by immunoblotting with PLA$_2$ monoclonal antibody was reduced. Cotransfection of CEC with cPLA$_2$ sense oligonucleotide prevented the decrease in PLA$_2$ protein level produced by transfection of CEC with cPLA$_2$ AS oligonucleotide (Fig. 3).

Characteristic of PLA$_2$ in CEC lysates. To further characterize the PLA$_2$ involved in ACh-induced PGI$_2$ synthesis in CEC, we compared its substrate specificity and sensitivity to DTT of PLA$_2$ in cell lysate exposed to ACh with that of *N. Naja* PLA$_2$, a secretory PLA$_2$ (30) from snake venom. *N. Naja* PLA$_2$ hydrolyzed phosphatidylcholine containing either AA or oleic acid at the *sn*-2 position; however, cell lysates prepared from CEC exposed to ACh hydrolyzed only phosphatidylcholine containing AA at its *sn*-2 position (Fig. 4A). In addition, 1 mM DTT decreased the ability of *N. Naja* PLA$_2$ to hydrolyze the phospholipid containing AA but had no effect on PLA$_2$ activity in lysates of CEC exposed to ACh (Fig. 4B).

Effects of calphostin C, PMA, and PDBu on 6-keto-PGF$_{1\alpha}$ synthesis and PKC activity in CEC. Calphostin C (1 μM) did not alter the effect of ACh in stimulating 6-keto-PGF$_{1\alpha}$ production (Fig. 5A) but prevented activation of PKC activity by ACh (Fig. 5B). Exposure of CEC to 100 nM PMA or 100 nM PDBu for 10 min did not change basal 6-keto-PGF$_{1\alpha}$ production in CEC (Fig. 3A), but both agents enhanced PKC activity in cell lysates (Fig. 5B).

Effect of AG126 and KN62 on ACh-induced 6-keto-PGF$_{1\alpha}$ production and MAPK and cPLA$_2$ activity. AG126 (1–100 μM) but not KN62 reduced ACh-induced 6-keto-PGF$_{1\alpha}$ production in a dose-dependent manner in CEC (Fig. 6A). ACh increased MAPK activity in CEC (Fig. 6B); this activity was not altered in the absence of extracellular Ca^{2+}. AG126 (100 μM) also reduced ACh-stimulated cPLA$_2$ activity (Fig. 6B) and MAPK activity in cell lysates prepared from CEC (Fig. 6C).

Effect of okadaic acid on 6-keto-PGF$_{1\alpha}$ synthesis and cPLA$_2$ activity. Okadaic acid simulates cPLA$_2$ activity and increases AA release in macrophages (13). In our study, okadaic acid (10 nM to 1 μM) incubated alone with CEC for 30 min did not change basal 6-keto-PGF$_{1\alpha}$ production; however, ACh-induced 6-keto-PGF$_{1\alpha}$ production was slightly but significantly increased by a higher concentration of okadaic acid (Fig. 7A). Okadaic acid also increased cPLA$_2$ activity in cell lysates prepared from CEC (Fig. 7B).

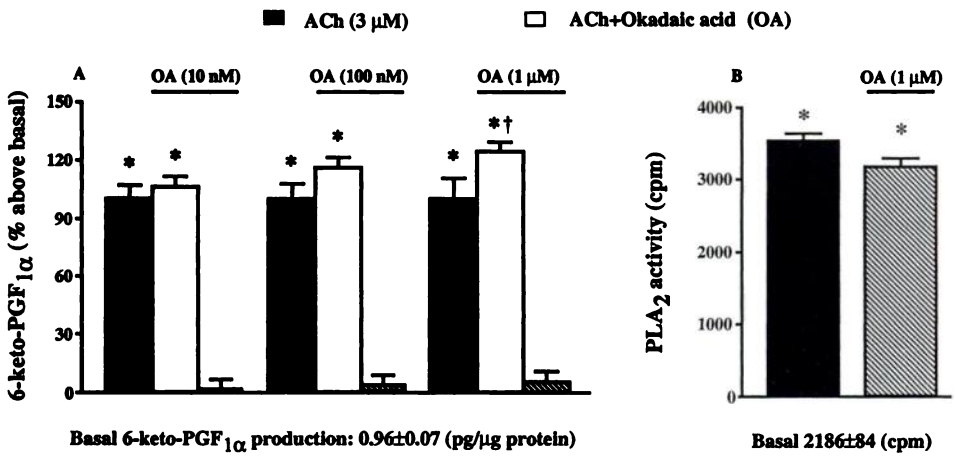

Fig. 7. Effect of okadaic acid (A) on 6-keto-$PGF_{1\alpha}$ production in cultured CEC and (B) on $cPLA_2$ activity in cell lysate prepared from cultured CEC. Data are mean ± standard error of nine samples from three separate experiments. *, Value significantly different from basal; †, value significantly different from the corresponding value obtained in the presence of ACh ($p < 0.05$).

Effect of ACh, AG126, and okadaic acid on phosphorylation of MAPK and $cPLA_2$. Semiconfluent quiescent CEC preincubated with [^{32}P]orthophosphate for 4 hr were treated with ACh for 10 min in the presence or absence of 100 μM AG126. ACh increased incorporation of ^{32}P into p42 MAP kinase (Fig. 8A) and $cPLA_2$ (Fig. 8B). AG126 inhibited incorporation of ^{32}P into p42 MAPK (Fig. 8A) and $cPLA_2$ elicited by ACh (Fig. 8B). Okadaic acid (1 μM) alone increased both p42 MAPK and $cPLA_2$ phosphorylation level in CEC (Fig. 8, A and B).

Effects of ACh, AG126, okadaic acid, and extracellular Ca^{2+} on translocation of $cPLA_2$. In a previous study, we demonstrated that extracellular Ca^{2+} is required for ACh-stimulated 6-keto-$PGF_{1\alpha}$ production in CEC (20). Nonselective binding of TRITC conjugate anti-mouse IgG (Fc specific) with CEC is shown in (Fig. 9A). In CEC exposed to the vehicle of ACh, no translocation of $cPLA_2$ from cytosol to the nuclear envelope was observed (Fig. 9B). In CEC treated with 3 μM ACh in the presence (Fig. 9C) but not in the absence of extracellular Ca^{2+} (Fig. 9E), $cPLA_2$ was translocated from cytosol to the nuclear envelope. AG126 (100 μM), which reduced phosphorylation of $cPLA_2$, did not affect translocation of $cPLA_2$ to the nuclear envelope induced by 3 μM ACh (Fig. 9D). Okadaic acid (1 μM), which increased $cPLA_2$ activity in cell lysates, did not promote translocation of $cPLA_2$ to the nuclear envelope (Fig. 9F).

Discussion

Results of the current study indicate that ACh stimulates PGI_2 synthesis in CEC of rabbit heart by activation of $cPLA_2$ in a PKC-independent mechanism and that both phosphorylation of $cPLA_2$ and translocation of $cPLA_2$ to the nuclear envelope in response to extracellular Ca^{2+} are required for ACh-induced PGI_2 synthesis. Furthermore, phosphorylation of $cPLA_2$ increases its activity but does not cause its translocation to the nuclear envelope.

In mammalian cells, both $sPLA_2$ and $cPLA_2$ have been reported to mediate liberation of AA from tissue lipids in response to chemical signals (10, 29). Current results demonstrate that ACh-induced 6-keto-$PGF_{1\alpha}$ production in CEC is reduced by transfection of cells with $cPLA_2$ AS oligonucleotides, indicating the involvement of $cPLA_2$ in releasing AA for 6-keto-$PGF_{1\alpha}$ synthesis. This was further confirmed by our finding that PLA_2 in cell lysates prepared from CEC exposed to ACh selectively hydrolyzed the lecithin containing AA at its *sn*-2 position and not that containing oleic acid and that the activity of PLA_2 in cell lysates was not altered by the reducing agent DTT. These characteristics are consistent with those reported for $cPLA_2$ but not those for $sPLA_2$ (12).

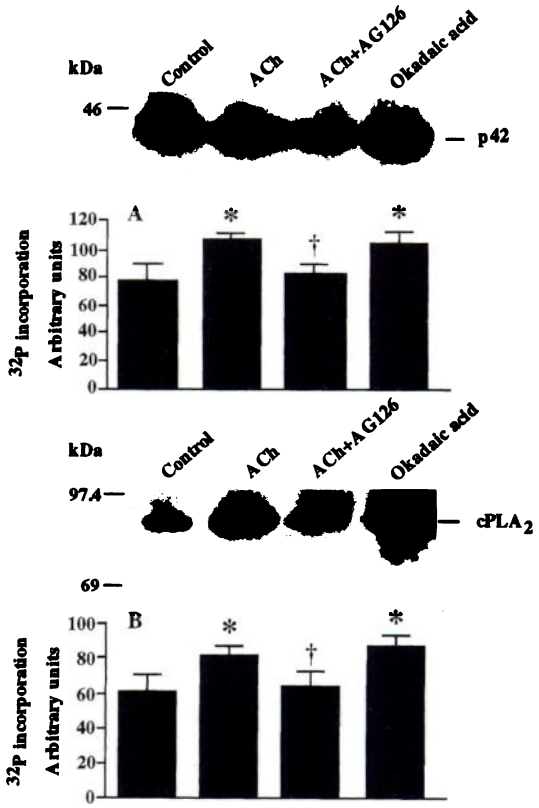

Fig. 8. ACh- and okadaic acid-stimulated phosphorylation of (A) p42 MAPK and (B) $cPLA_2$ in CEC and the effect of AG126 on ACh-induced ^{32}P corporation into (A) $cPLA_2$ and (B) p42 MAPK. The samples were prepared as described in Experimental Procedures and immunoprecipitated by monoclonal anti-$cPLA_2$ and anti-MAP_2 kinase antibodies. The immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Four experiments were performed that showed similar results. The amount of ^{32}P incorporation into the proteins was quantified by densitometric analysis and expressed in arbitrary units. *, Value significantly different from control; †, value significantly different from ACh alone.

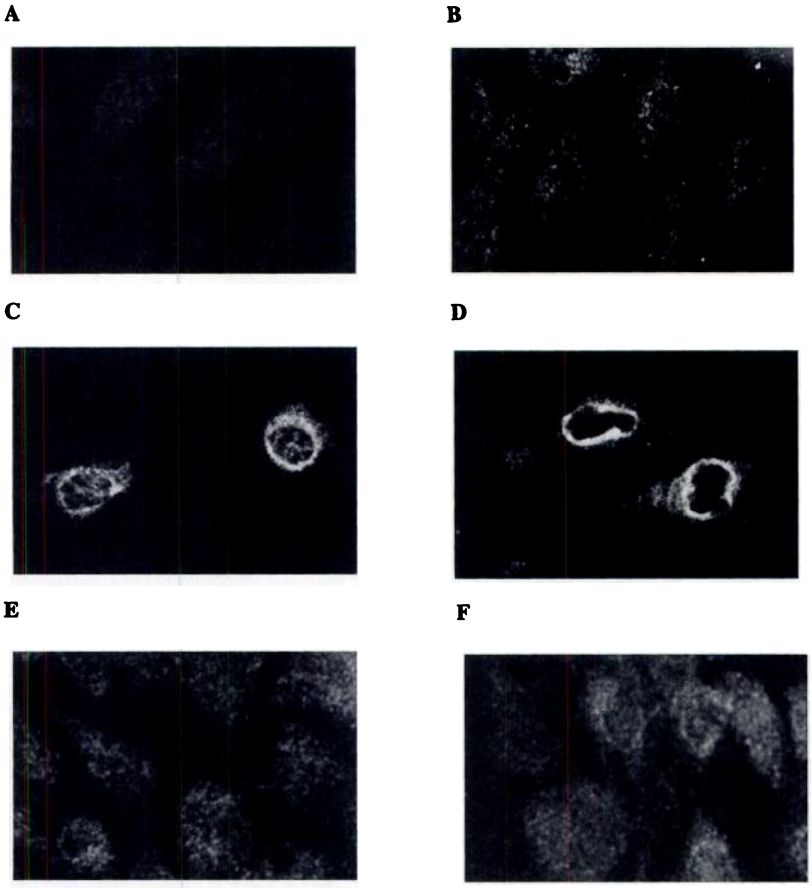

Fig. 9. Localization of $cPLA_2$ by confocal fluorescence microscopy in CEC. A, Nonselective binding of TRITC-conjugated anti-mouse IgG (Fc specific) with the cells. B, Nonstimulated CEC. C, ACh-stimulated CEC. D, ACh-stimulated CEC in the presence of AG126 (100 μM). E, ACh-stimulated CEC in the absence of extracellular Ca^{2+}. F, Okadaic acid (1 μM)-stimulated CEC. $cPLA_2$ was visualized with TRITC-conjugated anti-mouse IgG (Fc specific).

It has been reported that the PKC family plays an important role in a number of cellular functions mediated by activation of mAChR, including activation of PLA_2, PLC, and PLD (30). The mechanism by which stimulation of M_3 mAChR by ACh results in activation of $cPLA_2$ could involve PKC activity. ACh enhances PKC activity and PKC increases Ca^{2+} channel activity (31) and PLA_2 activities in some cell types (14, 32). Furthermore, PKC has been reported to be involved in PGI_2 synthesis or AA release in response to some agents (33). However, in our study in CEC, phorbol esters PMA and PDBu, which increased PKC activity, failed to stimulate 6-keto-$PGF_{1\alpha}$ production. Also, the PKC inhibitor calphostin C that attenuated the ACh-induced increase in PKC activity did not alter 6-keto-$PGF_{1\alpha}$ synthesis elicited by ACh. These observations rule out the involvement of PKC in 6-keto-$PGF_{1\alpha}$ synthesis via activation of $cPLA_2$ by ACh in the CEC of the rabbit heart.

Because ACh increases the activity of MAPK and MAPK has been shown to stimulate $cPLA_2$ activity in some cell types (13, 14, 24), we investigated the possible involvement of MAPK in ACh-induced increase in $cPLA_2$ activity in the CEC. Our study indicated that ACh increased MAPK activity and that AG126, which inhibited MAPK activity, reduced 6-keto-$PGF_{1\alpha}$ synthesis in the CEC and $cPLA_2$ activity in the cell lysates. These data combined with our finding that the calmodulin-dependent kinase inhibitor KN62 in concentrations known to inhibit the activity of this enzyme in other cell systems (27) failed to alter ACh-induced 6-keto-$PGF_{1\alpha}$ synthesis suggest that MAPK mediates ACh-induced $cPLA_2$ activation in CEC. However, the mechanism by which MAPK activity is regulated by mAChR stimulation in CEC is not known. MAPK activity has been reported to be regulated by PKC-dependent or -independent pathways (13) and by G protein or proteins (34). Because we ruled out the involvement of PKC in the activation of $cPLA_2$ in the CEC by ACh, activation of MAPK through mAChR by ACh could be regulated by a G protein (20). There is an evidence that MAPK is activated by $\beta\gamma$ subunits of heterotrimeric G proteins when mAChR is stimulated in COS-7 cells (34).

It has been shown that phosphorylation is required for the full activation of $cPLA_2$. Our findings in CEC that ACh increased incorporation of ^{32}P into both MAPK and $cPLA_2$ and that AG126, which decreased ACh-induced MAPK activity, also inhibited ACh-stimulated incorporation of ^{32}P into MAPK and $cPLA_2$ suggest that MAPK activation is required for $cPLA_2$ phosphorylation and activation by ACh in CEC.

$cPLA_2$ has been reported to be translocated from the cytosol to the nuclear envelope and/or to endoplasmic reticulum in response to Ca^{2+} ionophore or IgE/antigen in Chinese hamster ovary cells or rat basophilic leukemia cells (18, 35). The translocation of the $cPLA_2$ to these cell structures seems to be crucial for its function because of the localization of its substrate and of AA metabolizing enzymes to the same cell structures (19). An increase in intracellular Ca^{2+} concentration seems to be the crucial step for $cPLA_2$ translocation because many agents stimulate the activity of this enzyme by increasing influx of extracellular Ca^{2+} (15). In a previous study, we demonstrated that ACh increased cytosolic Ca^{2+}

levels via activation of M_3 mAChR and that removal of extracellular Ca^{2+} abolished ACh-stimulated 6-keto-$PGF_{1\alpha}$ in CEC (20). The current study demonstrates that ACh promoted $cPLA_2$ translocation from cytosol to nuclear envelope in the presence but not the absence of extracellular Ca^{2+}. Furthermore, ACh-induced $cPLA_2$ translocation to nuclear envelope was not affected by AG126, which attenuated $cPLA_2$ phosphorylation by inhibiting MAPK activity. Furthermore, removal of extracellular Ca^{2+}, which prevented ACh-induced translocation of $cPLA_2$ to nuclear envelope, did not alter MAPK activity. These observations suggest that ACh-induced Ca^{2+} influx is required for $cPLA_2$ translocation to the nuclear envelope but not $cPLA_2$ phosphorylation. That $cPLA_2$ phosphorylation is not necessary for its translocation to the nuclear envelope was further supported by our finding that okadaic acid, a protein phosphatase inhibitor that increased $cPLA_2$ activity and its phosphorylation, did not cause translocation of $cPLA_2$ to the nuclear envelope or increase 6-keto-$PGF_{1\alpha}$ production in the CEC. However, okadaic acid enhanced the synthesis of 6-keto-$PGF_{1\alpha}$ elicited by ACh, which is associated with increased influx of extracellular Ca^{2+} and translocation of $cPLA_2$ to the nuclear envelope. These observations, together with our finding that depletion of extracellular Ca^{2+} [which inhibits ACh induced 6-keto-$PGF_{1\alpha}$ synthesis (20)] support our contention that Ca^{2+} influx is essential for the synthesis of PGI_2 elicited by ACh in CEC. It has recently been reported that mutation at the MAPK phosphorylation site of $cPLA_2$ did not affect A23187-induced translocation of the enzyme to the nuclear envelope and endoplasmic reticulum in Chinese hamster ovary cells overexpressing $cPLA_2$ (18). However, deletion of the Ca^{2+}-dependent phospholipid-binding domain, which is required for the membrane binding of $cPLA_2$, inhibited translocation to the nuclear envelope and endoplasmic reticulum elicited by A23187 in these cells (18).

In conclusion, the current results demonstrate that ACh stimulates PGI_2 synthesis by activation of $cPLA_2$ through a PKC-independent pathway in CEC of rabbit heart. Furthermore, ACh-induced Ca^{2+} influx, but not phosphorylation, is required for translocation of $cPLA_2$ to the nuclear envelope. In addition to $cPLA_2$ translocation, ACh-induced increase in MAPK activity promotes phosphorylation and activation of $cPLA_2$, which are required for 6-keto-$PGF_{1\alpha}$ production in CEC elicited by ACh.

Acknowledgments

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