Angiotensin II stimulates phosphorylation of high-molecular-mass cytosolic phospholipase A_2 in vascular smooth-muscle cells

Gadiparthi N. RAO,* Bernard LASSÈGUE, R. Wayne ALEXANDER and Kathy K. GRIENDLING
Division of Cardiology, Emory University School of Medicine, Atlanta, GA 30322, U.S.A.

Phospholipase A_2 (PLA_2) may be one of the major components involved in cell signalling and proliferation, as suggested by recent studies. In this paper we show that the potent vasoconstrictor and hypertrophic agent angiotensin II (AngII) activates cytosolic PLA_2 ($cPLA_2$) in vascular smooth-muscle cells. AngII induced a rapid time-dependent release of [^{3}H]arachidonic acid from prelabelled cells that was inhibited by mepacrine, a PLA_2 inhibitor. AngII treatment of intact cells also activated a $cPLA_2$, as measured in cell-free extracts by the release of radiolabelled arachidonic acid from exogenously added 1-stearoyl-2-[1-^{14}C]arachidonoyl phosphatidylcholine. This AngII-stimulated $cPLA_2$ activity was also significantly inhibited by mepacrine. AngII induced a rapid and time-dependent increase in $cPLA_2$ phosphorylation. Protein kinase C (PKC) depletion inhibited both AngII-induced [^{3}H]arachidonic acid release and $cPLA_2$ phosphorylation. Together, these results suggest strongly that (1) AngII phosphorylates and activates $cPLA_2$ in a PKC-dependent manner, and that (2) $cPLA_2$ mediates the AngII-induced [^{3}H]arachidonic acid release in vascular smooth-muscle cells.

INTRODUCTION

Phospholipases A_2 (PLA_2) are enzymes that hydrolyse the *sn*-2 acyl ester bond of phospholipids, generating free fatty acids and lysophospholipids [1–3]. The PLA_2 products arachidonic acid and lysophosphatidylcholine are rate-limiting precursors of eicosanoids and platelet-activating factor, respectively, which are potent mediators of functions such as vasoactivity and inflammation [1–4].

For many years arachidonic acid was thought to be mainly generated by PLA_2s of the secretory type. These constitute a class of closely related enzymes with molecular masses ranging from 14 to 20 kDa [5–7]. Major features of secretory PLA_2s include their sensitivity to thiol reagents [4], their requirement for millimolar concentrations of Ca^{2+} and their lack of specificity for substrates with arachidonic acid in the *sn*-2 position. Their function is now thought to be limited to lipid digestion and maintenance of cell membrane homoeostasis [7].

Recent investigations in various cell types revealed another class of PLA_2s, with molecular masses in the range 85–110 kDa [8–17]. These enzymes are mainly cytosolic ($cPLA_2$s), are active at micromolar Ca^{2+} concentrations [4,18] and preferentially hydrolyse phospholipids with arachidonic acid at the *sn*-2 position [8–17]. These properties suggest that $cPLA_2$s may be specialized in cellular signalling [19]. Indeed, they are activated by hormones [10,11,20] and growth factors [20] and provide the precursor for generation of growth-promoting eicosanoids [21–23].

In vascular smooth-muscle cells (VSMC), the potent vasoconstrictor angiotensin II (AngII) is known to increase the synthesis of arachidonic acid derivatives such as prostaglandins [24] and leukotrienes, via activation of the cyclo-oxygenase and lipoxygenase pathways, respectively. However, the enzyme responsible for the initial production of arachidonic acid has not been identified. In the present paper we show that AngII stimulates [^{3}H]arachidonic acid release via protein kinase C (PKC) and $cPLA_2$ activation in VSMC. These observations raise the possibility that $cPLA_2$ activation may be involved in AngII-induced hypertrophy.

MATERIALS AND METHODS

Materials

Arachidonic acid and mepacrine were obtained from Sigma (St. Louis, MO, U.S.A.). Staurosporine was from Biomol (Philadelphia, PA, U.S.A.). [5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (210 Ci/mmol) and [^{32}P]orthophosphoric acid (8500 Ci/mmol) were purchased from NEN (Boston, MA, U.S.A.). 1-Stearoyl-2-[1-^{14}C]arachidonoyl phosphatidylcholine (55.6 mCi/mmol) was from Amersham (Arlington Heights, IL, U.S.A.).

Cell culture

VSMC were isolated from the thoracic aortae of 200–250 g male Sprague–Dawley rats by enzymic dissociation as described elsewhere [25]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained in humidified air/CO_2 (19:1) at 37 °C by passage of (1–3) $\times$ 10^5 cells/ml on reaching confluence. For all experiments, cells at 70–80% confluence were made quiescent by incubation for 48 h in fresh DMEM containing 0.1% heat-inactivated calf serum. Throughout the course of these experiments, cells were used at passage numbers 8–15.

[^{3}H]Arachidonic acid release

VSMC were grown for 24 h in DMEM containing 10% calf serum, supplemented with 0.5 μCi/ml [^{3}H]arachidonic acid [26]. Cells were then growth-arrested by incubation for 48 h in fresh DMEM containing 0.1% serum and 0.5 μCi/ml [^{3}H]arachidonic acid. To initiate the experiment, cells were rinsed four times with, and incubated at 37 °C in 1 ml of 0.1% calf serum/DMEM with or without agonist. The medium was removed and extracted

Abbreviations used: PLA_2, phospholipase A_2; $cPLA_2$, cytosolic PLA_2; VSMC, vascular smooth-muscle cells; AngII, angiotensin II; PKC, protein kinase C; DMEM, Dulbecco's modified Eagle's medium; PDBu, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate.

* To whom correspondence should be addressed.

with 2 vol. of chloroform/methanol (1:1, v/v). The organic phase was evaporated under nitrogen, resuspended in 200 μl of chloroform, spotted on Whatman silica-gel 60A LK6D t.l.c. plates and developed with the organic phase of the mixture ethyl acetate/hexane/acetic acid/water (17:7:3:18, by vol.); 1 μmol of unlabelled arachidonic acid standard was added as a carrier. The spot corresponding to arachidonic acid was made visible with iodine vapour, scraped, and its radioactivity was measured by liquid-scintillation spectrometry.

cPLA$_2$ assay

cPLA$_2$ activity was measured with 1-stearoyl-2-[1-^{14}C]arachidonoyl phosphatidylcholine as substrate as described by Gronich et al. [9]. Substrate was dried down under N_2 and resuspended in dimethyl sulphoxide by vigorous vortex-mixing. The reaction mixture consisted of 34 μl of cell extract in 50 mM Hepes (pH 7.4), 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, 200 μM pepstatin, 20 μM leupeptin and 0.1 mM phenylmethanesulphonyl fluoride, 2 μl of 54 mM $CaCl_2$ and 2 μl of 40 mM 2-mercaptoethanol. Reactions were initiated at 37 °C by addition of 2 μl of 200 μM substrate. The assay mixture had a final concentration of 1 mM free Ca^{2+} (in excess of EDTA and EGTA), 2 mM 2-mercaptoethanol and 10 μM substrate. After incubation for 30 min, reactions were terminated by addition of 40 μl of ethanol/acetic acid (49:1, v/v) containing 100 μg/ml unlabelled arachidonic acid. Release of radiolabelled arachidonic acid was measured by t.l.c. as described above.

Phosphorylation of cPLA$_2$

Growth-arrested VSMC were labelled with 300 μCi/ml [^{32}P]P_i for 3 h at 37 °C in Hepes-buffered salt solution (130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 20 mM Hepes, pH 7.4) before exposure to 100 nM AngII. At the end of the stimulation period, cells were rinsed with ice-cold PBS and lysed on ice for 15 min in 1.0 ml of 150 mM NaCl/1% SDS/50 mM Tris/HCl (pH 7.5)/1 mM sodium orthovanadate/0.5 mM phenylmethanesulphonyl fluoride/50 mM NaF/2 mM sodium pyrophosphate/80 mM β-glycerophosphate/2 mM EGTA. The cell lysates were then clarified by centrifugation for 30 min at 14000 rev./min in a microfuge at 4 °C. Cell lysates containing equal amounts of trichloroacetic acid-precipitable radioactivity were then incubated with 10 μl of polyclonal antiserum against cPLA$_2$ overnight at 4 °C. Then 50 μl of Protein A–Sepharose bead suspension in water (50%, v/v) was added to the samples and incubated for 90 min on ice with rocking. The beads were then collected by centrifugation at 6000 rev./min for 1 min at 4 °C in a microfuge, and washed five times with ice-cold lysis buffer and once with ice-cold PBS. The immunoprecipitated proteins were dissolved by heating the beads at 100 °C for 5 min in 40 μl of Laemmli sample buffer and were separated by electrophoresis on 0.1%-SDS/10%-polyacrylamide gels under reducing conditions [27]. The gels were dried and exposed to Kodak X-Omat AR X-ray film with an intensifying screen at −70 °C for 2 days.

Phosphoamino acid analysis

Cells were treated as described above for measurement of cPLA$_2$ phosphorylation. After cell lysis, immunoprecipitation and SDS/PAGE, proteins were transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA, U.S.A.) by electroblotting overnight at 100 mA [28]. After transfer, the membrane was autoradiographed. The cPLA$_2$ band was excised and exposed to vapours of 5.7 M HCl at 110 °C for 4 h in a Reactivial (Pierce, Rockford, IL, U.S.A.). After acid hydrolysis, one drop of methanol was placed on the membrane patches, and amino acids and peptide fragments were extracted with 1 ml of water [29]. A mixture of phosphoserine, phosphothreonine and phosphotyrosine (100 nmol each) was added to the aqueous extract before freeze-drying over NaOH. Samples were dissolved in 20 μl of water and spotted on a 20 cm × 20 cm cellulose t.l.c. plate (J. T. Baker, Phillipsburg, NJ, U.S.A.). The phosphoamino acids were separated by one-dimensional electrophoresis in 7.8% acetic acid/2.2% formic acid (pH 1.9) at 500 V for 4 h. Standards were revealed with ninhydrin before autoradiography.

RESULTS AND DISCUSSION

AngII induced a time-dependent release of [^{3}H]arachidonic acid from prelabelled VSMC, up to 2.4 times the control level after 30 min (Figure 1). The following biochemical pathways could account for the observed release of arachidonic acid: (1) direct phospholipid hydrolysis by PLA$_2$ [30]; (2) phospholipid hydrolysis by phospholipase C, generating diacylglycerol, followed by hydrolysis of diacylgycerol by diacylglycerol lipase [31]; (3) phospholipid hydrolysis by phospholipase D, generating phosphatidic acid [31–34], and subsequent hydrolysis by a specific lipase or PLA$_2$. Although PLA$_2$ is usually the major source of free fatty acids, the relative contributions of these pathways vary with each cell type [15]. In order to ascertain the pathway accounting for [^{3}H]arachidonic acid release in VSMC, we used mepacrine, a widely used PLA$_2$ inhibitor [35,36]. As shown in Table 1, mepacrine (5 μM) significantly inhibited (66%) AngII-induced [^{3}H]arachidonic acid release. Previous studies have reported that AngII does not stimulate secretory PLA$_2$ activity in rat aortic smooth-muscle cells [37]. Therefore it is likely that mepacrine-inhibitable [^{3}H]arachidonic acid release in VSMC is mediated by cPLA$_2$. However, because of lack of specificity of the inhibitor [38], we also measured cPLA$_2$ activity using a specific substrate, 1-stearoyl-2-[1-^{14}C]arachidonoyl phosphatidylcholine, in cell-free extracts of AngII-treated and untreated VSMC. AngII caused a 2-fold increase in cPLA$_2$ activity, which was significantly inhibited (71%) by mepacrine (Table 1). Together, these findings suggest that AngII-induced [^{3}H]arachidonic acid release is mediated by cPLA$_2$.

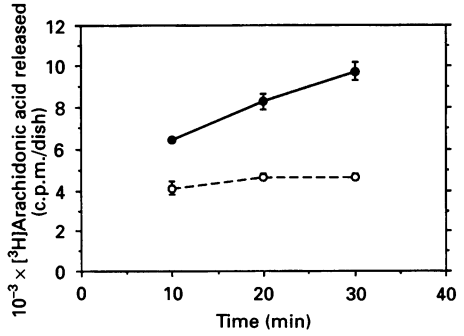

Figure 1 Time course of AngII-induced [^{3}H]arachidonic acid release

VSMC were labelled with [^{3}H]arachidonic acid and growth-arrested before exposure to vehicle (○) or 100 nM AngII (●) for the indicated times. [^{3}H]Arachidonic acid released in the medium was separated and identified by t.l.c. Points represent means ± S.E.M. of values from two independent experiments performed in duplicate.

Table 1 Effect of mepacrine on AngII-induced [^{3}H]arachidonic acid release and cPLA$_2$ activity

For measurement of [^{3}H]arachidonic acid release, prelabelled VSMC were growth-arrested and treated with or without 100 nM AngII or 5 μM mepacrine or both substances for 30 min. Released [^{3}H]arachidonic acid was measured as described in the Materials and methods section. For measurement of cPLA$_2$ activity, growth-arrested VSMC were treated with or without 100 nM AngII for 30 min and cell extracts were prepared. cPLA$_2$ activity was measured with 1-stearoyl-2-[1-^{14}C]arachidonoyl phosphatidylcholine as substrate. Mepacrine was added to both control and AngII-treated cell-free extracts before addition of substrate. Data are presented as means ± S.E.M. ($n = 4$).

Treatment	[^{3}H]Arachidonic acid release (c.p.m./dish)	cPLA$_2$ activity (pmol/30 min per mg of protein)
Control	7913 ± 143	48 ± 2
AngII	16629 ± 331	89 ± 1
Mepacrine + AngII	11004 ± 343	53 ± 2
Mepacrine	8205 ± 477	55 ± 3

Table 2 Role of PKC in AngII-induced [^{3}H]arachidonic acid release

Growth-arrested pre-labelled VSMC were treated with 200 nM PDBu for 24 h, washed with DMEM and exposed to 100 nM AngII or 200 nM PMA for 30 min. Staurosporine was added 15 min before addition of agonist. Released [^{3}H]arachidonic acid was measured as described in the Materials and methods section. Data are presented as means ± S.E.M. ($n = 6$).

Treatment	[^{3}H]Arachidonic acid released (c.p.m./dish)
Control	6533 ± 183
AngII	13193 ± 1085
PDBu + AngII	5380 ± 377
PMA	15608 ± 1106
PDBu ± PMA	4782 ± 989
Staurosporine + AngII	8188 ± 87
Staurosporine + PMA	7832 ± 281
Staurosporine	4601 ± 281

In other cell systems, activation of cPLA$_2$ is often secondary to PKC stimulation [20,36,39]. To determine if this was also the case in our system, growth-arrested VSMC, prelabelled with [^{3}H]arachidonic acid, were exposed to 200 nM phorbol 12,13-dibutyrate (PDBu) for 24 h to down-regulate PKC. Prolonged PDBu treatment has been shown to down-regulate PKC significantly in VSMC [40], as well as in other cell types [41,42]. PKC-depleted VSMC were then exposed to AngII for 30 min, and [^{3}H]arachidonic acid release was measured. PKC down-regulation blocked both AngII- and phorbol 12-myristate 13-acetate (PMA)-induced [^{3}H]arachidonic acid release (Table 2). Similar findings were observed by use of the PKC inhibitor staurosporine (Table 2). Our observations are consistent with data obtained in CHO cells over-expressing cPLA$_2$, in which PKC inhibition attenuated [^{3}H]arachidonic acid release induced by various agonists [20,39].

To investigate the mechanism of activation of cPLA$_2$, growth-arrested VSMC were prelabelled with [^{32}P]P_i and exposed to AngII for various times. Cell lysates were immunoprecipitated with anti-cPLA$_2$ polyclonal antibodies and analysed by SDS/PAGE. As shown in Figure 2, AngII caused a time-dependent phosphorylation of cPLA$_2$. Stimulation with AngII for 30 min induced a 3–4-fold increase in cPLA$_2$ phosphorylation. This time course is similar to that of AngII-induced [^{3}H]arachidonic acid release (Figure 1), supporting the idea that cPLA$_2$ may be activated by phosphorylation. This result is consistent with a recent report which showed that cPLA$_2$ is phosphorylated and

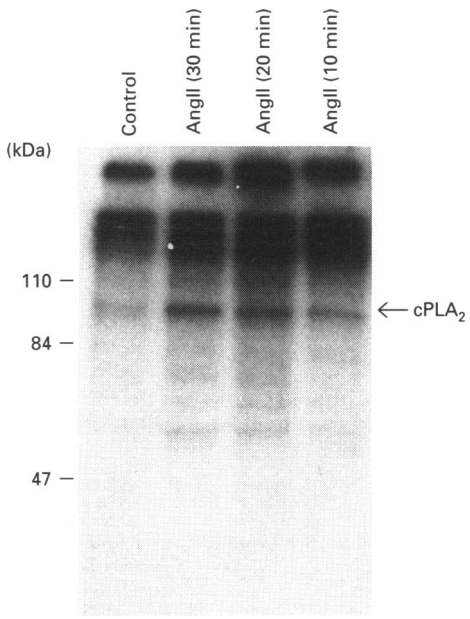

Figure 2 Time course of AngII-induced cPLA$_2$ phosphorylation

Growth-arrested VSMC were labelled with [^{32}P]P_i and exposed to 100 nM AngII for the indicated times. Samples of cell lysates containing equal amounts of trichloroacetic acid-precipitable radioactivity were incubated with polyclonal antiserum against cPLA$_2$. The immunoprecipitates were separated on a 0.1%-SDS/10%-polyacrylamide gel. ^{32}P-labelled cPLA$_2$ was identified by its apparent molecular mass (~ 97 kDa, similar to that of Rat-2 cells [20]) on the autoradiogram.

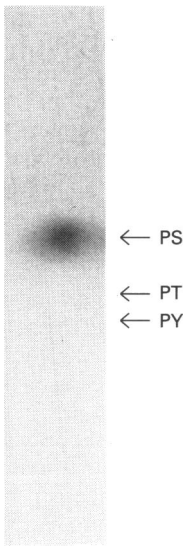

Figure 3 Phosphoamino acid analysis of cPLA$_2$

^{32}P-labelled VSMC were exposed to 100 nM AngII for 30 min. Cell lysates were immunoprecipitated, separated on SDS/PAGE, and transferred to a polyvinylidene difluoride membrane. The ^{32}P-labelled cPLA$_2$ was identified by autoradiography, excised and acid-hydrolysed. Phosphoamino acids were separated by electrophoresis on a cellulose t.l.c. plate. Standards were revealed with ninhydrin and ^{32}P-labelled amino acids were detected by autoradiography. Abbreviations: PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine.

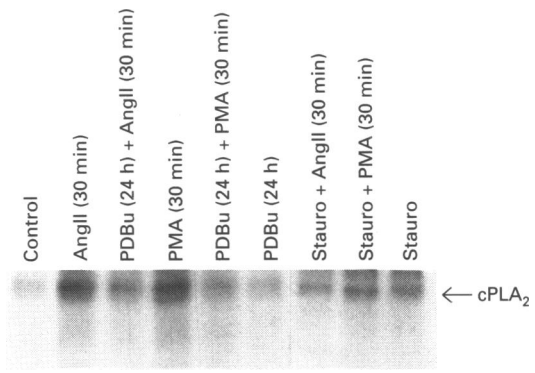

Figure 4 PKC dependency of AngII-induced cPLA$_2$ phosphorylation

Growth-arrested PKC-depleted VSMC were labelled with [^{32}P]P_i and exposed to 100 nM AngII or 200 nM PMA for 30 min. For PKC inhibition, growth-arrested [^{32}P]P_i-labelled VSMC were treated with 100 nM AngII or 200 nM PMA in the absence or presence of 200 nM staurosporine (Stauro). Cell lysates were treated as in Figure 2 and cPLA$_2$ was separated by SDS/PAGE.

activated in CHO cells in response to various agonists such as platelet-derived growth factor, epidermal growth factor, thrombin and ATP [20]. To determine which type of amino acid in cPLA$_2$ is phosphorylated by exposure to AngII, phosphoamino acid analysis was performed. Figure 3 indicates that cPLA$_2$ phosphorylation occurs on serine residues, a result consistent with previous observations in CHO cells [20].

Since [^{3}H]arachidonic acid release appeared to be dependent on PKC activation (Table 2), we determined whether cPLA$_2$ phosphorylation also required PKC activity. As indicated in Figure 4, PKC depletion blocked both AngII- and PMA-induced cPLA$_2$ phosphorylation. Staurosporine, a PKC inhibitor, also significantly attenuated cPLA$_2$ phosphorylation by these agonists. These results further support the idea that cPLA$_2$ is activated by phosphorylation and are consistent with the reported inhibition of cPLA$_2$ phosphorylation by staurosporine in CHO and HL60 cells [20,36].

The activation of cPLA$_2$ by AngII may be the origin of numerous signalling events, since arachidonic acid and its metabolites are known to have second-messenger functions in G-protein-coupled receptor signal transduction [19]. Arachidonic acid is likely to be involved in AngII-induced contraction and, perhaps more importantly, in cell growth.

The modulation of vascular tone by AngII-stimulated PLA$_2$-mediated arachidonic acid formation may be complex. Arachidonic acid might be expected to decrease vasoconstriction, since inhibition of prostaglandin synthesis potentiates AngII-induced vasoconstriction and decreases bradykinin-induced vasodilation [43]. However, it is also likely to enhance vasoconstriction if it is converted into eicosanoids, since inhibitors of lipoxygenase decreased the contractile response to AngII *in vivo* and in isolated vascular tissue [44]. The net role of PLA$_2$ activation in AngII-induced vasoconstriction remains to be determined.

The metabolites of arachidonic acid also appear to be involved in cell growth. Indeed, arachidonic acid metabolism was required for growth stimulation of mesangial cells by serum [45] and of BALB 3T3 cells by epidermal growth factor [46]. Similarly, the epoxygenase-dependent metabolites of arachidonic acid, epoxyeicosatrienoic and prostaglandin E_1, induced mesangial-cell division [47] and VSMC DNA synthesis [48], respectively. In VSMC, AngII phosphorylates and activates mitogen-activated protein (MAP) kinases [49], and MAP kinases phosphorylate and activate cPLA$_2$ in other cell types [50,51]. In addition, Nemenoff et al. [51] showed that PKC also phosphorylates and activates cPLA$_2$. Our present results indicate that AngII phosphorylates and activates cPLA$_2$ in VSMC via a PKC-dependent pathway. Considering these findings in light of the known function of PKC, MAP kinase and eicosanoids in cell growth, it is likely that cPLA$_2$ plays an important role in VSMC growth by AngII. Future studies will determine which metabolites of arachidonic acid are increased by AngII stimulation and whether the administration of these compounds can mimic the effect of the agonist.

In summary, our results demonstrate that AngII activates cPLA$_2$ in vascular smooth muscle and raise the possibility that this enzyme may be a key component in the regulation of contraction and pathological growth.

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