Calcium/Calmodulin-dependent Protein Kinase IIα Mediates Activation of Mitogen-activated Protein Kinase and Cytosolic Phospholipase A2 in Norepinephrine-induced Arachidonic Acid Release in Rabbit Aortic Smooth Muscle Cells*

Mubarack M. Muthalif‡, Ibrahim F. Benter§, Mohammed R. Uddin¶, and Kafait U. Malik¶

From the ‡Department of Pharmacology, College of Medicine, The University of Tennessee Center for Health Sciences, Memphis, Tennessee 38163, §Southern College of Optometry, Memphis, Tennessee 38104, and ¶LeMoyne Owen College, Memphis, Tennessee 38126

We have investigated the contribution of Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaM kinase II) and mitogen-activated protein kinase (MAP kinase) in norepinephrine (NE)-induced arachidonic acid (AA) release in rabbit aortic vascular smooth muscle cells (VSMC). NE enhanced release of AA via activation of cytosolic phospholipase A\textsubscript{2} (cPLA\textsubscript{2}) but not secretory PLA\textsubscript{2} in VSMC prelabeled with \([\text{H}]\text{AA}. NE (10 \mu M) enhanced CaM kinase II and MAP kinase activity. In cells transiently transfected with antisense oligonucleotides complementary to the translation initiation sites of CaM kinase II and MAP kinase, NE-induced AA release was inhibited by 100 and 35\% respectively. Treatment of cells with PD-098059, a MAP kinase inhibitor, or with MAP kinase antisense oligonucleotide reduced NE-induced activation of MAP kinase and cPLA\textsubscript{2}. NE-induced MAP kinase and cPLA\textsubscript{2} activation was also inhibited in cells treated with a CaM kinase II inhibitor, KN-93, or with CaM kinase II antisense oligonucleotide. On the other hand, inhibition of MAP kinase with PD-098059 or of MAP kinase with antisense oligonucleotides did not alter the NE-induced increase in CaM kinase II activity. Phosphorylation of MAP kinase and CaM kinase II by NE, studied by \textsuperscript{32}P incorporation and immune complex kinase assays, was inhibited by KN-93. Collectively, these data suggest that CaM kinase II can activate MAP kinase, which in turn activates cPLA\textsubscript{2} to release AA for prostacyclin synthesis in the rabbit VSMC. This novel pathway for activation of MAP kinase by CaM kinase II appears to be mediated through stimulation of MAP kinase activity. Activation of adrenergic receptors with NE in VSMC caused translocation of CaM kinase II, MAP kinase, and cPLA\textsubscript{2} to the nuclear envelope only in the presence of extracellular Ca\textsuperscript{2+}. Okadaic acid, which increased phosphorylation and activity, did not translocate these enzymes. Therefore, it appears that in rabbit VSMC, NE, by promoting extracellular Ca\textsuperscript{2+} influx, increases CaM kinase II activity, leading to activation of MAP kinase and cPLA\textsubscript{2} and translocation to the nuclear envelope, resulting in release of AA from the nuclear envelope for prostacyclin synthesis.

Norepinephrine (NE)\textsuperscript{1} stimulates prostaglandin synthesis in the cardiovascular system via activation of distinct types of adrenergic receptor (AR) at the postjunctional effector cells, e.g. \(\beta_1\) AR in the heart, \(\alpha_1\) AR in the kidney and spleen, and \(\alpha_2\) AR in blood vessels (1, 2). PGI\textsubscript{2} synthesis elicited by NE in VSMC is primarily due to activation of \(\alpha_2\) and to a lesser extent \(\alpha_1\) AR (2). Activation of both \(\alpha_1\) and \(\alpha_2\) AR in VSMC promotes PGI\textsubscript{2} synthesis by increasing Ca\textsuperscript{2+} influx, primarily through voltage-dependent Ca\textsuperscript{2+} channels, via a pertussis toxin-sensitive \(G_{\text{m}}\)-like protein (2). The increased Ca\textsuperscript{2+} influx, by interacting with calmodulin, activates PLA\textsubscript{2}, which releases AA from tissue lipids for PGI\textsubscript{2} synthesis (2). The release of AA for prostaglandin synthesis in response to various stimuli has been reported to be due to the activation of cPLA\textsubscript{2} or sPLA\textsubscript{2} species. sPLA\textsubscript{2} can hydrolyze phospholipids containing different fatty acids at the sn-2-position, requires millimolar Ca\textsuperscript{2+} concentrations for activation, and is sensitive to disulfide reducing agents (3). On the other hand, cPLA\textsubscript{2} selectively hydrolyzes phospholipids containing AA at the sn-2-position, is activated by micromolar Ca\textsuperscript{2+} concentrations, and is not sensitive to disulfide reducing agents (4, 5). Whether NE stimulates AA release for PGI\textsubscript{2} synthesis in the VSMC by activation of cPLA\textsubscript{2} and/or sPLA\textsubscript{2} is not known.

cPLA\textsubscript{2} activity has been reported to be regulated by phosphorylation by MAP kinase (6–8) and by Ca\textsuperscript{2+}-dependent translocation to the nuclear envelope (9, 10), allowing its access to arachidonoyl-containing phospholipid substrate. However, recent studies have provided evidence for cPLA\textsubscript{2} activation independent of MAP kinase in human platelets in response to the thrombin agonist SFLLRN (11) and in human neutrophils in response to TNF-\(\alpha\) (12). Our previous findings in the VSMC of rabbit aorta that PGL\textsubscript{2} synthesis elicited by activation of \(\alpha_1\) and \(\alpha_2\) AR was attenuated by the calmodulin (CaM) inhibitor W-7 (2) raises the possibility that Ca\textsuperscript{2+}/CaM might stimulate cPLA\textsubscript{2} directly or indirectly via activation of CaM kinase II or MAP kinase.

CaM kinase II is abundant in the brain and has been implicated in neurotransmitter release (13). In this study, we report that CaM kinase IIα is also expressed in VSMC and report for the first time that CaM kinase II promotes MAP kinase-induced activation of cPLA\textsubscript{2}. We further show that, upon NE

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1 The abbreviations used are: NE, norepinephrine; AA, arachidonic acid; AR, adrenergic receptor; BSA, bovine serum albumin; CaM, calmodulin; CaM kinase II, Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II; PLA\textsubscript{2}, phospholipase A\textsubscript{2}; cPLA\textsubscript{2}, cytosolic PLA\textsubscript{2}; DTT, dithiothreitol; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase kinase; sPLA\textsubscript{2}, secretory PLA\textsubscript{2}; TBS, Tris-buffer saline; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; PGI\textsubscript{2}, prostacyclin; VSMC, vascular smooth muscle cells.
treatment, cPLA2 translocates to the nucleus along with CaM kinase II and MAP kinase.

EXPERIMENTAL PROCEDURES

Materials—[3H]AA (100 Ci/mmol) was purchased from DuPont NEN. Hanks’ balanced salt solution, M-199, phosphate-buffered saline, bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), leupeptin, and DTT were from Boehringer Mannheim, and 1,2-dioleoyl-sn-glycerol was from Avanti Polar Lipids (Alabaster, AL).

Preparation of VSMC—Male New Zealand rabbits (1–2 kg) were anesthetized with 30 mg/kg pentobarbital (Abbott Laboratories, North Chicago, IL), and the thorax and abdomen were opened by a midline incision. The aorta was rapidly removed, and VSMC were isolated as described previously (15). Cells between four and eight passages were plated in 12 or 24 wells or 100-mm plates. Cells were maintained under 5% CO2 in M-199 medium (Sigma) with penicillin, streptomycin, and 10% FBS.

Preparation of Thioligonucleotides and Transient Transfection of VSMC—Antisense oligonucleotides directed against the translation initiation sites of cPLA2, pPLA2, CaM kinase IIα, and MAP kinase were designed (Table I). VSMC were transfected with sense and/or antisense oligonucleotides complexed with 4 μg/ml of lipofectamine and incubated in serum-free M-199 for 6 h. Thereafter, fresh M-199 containing 10% FBS and oligonucleotides was added, and the cells were incubated with [3H]AA for another 18 h to label tissue lipids.

[3H]AA Release—After transient transfection, cells were washed with Hanks’ balanced salt solution and exposed to NE in balanced salt solution containing BSA for 15 min at 37°C. [3H]Released into extracellular medium and that remaining in the VSMC was measured by liquid scintillation spectroscopy. Total radioactivity in the cells was determined after treating the cells with 1 N NaOH overnight. [3H]Released into the medium was expressed as percentage of the total cellular radioactivity and referred to as fractional release.

Phospholipase A2 Assay—Cells grown in 100-mm plates were harvested for 24 h and stimulated with or without NE and lysed in HEPES buffer containing protease and phosphatase inhibitors (350 μM sucrose, 1 mM EDTA, 1 mM PMSF, 10 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 20 μg/ml soybean trypsin inhibitor). The concentration of protein was determined by Bradford assay (Bio-Rad). PLA2 activity in lysates of VSMC fractions (20–30 μg of protein) assay was measured using [14C]arachidonyl phosphatidylcholine as substrate as described previously (16). 11 μl of radiolabeled phospholipid stock was dried under N2 and added to 0.5 ml of reaction mixture (9 μM dioleoylglycerol, 25 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM CaCl2, 1 mM DTT, 1 mg/ml BSA) and sonicated for 15 min on ice. The reaction mixture (50 μl) containing 25 μg of protein from cell lysate was incubated at 37°C for 1 h. The reaction was stopped by adding 2.5 ml of 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM CaCl2, and 1 mM phenylmethylsulfonyl fluoride. The heptane phase containing radiolabeled phospholipase A2 product was dried under N2 and added to 0.5 ml of this mixture was spotted onto phosphocellulose papers (Whatman) and eluted with 95% ethanol and 5% acetic acid. The eluates were collected in a scintillation vial and air-dried, and radioactivity was determined in a scintillation vial and air-dried, and radioactivity was determined in a scintillation vial.

CaMK Kinase Phosphorylation—VSMC were washed three times with phosphate-free DMEM and then prelabeled for 4 h with [32P]orthophosphate (300 μCi/ml) along with inhibitors and treated with NE (10 μM) for 10 min. The cells were washed three times with ice-cold phosphate-buffered saline and resuspended in cell lysis buffer containing 10 mM Tris (pH 7.4), 10 mM CaCl2, 1 mM EGTA, 1 mM DTT, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 0.5% BSA. The cells were scraped and sonicated in buffer containing 10 mM HEPES, 250 mM sucrose, 5 mM EDTA, protease inhibitors (100 μg/ml phenylmethylsulfonyl fluoride, 100 μg/ml leupeptin, 10 μg/ml aprotinin, and 20 μg/ml soybean trypsin inhibitor), and phosphatase inhibitors (5 μM phosphoserine, phosphothreonine, phosphotyrosine, β-γ-eliminase, pep-nitrilotripeptide, and sodium vanadate). The amount of protein was adjusted to 1 mg/ml and split into two halves. One half was incubated with rat monoclonal CaM kinase IIα antibody, and the other half was incubated with mouse IgG for 4 h at 4°C and then with protein A-agarose beads for 1 h. The immune complexes were washed at 12,000 rpm for 2 min, and the pellets were washed with ice-cold phosphate-buffered saline containing phosphatase inhibitor. The pellets were resuspended in Laemmli buffer, and the supernatants were subjected to SDS-PAGE (10% gel) and autoradiography.

MAP Kinase Phosphorylation—Phosphospecific MAP kinase antibody (New England Biolabs) that detects phosphorylated Tyr residues of p44 and/or p42 MAP kinases but does not appreciably cross-react with the thiolated phosphorylated forms was used. Lysates from cells that were stimulated by NE in the presence or absence of inhibitors were resolved on an SDS-PAGE and transferred to polyvinylidine difluoride membrane. The blots were processed as per the manufacturer’s instructions.

Immune Complex Kinase Assays—Cell lysates containing equal amounts of proteins (500 μg) from control and NE-treated samples in the presence and absence of inhibitors were incubated with 5 μl of ECL Western blotting detection reagents (Amersham), using a 1:200 dilution. After a 45-min incubation in the dark, the cells were washed three times with 1 ml of radioimmunoprecipitation buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 1% Nonidet P-40, 0.25% sodium deoxycholate containing 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 2 mM EGTA, 50 μg/ml leupeptin, and 0.5% aprotinin). The pellets were suspended in 50 μl of protein kinase assay buffer containing 10 μl of 10 mM ATP and assayed for MAP kinase and CaM kinase II as described above.

Western Blot Analysis—Lysates from transfectants and parental cells were prepared in buffer. Samples containing 30 μg of protein were resolved by SDS-polyacrylamide gel electrophoresis before transfer to nitrocellulose. The blots were blocked with 3% BSA in TBS at room temperature for 2 h and then incubated for 2 h with primary monoclonal antibodies (1:1000 dilution). The blots were developed using biotinylated secondary antibodies and horseradish peroxidase, and signals were detected using ECL Western blotting detection reagents. Western blotting experiments were carried out at least three times on the transfectants.

Confocal Microscopy—Cells were grown to approximately 70% confluency on chamber slides (Nunc Inc., Naperville, IL) and arrested for 24 h. Then the cells were washed with 1 ml of balanced salt solution containing CaCl2 and treated with NE (10 μM) for 10 min in the presence or absence of extracellular Ca2+ or okadacid acid, a phosphatase inhibitor. Cells were fixed in cold methanol/acetone solution (1:1) for 3 min at room temperature. The cells were then washed in TBS and blocked in TBS containing 3% BSA for 30 min. Monoclonal antibodies (cPLA2 or CaM kinase II or MAP kinase, diluted 200-fold with 3% BSA in TBS containing 0.1% Tween 20 (TBST)) were applied to each well. After 1 h, the cells were washed three times (10 min each) and exposed to tetramethyl rhodamine (TRITC)-conjugated goat anti-mouse IgG (1:200 dilution). After a 45-min incubation in the dark, the cells were...
washed three times (10 min each) with TBST and rinsed quickly with water. 10 μl of Galvetol (Sigma) was applied to the cell surface, and coverslips were mounted. Controls were carried out, replacing the primary antibody with IgG. Nuclei were visualized with 4',6-diamidino-2-phenylindole (Sigma). Slides were viewed by confocal fluorescence microscopy (Bio-Rad MRC-1000 Laser Scanning Confocal Imaging system using an argon/krypton lamp) with a × 100 objective lens.

**RESULTS**

Effect of NE on AA Release in the VSMC of Rabbit Aorta—Rabbit aortic VSMC contain α1 and α2 AR, and both are coupled to prostacyclin synthesis (2). To determine the effect of AR agonist NE on release of AA from tissue lipids, the [3H]AA-labeled VSMC were incubated for different time periods with NE, which acts on both α1 and α2 AR. The release of [3H]AA and its metabolites into the medium and the cell content of tritium were measured. NE enhanced release of [3H]AA in a time-dependent manner, with the maximal release at 15 min (Fig. 1). NE was more efficacious than methoxamine, an α1 AR

**TABLE I**

| Oligonucleotide design | Sequence |
|------------------------|----------|
| 1. cPLA2 AS^a          | TAC AGT AAA TAT CTA GGA ATG |
| 2. cPLA2 S^b           | ATG TCA TTT ATA GAT CCT TAC |
| 3. cPLA2 random        | GAT GAT AGA AAA TAT CTA GGA |
| 4. sPLA2 AS            | AAG GAA GAA GAA TAC TTA CAT |
| 5. sPLA2 S             | ATG AAA TTC CTG TGC CTG GCT |
| 6. sPLA2 random        | AGC CAG CTG AGA ATA CTA GAT |
| 7. MAPK (ERK-1)^c AS   | AGC CGC CGC CGC CGC CGC CAT |
| 8. MAPK (ERK-1) S      | ATG GCG GCG GCG GCG GCG GCT |
| 9. MAPK random         | GCA CAG CCG CCT GCC GCC GCC |
| 10. CaMK II^e AS       | GCA GGC GGC GGC GGC GGC CAT |
| 11. CaMK II S          | ATG GAG ACC GCC ACC ACC TGC |
| 12. CaMK II random     | CCA TGC GTG GTC GTG CGA TGG |

^a AS, antisense.
^b S, sense.
^c MAPK, MAP kinase.
^d ERK, extracellular regulated kinase.
^e CaMK, CaM kinase II.

**FIG. 1.** [3H]AA release in response to AR agonist NE in rabbit aortic VSMC. Cells grown to 80% confluency on a 24-well plate were prelabeled with medium containing 0.3 μCi of [3H]AA for 24 h. The cells were then incubated with NE for various time intervals at 37°C. Fractional release is the percentage of tritium released into the medium from the total cellular radioactivity. Data represent the means ± S.E. of nine experiments from three different batches of cells and are expressed as an increase in fractional release (%) over basal. *, value significantly different from the basal value.

**FIG. 2.** Effect of cPLA2 antisense oligonucleotides or their vehicle (VEH) on NE-induced [3H]arachidonic acid release (A) and PLA2 activity (B) in rabbit aortic VSMC. Cells were transiently transfected with sense (S) and antisense (AS) oligonucleotides of cPLA2 and sPLA2, using lipofectamine and exposed to NE (10 μM) for 15 min. Cell homogenates were prepared, and PLA2 activity was measured by hydrolysis of [14C]arachidonoyl phosphatidylcholine. Data represent the mean ± S.E. of six wells from two batches of cells. *, value significantly different from vehicle of NE; †, value significantly different from that obtained with NE alone; ††, value significantly different from that obtained with cPLA2 antisense alone (p < 0.05).
agonist, and UK-14304, an α₂ AR agonist (data not shown).

Type of Lipases Involved in the NE-stimulated AA Release—To delineate the type of PLA₂ involved in the release of AA in response to NE, antisense oligonucleotides directed against the translation initiation sites of cPLA₂ and sPLA₂ were used. Phosphorothioate oligonucleotides have been successfully targeted to inhibit c-Myb, c-Fos, c-Myc, and many other signal transduction and effector molecules including PLA₂ (17). The phosphorothioate oligonucleotides penetrate the cell membrane easily and allow sequence-specific inhibition of the processes of translation of mRNA into protein. Moreover, they are more stable to endo- and exonucleases that degrade naked DNA. However, antisense oligonucleotides may also act by a nonantisense mechanism, particularly when continuous four-G-nucleotide sequences occur (18). After careful consideration of the above criteria, antisense oligonucleotides directed against translation initiation sites of cPLA₂, sPLA₂, CaM kinase II, and MAP kinase were designed (Table I). The PLA₂ antisense oligonucleotide has been used to block lipopolysaccharide- and platelet-activating factor induced prostaglandin production in macrophage-like P388D1 cells (19) and lipopolysaccharide-induced prostaglandin production in monocytes (20).

VSMC were transfected with either cPLA₂ or sPLA₂ sense and antisense oligonucleotides complexed with lipofectamine (4 μg/ml) and incubated with medium (Opti-MEM from Life Technologies, Inc.) for 6 h. Thereafter, fresh M-199 medium containing 10% FBS was added, and the cells were incubated for another 18 h. Treatment of VSMC with cPLA₂, but not with sPLA₂, antisense oligonucleotides decreased the release of [³H]AA elicited by NE. The inhibitory effect of cPLA₂ antisense on [³H]AA release elicited by NE did not occur when VSMC cotransfected with cPLA₂ sense oligonucleotides (Fig. 2A). The effect of cPLA₂ antisense oligonucleotides on PLA₂ activity was also examined. VSMC transfected with sense or antisense oligonucleotides were exposed to NE for 15 min, and cell lysates were prepared to study AA release from [¹⁴C]arachidonylphosphatidylcholine (Fig. 2B). The results show that in cells exposed to NE there was an increase in the hydrolysis of [¹⁴C]arachidonyl phosphatidylcholine by the cell lysate over the unstimulated control cells. Transfection of VSMC with cPLA₂

![Figure 3](image_url1)

**Fig. 3.** Inhibition of cPLA₂ and CaM kinase II protein levels by their respective antisense oligonucleotides in rabbit VSMC. Cells were transiently transfected for 6 h with 1 μM oligonucleotides or vehicle (Veh) in medium containing lipofectamine. Cells were allowed to recover in 0.1% FBS/M-199 for 18 h and then stimulated with NE (10 μM) for 30 min. Total proteins were separated by 12% SDS-PAGE and examined by Western blot analysis using mouse monoclonal cPLA₂ and CaM kinase II antibodies.

![Figure 4](image_url2)

**Fig. 4.** Effect of CaM kinase II antisense (AS) and sense (S) oligonucleotides, CaM kinase inhibitor, KN-93, or their vehicle (VEH) on NE-stimulated [³H]Arachidonic acid release (A) and CaM kinase II activity (B). Cells were transiently transfected with sense and antisense oligonucleotides for CaM kinase II using lipofectamine or preincubated with KN-93 (20 μM) for 3 h and exposed to NE (10 μM) for 15 min. CaM kinase activity was measured in 10 μg of proteins, using a synthetic substrate. Data represent the means ± S.E. of six experiments from two batches of cells. *, value significantly different from vehicle of NE; †, value significantly different from that obtained with NE alone; ††, value significantly different from that obtained with antisense treatments (p < 0.05).
antisense, but not sense, oligonucleotides reduced PLA2 activity in the lysate of VSMC exposed to NE. These data suggest that NE stimulates AA release for prostanoid synthesis in the VSMC via activation of cPLA2.

Fig. 3 shows an immunoblot of VSMC total lysate proteins probed with monoclonal anti-cPLA2. The cPLA2 migrates at ~100 kDa on SDS-PAGE. Western analysis of cells transfected with cPLA2 antisense oligonucleotides for a longer duration (24 h) showed a significant reduction in the immunoreactive protein. These data positively correlate with the observed PLA2 activity (Fig. 2B) and also indicate that the action of cPLA2 antisense was specific for the cPLA2. The sense oligonucleotide had little or no effect on the 85-kDa PLA2 immunoreactive protein.

CaM Kinase II and MAP Kinase Mediate NE-stimulated AA Release—It has been shown that MAP kinase can activate cPLA2 \textit{in vitro} by phosphorylating Ser-505 in response to platelet-derived growth factor (7). MAP kinase activation can occur through both protein kinase C-dependent and protein kinase C-independent mechanisms (21, 22). MAP kinase-independent regulation of cPLA2 has also been reported (23). To study the type of kinases involved in NE-induced AA release, VSMC were transiently transfected for 6 h with CaM kinase II or MAP kinase antisense oligonucleotides and then exposed to NE (10 μM) for 15 min. MAP kinase activity was determined in 10 μg of lysates, using a synthetic substrate. Data represent the means ± S.E. of four experiments from two batches of cells. *, value significantly different from vehicle (VEH) of NE; †, value significantly different from that obtained with NE alone; ††, value significantly different from that obtained with antisense treatments ($p < 0.05$).

CaM Kinase II Activates cPLA2 via MAP Kinase

Fig. 5. Effect of MAP kinase antisense (AS) and sense (S) oligonucleotides, MEK inhibitor PD-098059, or their vehicle (VEH) on NE-stimulated [3H]arachidonic acid release (A) and MAP kinase activity (B). Cells were transiently transfected with MAP kinase antisense oligonucleotides (1 μM) or preincubated with PD-098059 (50 μM) for 3 h and exposed to NE (10 μM) for 15 min. MAP kinase activity was determined in 10 μg of lysates, using a synthetic substrate. Data represent the means ± S.E. of four experiments from two batches of cells. * value significantly different from vehicle (VEH) of NE. ** value significantly different from that obtained with NE alone; ††, value significantly different from that obtained with antisense treatments ($p < 0.05$).
port our finding that cPLA₂ is activated by both CaM kinase II and MAP kinase. The activity of purified cPLA₂ is insensitive to reduction by DTT (7); likewise, NE-stimulated PLA₂ activity in VSMC was found to be insensitive to DTT included in the buffer. This provides further confirmation that cPLA₂ and not sPLA₂ mediates the ability of NE to release AA in VSMC (data not shown).

CaM Kinase II Acts Upstream of MAP Kinase in the NE-induced PLA₂ Activation—MAP kinase has been reported to activate cPLA₂ and AA release in response to various stimuli in different cell systems (6–8, 22). In most of the experiments with growth factors, MAP kinase has been implicated in the cPLA₂ activation and AA release. There are no reports on the involvement of any other kinase in cPLA₂ activation. Our results suggest that both CaM kinase II and MAP kinase are involved in NE-induced cPLA₂ activation in VSMC. To determine the sequence of events in cPLA₂ activation, experiments were designed to study the effect of NE on 1) MAP kinase activity in the presence of CaM kinase II antisense oligonucleotides or the CaM kinase II inhibitor, KN-93, and 2) CaM kinase activity in the presence of MAP kinase antisense oligonucleotides and MEK inhibitor, PD-098059. CaM kinase II antisense and KN-93 inhibited MAP kinase activity elicited by NE (Fig. 7A). The MEK inhibitor, PD-098059, also significantly reduced the NE-stimulated MAP kinase activity. On the other hand, MAP kinase antisense oligonucleotides and PD-098059 did not reduce NE-stimulated CaM kinase II activity (Fig. 7B). This suggests that MAP kinase does not activate CaM kinase II and that CaM kinase II acts upstream of MAP kinase in NE-stimulated AA release.

It has been reported that phosphorylation is required for the full activation of many enzymes. To determine phosphorylation and activation of MAP kinase and CaM kinase II in response to NE, two approaches were employed. The first approach was to measure 32P incorporation into these kinases, and the second approach was to measure kinase activity in MAP kinase and CaM kinase II immunoprecipitates using synthetic substrates. Fig. 8 shows that NE increased MAP kinase and CaM kinase II phosphorylation and that MAP kinase phosphorylation was inhibited by CaM kinase II inhibitor, KN-93, and MEK inhib-
CaM Kinase II Activates cPLA₂ via MAP Kinase

Table II

| Treatments       | CaM kinase II | MAP kinase |
|------------------|---------------|------------|
| Vehicle          | 4.6           | 1.74       |
| NE               | 9.4           | 3.34       |
| PD-098059 + NE   | 10.0          | 1.52       |
| KN-93 + NE       | 6.8           | 2.18       |

FIG. 9. Translocation of cPLA₂ (without Ca²⁺ (A) and with Ca²⁺ (B)), CaM kinase II (without Ca²⁺ (C) and with Ca²⁺ (D)), and MAP kinase (without Ca²⁺ (E) and with Ca²⁺ (F)) in response to NE as visualized by confocal microscopy. Arrested VSMC that were exposed to NE (10 μM) were visualized using anti-cPLA₂, CaM kinase II, MAP kinase, and TRITC-conjugated goat anti-mouse IgG.

Fig. 8. Effect of CaM kinase II and MAP kinase inhibitors on NE-induced MAP kinase (A) and CaM kinase II (B) phosphorylation. A, Western blot using phosphospecific antibody raised against the activation site of MAP kinase. Cells were incubated for 3 h with inhibitors and then stimulated with NE for 10 min. The lysates were separated by 10% SDS-PAGE and examined by Western blotting. B, NE-stimulated phosphorylation of CaM kinase II and the effect of KN-93 and PD-098059 on NE-stimulated ³²P incorporation into CaM kinase II. The samples were immunoprecipitated by anti-CaM kinase antibody and separated on an SDS-PAGE and subjected to autoradiography.

Table II shows the transfer of phosphate by MAP kinase and CaM kinase II to their respective substrates with NE treatment, and this kinase activity was inhibited by KN-93. In contrast, PD-098059 did not affect the transfer of phosphate to CaM kinase II substrate in VSMC exposed to NE. Collectively, enzyme assays, ³²P incorporation, and immune complex kinase assay confirm the activation of MAP kinase by CaM kinase II. Okadaic acid, a phosphatase inhibitor, which increased phosphorylation of MAP kinase, CaM kinase II (Fig. 8, A and B), and cPLA₂ did not increase [³H]AA release in VSMC (data not shown).

Translocation of cPLA₂, CaM Kinase II, and MAP Kinase to the Nucleus by NE—cPLA₂, CaM kinase II, and MAP kinase translocate in response to various agents. MAP kinase and CaM kinase II exhibit isoform-specific targeting to the nucleus (26, 27). On the other hand, cPLA₂ is targeted to nuclear membrane (9), and this translocation is Ca²⁺-mediated (10). The contribution of phosphorylation and translocation of these enzymes, particularly cPLA₂, in response to endogenous ligands including NE has not yet been characterized. Therefore, we performed immunofluorescence experiments, and the confocal images were obtained using anti-cPLA₂, anti-CaM kinase II, and anti-MAP kinase antibodies in NE-stimulated and unstimulated cells in the presence or absence of extracellular Ca²⁺. We also examined the effect of okadaic acid on the translocation of these enzymes in VSMC. It can be clearly seen that these enzymes are initially dispersed throughout the cytoplasm and that, upon stimulation with NE, they translocate to the nucleus. However, in cells that were stimulated with NE in the absence of extracellular Ca²⁺, cPLA₂, CaM kinase II, and MAP kinase did not translocate to the nuclear envelope. The confocal images of VSMC exposed to vehicle of NE (not shown in Fig. 9) were similar to those that were exposed to NE in the absence of extracellular Ca²⁺. Okadaic acid, which increased phosphorylation and activities of these enzymes, also did not translocate cPLA₂, MAP kinase, or CaM kinase II to the nuclear envelope (data not shown). In control experiments, in which the cells were treated with secondary antibody (TRITC-conjugated goat anti-mouse IgG) in the absence of primary antibody but in the presence of IgG, or in cells that were treated with secondary antibody alone, only faint background fluorescence was observed (data not shown).

DISCUSSION

The present study has led to the following conclusions. 1) NE-induced AA release is mediated via activation of cPLA₂. 2) This requires concomitant activation of CaM kinase II and...
CaM Kinase II Activates cPLA<sub>2</sub> via MAP Kinase

Fig. 10. Schematic diagram illustrating proposed model of CaM kinase II-dependent MAP kinase and cPLA<sub>2</sub> activation in response to α-AR stimulation with NE. G<sub>i</sub>, inhibitory guanine nucleotide binding protein. In this simplified model, activation of AR leads to an influx of Ca<sup>2+</sup> ions through Ca<sup>2+</sup>-channels. Ca<sup>2+</sup> binds to CaM and activates CaM kinase II. CaM kinase II, a Ser/Thr kinase, directly activates MAP kinase through MEK. cPLA<sub>2</sub> upon activation by either CaM kinase II or MAP kinase, translocates to act on its substrate at the nuclear membrane. The nature of the phospholipid and various pathways involved in AA and PGI<sub>2</sub> release are not indicated. The main point illustrated is the role of CaM kinase II in the activation of MAP kinase and cPLA<sub>2</sub>.

MAP kinase. CaM kinase II stimulates cPLA<sub>2</sub> by activation of MAP kinase, most probably via MEK. 3) Upon exposure of VSMC to NE, cPLA<sub>2</sub>, CaM kinase II, and MAP kinase translocate to the nuclear membrane in a Ca<sup>2+</sup>-dependent manner. We have proposed a novel signaling pathway of MAP kinase activation by CaM kinase II (Fig. 10).

Our results provide evidence that in rabbit VSMC, NE stimulates AA release by activating the 85-kDa cPLA<sub>2</sub>. NE-stimulated cPLA<sub>2</sub> activity in VSMC was not altered by the reducing agent DTT, which inactivates sPLA<sub>2</sub>, but not cPLA<sub>2</sub>. cPLA<sub>2</sub>, but not sPLA<sub>2</sub> antisense oligonucleotides attenuated NE-induced AA release in VSMC. Moreover, cotransfection of VSMC with cPLA<sub>2</sub> but not sPLA<sub>2</sub> sense oligonucleotides prevented the inhibitory effect of cPLA<sub>2</sub> AS on NE-induced AA release.

We also investigated the contribution of CaM kinase II and MAP kinase to the activation of cPLA<sub>2</sub>. MAP kinase has been shown to phosphorylate and activate cPLA<sub>2</sub> in vitro (7, 22, 28). Several studies have shown that AA release in response to various stimuli is mediated via the activation of cPLA<sub>2</sub> by MAP kinase (7, 8, 28). However, it has recently been suggested that other uncharacterized kinases are involved in the activation of cPLA<sub>2</sub> (11, 12). The demonstration that prostacyclin synthesis elicited by NE in VSMC was inhibited by a calmodulin inhibitor, W-7 (2), suggests that Ca<sup>2+</sup>/calmodulin can activate cPLA<sub>2</sub> by stimulating CaM kinase II. This kinase is known to be activated by Ca<sup>2+</sup> influx as well as by Ca<sup>2+</sup> released from intracellular stores (13). Our results indicate that activation of α<sub>1</sub>β<sub>2</sub> AR with NE in VSMC leads to activation of CaM kinase II and MAP kinase, which in turn stimulate cPLA<sub>2</sub> to release AA by promoting the influx of extracellular Ca<sup>2+</sup>. Inhibition of NE-induced release of AA in VSMC transiently transfected with CaM kinase II, MAP kinase antisense oligonucleotides, CaM kinase II inhibitor, KN-93, and MEK inhibitor, PD-098059, strongly support this conclusion. This observation is further supported by our findings that CaM kinase II and MAP kinase antisense oligonucleotides and their respective inhibitors reduced the activity of CaM kinase II and MAP kinase, respectively. In addition, CaM kinase II inhibitor and antisense oligonucleotide reduced the MAP kinase activity. However, MAP kinase antisense and the MEK inhibitor, PD-098059, failed to decrease CaM kinase II activity. This suggests the sequential activation of MAP kinase and cPLA<sub>2</sub> by CaM kinase II. Our findings that NE increased 32P incorporation into both MAP kinase and CaM kinase II and transfer of phosphates into their respective substrates and that CaM kinase II inhibitor, KN-93, reduced these effects suggest that CaM kinase II mediates MAP kinase phosphorylation. Since MAP kinase antisense oligonucleotides abolished NE-induced MAP kinase activity but reduced only partially the release of AA, we cannot exclude the possibility that CaM kinase II may also directly activate cPLA<sub>2</sub>. Supporting this view was our finding that CaM kinase II antisense or its inhibitor, KN-93, abolished NE-induced PLA<sub>2</sub> activity, whereas a MEK inhibitor, PD-098059, reduced PLA<sub>2</sub> activity by −50%.

For AA to be released from phospholipids, cPLA<sub>2</sub> has to translocate to nuclear membrane, and a kinase must phosphorylate cPLA<sub>2</sub>. However, the sequence of events is not well defined. Translocation of cPLA<sub>2</sub> from cytosol to nuclear membrane has been reported in response to ionophore or IgE/antigen (9, 10). This translocation of cPLA<sub>2</sub> appears to be very crucial for its function because of the localization of its substrate and of AA-metabolizing enzymes. The nuclear membrane is an important compartment for uptake and release of arachidonate. EM autoradiography studies have shown that the nuclear membrane exhibits the highest specific activity of [3H]arachidonate labeling (29). Arachidonate compartmentalization within the nuclear membrane, and possibly within certain phospholipids in this membrane, is important for AA release and conversion to eicosanoids (30). Our finding that cPLA<sub>2</sub> and the enzymes that increase its activity, CaM kinase II and MAP kinase, translocate to the nuclear envelope strongly suggests that NE promotes AA release from the nuclear membrane. It has been demonstrated that the Ca<sup>2+</sup>-dependent lipid binding domain exists in cPLA<sub>2</sub> with homology to protein kinase C, p65, GTPase-activating protein, and phospholipase C (5). This domain facilitates agonist-stimulated translocation to membranes. Our results showed that cPLA<sub>2</sub>, CaM kinase II, and MAP kinase translocated to the nuclear envelope in a Ca<sup>2+</sup>-dependent manner. It has also been reported that mutation at the MAP kinase phosphorylation site of cPLA<sub>2</sub> did not affect ionophore-induced translocation of the enzyme to the nuclear envelope (10), suggesting that translocation is phosphorylation-independent. Okadaic acid, a protein phosphatase inhibitor, which increased MAP kinase, CaM kinase II, and cPLA<sub>2</sub> phosphorylation did not cause release of [3H]AA or translocation of cPLA<sub>2</sub> to the nuclear envelope. Thus phosphorylation of these enzymes does not appear to require translocation to the nuclear envelope. On the other hand, upon
NE stimulation, cPLA₂, CaM kinase II, and MAP kinase translocate to the nuclear envelope in a Ca²⁺-dependent manner. Thus Ca²⁺ may play a significant role in the translocation of these enzymes and in interaction of cPLA₂ with the phospholipid substrate, which is mediated by a Ca²⁺-dependent phospholipid binding region.

Ca²⁺-dependent MAP kinase activation has been documented in rat cardiac myocytes upon angiotension II stimulation (31). Recently, Ca²⁺-dependent MAP kinase activation by angiotension II was demonstrated to be mediated by intracellular Ca²⁺ and CaM (32). Our results provide evidence that CaM kinase II activates MAP kinase in rabbit VSMC. It has been shown that a protein-tyrosine kinase (Pyk2) is involved in relaying messages from protein kinase C and Ca²⁺, thus activating Ras-mediated regulation of MAP kinase (33). In G-protein-coupled muscarinic acetylcholine receptors, tyrosine kinases Lyn and Syk are involved in the MAP kinase signaling cascade (25). Our results indicate that Ca²⁺-dependent MAP kinase activation by NE is mediated by CaM kinase II, most probably via MEK. CaM kinase is a Ser/Thr kinase that may activate MEK. The protein-tyrosine kinase that has been implicated in the MAP kinase activation does not have a Ca²⁺-sensing region and CaM-binding motif (32), suggesting the possible involvement of CaM kinase II, which fulfills both criteria. In conclusion, we have demonstrated that NE-induced MAP kinase activation in VSMC is mediated by CaM kinase II. CaM kinase II may be a key player orchestrating several mitogenic signaling pathways via activation of MAP kinase and cPLA₂, leading to uncontrolled cell growth in VSMC.

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