Vascular smooth-muscle cells contain AT₁ angiotensin receptors coupled to phospholipase D activation

Ernest J. FREEMAN* and E. Ann TALLANT†‡

*Calhoun Research Laboratory, Department of Internal Medicine, Akron General Medical Center, 400 Wabash Ave., Akron, OH 44307, and

†The Hypertension Center, Bowman Gray School of Medicine of Wake Forest University, Medical Center Boulevard, Winston-Salem, NC 27157-1032, U.S.A.

We previously showed that angiotensin II (Ang II) and angiotensin-(2–8)-peptide [Ang-(2–8)] activate a phosphoinositide-specific phospholipase C (PLC) and cause calcium mobilization in rat aortic vascular smooth-muscle cells (VSMC), while Ang II and Ang-(1–7) produce prostaglandins. To define further the signal-transduction mechanisms activated by angiotensin peptides in smooth-muscle cells, we measured diacylglycerol (DAG) accumulation in response to different angiotensin peptides and its inhibition by subtype-selective receptor antagonists. Both an initial (10 s) and secondary (10 min) phase of DAG production in response to 100 nM Ang II were inhibited by 1 μ M losartan (DuP 753), an AT₁ antagonist, while 1 μ M PD 123177, an AT₂ antagonist, was ineffective. In contrast, the heptapeptide Ang-(1–7) did not produce DAG in VSMC. Ang II also caused the hydrolysis of phosphatidylinositol and phosphatidylcholine, the formation of phosphatidic acid and the formation of phosphatidylethanol (PEt) in the presence of ethanol, through activation

of a PLD and a PLD-induced transphosphatidyl reaction. A similar concentration of Ang-(2–8) also activated PLD; in contrast, Ang-(1–7) was ineffective. PEt production by 100 nM Ang II was significantly attenuated by the AT₁ antagonists losartan, its metabolite EXP 3174 or L-158,809 (all at 1 μ M), whereas a similar concentration of the AT₂ antagonists CGP 42112A or PD 123177 was ineffective. The production of PEt by Ang II was also partially attenuated by the removal of extracellular calcium and potentiated by increasing calcium concentrations, indicating that PLD activity is partially dependent on extracellular calcium. Thus VSMC PLD is coupled to an AT₁ receptor and occurs in response to Ang II or Ang-(2–8), but not Ang-(1–7). Since AT₁ receptors in VSMC are also coupled to activation of PLC, both PLC and PLD may be coupled to the same or a different AT₁ receptor. Alternatively, PLD may be sequentially activated in response to Ang II activation of PLC and a subsequent increase in calcium concentration.

INTRODUCTION

Vascular smooth-muscle cells (VSMC) contain many components of the renin–angiotensin system, including high-affinity angiotensin II (Ang II) receptors [1]. Furthermore, Ang II participates in the regulation of blood pressure [2] and has been suggested to act as a partial mitogen in vascular smooth-muscle tissue [3,4]. VSMC isolated from rat aorta possess functional Ang II receptors which are coupled to the activation of a phosphoinositide-specific phospholipase C (PLC), the production of inositol 1,4,5-trisphosphate and diacylglycerol (DAG), the mobilization of intracellular Ca²⁺ and the activation of protein kinase C (PKC) [2,5,6]. The production of DAG by Ang II in VSMC from rat aorta is biphasic [7]. The initial phase of DAG production most likely results from phosphoinositide hydrolysis. However, the hydrolysis of phosphatidylcholine or other phospholipids by phospholipase D (PLD) may account for the secondary, more sustained formation of DAG, since Ang II activates PLD in VSMC and mesangial cells [8,9].

At least two subtypes of Ang II receptors have been identified by their selectivity for different peptide and non-peptide receptor antagonists. AT₁ receptors display high-affinity binding for the non-peptide antagonist losartan (DuP 753), its metabolite EXP 3174 [10] and L-158,809 [11]. The available AT₂ receptor

antagonists include the peptide CGP 42112A and the non-peptide PD 123177 [10,12]. Using these antagonists we have shown that AT₁ and AT₂ receptors on rat VSMC and cultured human astrocytes are coupled to different mechanisms of signal transduction (phosphoinositide hydrolysis and prostaglandin release respectively) [13–16]. Although AT₁ receptors coupled to phosphoinositide hydrolysis and the mobilization of intracellular Ca²⁺ are most likely responsible for the vasoconstrictive responses to Ang II, both AT₁ and AT₂ receptors participate in neointimal development after injury [17–20]. Thus both AT₁ and AT₂ receptors have been functionally identified on VSMC. However, rat aortic VSMC in culture have been reported to contain only AT₁ receptors, on the basis of competition by subtype-selective receptor antagonists in binding assays [12,21]. In contrast, Viswanathan et al. [22] identified both AT₁ and AT₂ receptors in the rat aorta by quantitative autoradiography *in vitro*. Moreover, the relative expression of AT₁ and AT₂ receptors changed during aortic development, with AT₂ receptors predominating in embryonic and 2-week postnatal rats compared with a predominance of AT₁ receptors in 8-week-old rats. These results can be interpreted as a loss of AT₂ receptors on placing VSMC into culture, or a developmental loss of AT₂ receptors such that they are undetectable in binding assays of cells isolated from 12–14-week-old rats. The latter interpretation suggests that, even though

Abbreviations used: VSMC, vascular smooth-muscle cells; Ang, angiotensin; PLC, phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; PLD, phospholipase D; PG, prostaglandin; PA, phosphatidic acid; PP, phosphatidate phosphohydrolase; FBS, fetal-bovine serum; KR, Krebs–Ringer; EtOH, ethanol; PEt, phosphatidylethanol; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; DMEM, Dulbecco's modified Eagle's medium.

‡ To whom correspondence should be sent.

AT₂ receptors are not detectable in binding assays, a small population may be present on cultured VSMC. In fact, in differentiated NG108-15 cells and pancreatic acinar AR42J cells, we find a predominance of AT₂ receptors upon measurement of receptor binding; however, functional assessment of AT₁ receptor activity (Ca²⁺ mobilization through activation of AT₁ receptors) revealed a small population of functionally active AT₁ receptors [23,24]. Collectively, these results suggest that rat aortic VSMC contain a small population of functional AT₂ receptors which are not detected in binding assays.

We recently showed that Ang II and the heptapeptide angiotensin-(2-8)-peptide [Ang-(2-8)] activate PLC in rat aortic VSMC, while Ang II and the heptapeptide Ang-(1-7) produce prostaglandins [13,14]. These results suggest that phosphoinositide hydrolysis and prostaglandin (PG) release can be selectively activated by the angiotensin heptapeptides Ang-(2-8) and Ang-(1-7). Furthermore, results that we obtained in glial cells [16] suggest that PG release in response to Ang-(1-7) occurs through activation of AT₂ receptors. Since Ang-(1-7) does not cause an increase in intracellular Ca²⁺ in rat aortic VSMC [13], PGs are not generated by activation of a Ca²⁺-dependent phospholipase A₂. As an alternative mechanism, DAG directly produced from PLC or indirectly generated by PLD, through the conversion of phosphatidic acid (PA) to DAG by phosphatidate phosphohydrolase (PP), could be hydrolysed by a DAG lipase to release arachidonic acid for PG production. To define further the responses to angiotensin peptides in VSMC, we investigated which peptides [i.e. Ang II, Ang-(2-8) or Ang-(1-7)] and what subtypes of receptors participate in DAG formation and activate PLD in rat aortic VSMC. Part of this work was presented at the 1992 Meeting of the Federation of American Societies for Experimental Biology [25].

MATERIALS AND METHODS

Materials

[³H]Oleic acid (7.2 Ci/mmol) and [γ -³²P]ATP (3000 Ci/mmol) were purchased from du Pont-New England Nuclear, Boston, MA, U.S.A. Dulbecco's modified Eagle's medium (DMEM) and F12 medium were obtained from JRH Bioscience, Lenexa, KS, U.S.A.; fetal-bovine serum (FBS), penicillin, streptomycin and trypsin/EDTA were from GIBCO, Grand Island, NY, U.S.A. DAG, phosphatidylserine (PS), PA, diethylenetriaminepenta-acetic acid, imidazole hydrochloride, ATP, DL-dithiothreitol and BSA were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bovine brain cardiolipin and PEt (sodium salt from egg) were from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Octyl β -D-glucoside was from Calbiochem (San Diego, CA, U.S.A.) and DAG kinase (from *Escherichia coli*) was from Lipidex (Middleton, WI, U.S.A.). Losartan (Losartan potassium; DuP 753), EXP 3174 and PD 123177 were gifts from Dr. P. Timmermans of du Pont (Wilmington, DE, U.S.A.), CGP 42112A was from Dr. M. DeGasparo of CIBA-GEIGY (Basle, Switzerland), and L-158,809 was a gift from Merck Sharp and Dohme Research Laboratories (West Point, PA, U.S.A.).

Culture of rat vascular smooth-muscle cells

VSMC were isolated from rat thoracic aorta by explant culture using a previously published procedure [5]. Cells were identified as VSMC by their characteristic morphology as well as the antibodies to VSMC-specific α -actin (Sigma). Cells were routinely used between passages 3 and 10.

DAG assay

For DAG assays, confluent monolayers of VSMC in six-well cluster dishes were rinsed two times in PBS and pretreated for 10 min with a Krebs-Ringer (KR) solution [components (in mM): NaCl, 125; KCl, 5; MgSO₄, 1.2; glucose, 6; Hepes, 25; pH 7.5] containing 1 mM Ca²⁺ and 0.1% BSA. Subsequently, the monolayers were incubated with Ang II for various time periods and the reactions were stopped by aspirating the medium and adding ice-cold methanol. Lipids were extracted with chloroform/methanol (1:2, v/v), essentially as described by Bligh and Dyer [26]. The DAG in lipid extracts prepared from cells was quantitatively converted into [³²P]PA by DAG kinase in the presence of [γ -³²P]ATP, and the mass content of DAG was measured as described by Preiss et al. [27].

Determination of phospholipid hydrolysis and PLD activity

The hydrolysis of various phospholipids and the measurement of PLD activity were determined in VSMC in which the phosphatidyl moieties were metabolically prelabelled with radiolabelled oleic acid. Pre-confluent VSMC in six-well plates were incubated for 24 h with [³H]oleic acid (2 μ Ci/well) in 2 ml of DMEM/F12 containing 10% FBS. After 24 h the growth medium was removed and replaced with KR solution containing 1 mM Ca²⁺. For determining the incorporation of radiolabel into individual phospholipids and their hydrolysis by Ang II, experiments were either terminated after the 30 min incubation with KR solution or incubated an additional 10 min with Ang II. For measurement of PLD activity, KR solution was removed after 30 min and KR with or without 100 mM ethanol (EtOH) was introduced for an additional 5 min. Wells were then treated with the appropriate agonists and/or antagonists in the continued presence or absence of 100 mM EtOH for various times. Experiments were terminated by aspirating the treatment buffer and adding ice-cold methanol. Lipids were extracted as described by Bligh and Dyer [26] and spotted on precoated 20 cm $\times$ 20 cm silica-gel LK6D TLC plates (Whatman). To determine the incorporation of radiolabel into various phospholipids, the plates were analysed by two-dimensional chromatography; the first dimension was developed in chloroform/methanol/7 M NH₄OH (90:54:11, by vol.) and the second dimension in chloroform/methanol/acetic acid/water (90:40:12:2, by vol.) [28]. For analysis of production of PA and phosphatidylethanol (PEt) by PLD, the plates were developed using the solvent system ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (13:2:3:10, by vol.). Phospholipids were revealed with iodine vapour and identified by the position of authentic standards. These areas were scraped from the plates into scintillation vials, extracted with 1 ml of methanol and counted for radioactivity in a liquid-scintillation spectrometer after the addition of 9 ml of Ecolume scintillant (ICN Bio-medicals, Irvine, CA, U.S.A.).

Statistics

The results are expressed as means $\pm$ S.E.M. The data were subjected to analysis of variance and the Newman-Keuls pairwise-comparison test. A value of $P < 0.05$ was considered as significant.

RESULTS

Effects of Ang peptides and Ang receptor antagonists on DAG formation

Ang II (100 nM) produced two phases of diacylglycerol accumulation in VSMC, as previously observed by others [7]. The

Table 1 Effect of receptor antagonists on Ang II-stimulated DAG accumulation

Cells growing as monolayers in six-well cluster dishes were preincubated with the indicated antagonist (1 μ M) for 2 min prior to the addition of Ang II (100 nM). Incubations were continued for either 10 s or 10 min as indicated, and terminated by removal of the incubation medium and addition of ice-cold methanol. DAG levels were quantified as described in the Methods and materials section. Results are means $\pm$ SEM for four experiments and are presented as percentages of control in the presence of buffer alone (214.7 ± 37.7 pmol of DAG formed/well at 10 s and 258.5 ± 52.7 pmol of DAG formed/well at 10 min). Values marginally significant from control are indicated * ($P = 0.06$); values significantly different from control are indicated ** ($P < 0.05$).

Period of incubation and component added	DAG formed (% of control)
At 10 s	
Ang II	132.0 ± 8.9
Ang II + DuP 753	$88.2 \pm 16.4^*$
Ang II + CGP 42112A	100.5 ± 17.7
Ang II + PD 123177	133.2 ± 7.1
At 10 min	
Ang II	215.1 ± 28.3
Ang II + DuP 753	$110.9 \pm 14.1^{**}$
Ang II + CGP 42112A	219.8 ± 17.6
Ang II + PD 123177	174.2 ± 18.4

initial peak of diacylglycerol production, at 10 s, was inhibited by the AT₁ receptor antagonist losartan (1 μ M), while the AT₂ antagonist PD 123177 (1 μ M) was ineffective (Table 1). Although the AT₂ antagonist CGP 42112A (1 μ M) tended to decrease the initial phase of DAG formation (from $132 \pm 8.9\%$ to $100.5 \pm 17.7\%$), CGP 42112A is known to be non-selective at higher concentrations [12]. The secondary more sustained peak of DAG formation, at 10 min, was also completely inhibited by 1 μ M losartan, while neither of the two AT₂ selective antagonists was effective. A similar concentration of the receptor antagonists alone had no significant effect on the levels of DAG. Finally, the addition of 1 μ M Ang-(1–7) did not result in any significant accumulation of DAG over the 20 min time course of incubation.

Hydrolysis of phospholipids by Ang II

Although VSMC DAG is formed by the hydrolysis of polyphosphoinositides by a phosphoinositide-specific PLC [7], DAG formation may also result from the hydrolysis of other phospholipids by an additional PLC or a PLD. To examine the hydrolysis of other phospholipids in response to Ang II stimulation, the phosphatidyl moieties of VSMC phospholipids were metabolically labelled by incubation with [³H]oleic acid. Although the major phospholipid labelled by this treatment was phosphatidylcholine (PC; $55.8 \pm 2.8\%$ of the total amount of radiolabelled phospholipids; $n = 3$), radiolabel was incorporated into other phospholipids, including phosphatidylinositol (PI; $13.2 \pm 0.7\%$ of total) and PS ($9.4 \pm 1.2\%$ of total). The remaining radiolabel was incorporated in various other phospholipids. Treatment with 100 nM Ang II for 10 min significantly decreased the amount of radiolabel associated with PC ($52.5 \pm 0.6\%$ of total, $P < 0.05$) and PI ($11.3 \pm 0.1\%$ of total, $P < 0.05$). The radiolabel associated with PS did not change ($9.1 \pm 0.9\%$ of total). Similar results were obtained after longer times of incubation.

Stimulation of PLD by Ang II

Activation of PLD results in phospholipid hydrolysis and the production of PA or, in the presence of EtOH, in the specific

Figure 1 Dose response and time course for Ang II-induced activation of PLD in VSMC

VSMC prelabelled with [³H]oleic acid were incubated with Ang II in the presence of 100 mM EtOH. The formation of both PET and PA was quantified as described in the Materials and methods section. In (a) cells were incubated with increasing concentrations of Ang II. Results are means $\pm$ SEM for five experiments and are reported as percentages above control in the presence of 100 mM EtOH alone. Control values for PA (○) were 1107 ± 109 c.p.m./well and 689 ± 74 c.p.m./well for PET (●). * $P < 0.05$ as compared with controls. In (b) VSMC were incubated in the presence or absence of 100 nM Ang II for increasing periods of time. Results are means $\pm$ SEM for at least four experiments and are presented as c.p.m./well. The symbols used are: PET in the presence (●) or absence (○) of Ang II, and PA in the presence (■) or absence (□) of Ang II.

transphosphatidylation of phospholipids to PET [5]. To measure PLD activity in VSMC, we initially monitored the production of [³H]PA in [³H]oleic acid-labelled cells. After incubation for 20 min in the absence of EtOH, Ang II (100 nM) increased [³H]PA production by $76 \pm 6\%$ above control ($n = 3$) with essentially no production of PET ($5 \pm 13\%$). The presence of EtOH had no significant effect on the unstimulated levels of PET or PA ($26 \pm 10.8\%$ and $1 \pm 9.8\%$ above control respectively) or on the production of PA in response to Ang II ($49 \pm 10\%$ above control). However, Ang II stimulated the production of [³H]PET, in the presence of 100 mM EtOH, to $368 \pm 13.2\%$ of control. Activation of PLD was dependent on the concentration of Ang II over the range 1 nM–1 μ M (Figure 1a), with an EC₅₀ of 7 nM. Maximal stimulation was observed with 100 nM Ang II, which was the concentration used in subsequent experiments.

Stimulation of PA and PET production by Ang II was also dependent on the period of incubation (Figure 1b). The formation of both PET and PA was significantly elevated after 30 s of incubation with Ang II, corresponding to increases of 77 and 53% respectively. The level of PET continued to rise for up to 10 min. At longer times of incubation (between 10 and 40 min), PET levels reached a plateau of approx. 4200 c.p.m./well (or 350% above basal; results not shown). In contrast, PA formation peaked at 1 min (3733 c.p.m./well or 190% above basal). The level of PA decreased with further incubation, but remained

Table 2 Effect of Ang peptides on the activation of PLD in VSMC

Cells prelabelled with [3 H]oleic acid were incubated for 20 min with the indicated concentrations of Ang peptides in the presence of 100 mM EtOH. Results are means $\pm$ SEM for at least five experiments and are expressed as percentages above control in the presence of 100 mM EtOH alone (689 ± 75 c.p.m./well). * $P < 0.05$ as compared with control.

Ang peptide	PEt formation (% above control)
Ang II (100 nM)	$270 \pm 13^*$
Ang-(2-8) (100 nM)	$103 \pm 21^*$
Ang-(1-7) (1 μ M)	5 ± 10
Ang I (1 μ M)	20 ± 16

Table 3 Effect of receptor antagonists on activation of PLD by Ang II

VSMC prelabelled with [3 H]oleic acid were preincubated with the indicated Ang II receptor antagonists for 5 min and then stimulated for 20 min by addition of Ang II in the presence of 100 mM EtOH. The receptor antagonists were used at a concentration of 1 μ M, and Ang II was at 100 nM. Results are means $\pm$ SEM for at least seven experiments and are expressed as percentages of control in the presence of 100 nM Ang II and 100 mM EtOH (2551 ± 338 c.p.m./well). * $P < 0.05$ as compared with control.

Antagonist	PEt formation (% of control)
[Sar ¹ , Ile ⁸]Ang II	$25 \pm 5^*$
[Sar ¹ , Thr ⁸]Ang II	$17 \pm 3^*$
Losartan	$24 \pm 4^*$
EXP 3174	$6 \pm 2^*$
L-158,809	$22 \pm 6^*$
CGP 42112A	82 ± 10
PD 123177	100 ± 5

significantly elevated above control values in the presence of EtOH alone.

Effect of Ang peptides on PLD activity

The effect of various Ang peptides on the activation of PLD is illustrated in Table 2. At a concentration of 100 nM, Ang II stimulated PLD activity $270 \pm 13\%$ above basal. An equivalent concentration of the C-terminal heptapeptide Ang-(2-8) also increased PLD activity; however, Ang-(2-8) was much less effective, only increasing PEt formation by $103 \pm 4\%$ above basal. In contrast, the N-terminal fragment of Ang II, Ang-(1-7), at concentrations up to 1 μ M, did not significantly increase PLD activity. Furthermore, concentrations of 1 or 10 μ M Ang I also had no effect on PLD activity.

Effect of Ang receptor antagonists on stimulation of PLD by Ang II

To demonstrate the specific involvement of an Ang II receptor in the stimulation of PLD, we incubated VSMC with Ang II (100 nM) in the presence of the non-selective receptor antagonists [Sar¹, Ile⁸]Ang II or [Sar¹, Thr⁸]Ang II (each at 1 μ M). Both antagonists were potent inhibitors of PEt formation, reducing the Ang II-evoked increases by 75 ± 4 and $83 \pm 3\%$ respectively (Table 3). Pretreatment with the AT₁ receptor antagonists losartan, its metabolite EXP 3174 or L-158,809 (each at 1 μ M) reduced 100 nM Ang II-stimulated PEt formation by at least 75%. EXP 3174 appeared to be the most potent antagonist, as

Table 4 Effect of extracellular Ca²⁺ on PLD activation

Cells preincubated with [3 H]oleic acid were washed for 30 min in a Ca²⁺-free KR prior to their exposure to 100 nM Ang II for 20 min in KR buffer containing the indicated concentrations of Ca²⁺ or EGTA. Results are mean $\pm$ SEM for at least four experiments and are presented as percentages above 100 mM EtOH-treated controls (1238 ± 62 c.p.m./well). Similar results were obtained in cells preincubated in Ca²⁺-free KR for only 10 min prior to their exposure to Ang II (results not shown). * $P < 0.05$ as compared with control.

Addition	PEt formation (% above control)
None	70 ± 4
Ca ²⁺	
1 mM	114 ± 8
2 mM	168 ± 7
4 mM	209 ± 2
6 mM	197 ± 2
1 mM EGTA	65 ± 9

it inhibited PEt formation by $95 \pm 2\%$. In contrast, CGP 42112A, the peptide which has been shown to compete for Ang II binding at AT₂ receptors, as well as the non-peptide AT₂ antagonist PD 123177 (each at 1 μ M), were ineffective at blocking the production of PEt evoked by 100 nM Ang II. None of the antagonists increased the formation of PEt or PA when incubated with VSMC alone.

Effect of extracellular Ca²⁺ on PLD activation by Ang II

Activation of PLD by Ang II was dependent upon the extracellular concentration of Ca²⁺, as shown in Table 4. In Ca²⁺-free medium (in the absence of additional Ca²⁺) or in Ca²⁺-free medium containing 1 mM EGTA, Ang II stimulated production of PEt by $70 \pm 4\%$ and $65 \pm 9\%$ above control respectively, indicating that a fraction of PLD activity is not dependent on extracellular Ca²⁺. However, Ang II stimulation of PLD was augmented in the presence of increasing concentrations of Ca²⁺, reaching a maximal stimulation of $209 \pm 2\%$ at 4 mM Ca²⁺. Activation of VSMC PLD by 100 nM Ang II was increased by 114% in the presence of 1 mM Ca²⁺ when compared with unstimulated controls, which is the concentration normally present in our KR solution.

DISCUSSION

VSMC contain high affinity Ang II receptors [1] that are coupled to multiple mechanisms of signal transduction, including activation of a phosphoinositide-specific PLC and a PLD as well as the production of PGs [2,9,13,29,30]. Activation of PLC and/or PLD could account for the biphasic generation of DAG that occurs in response to Ang II [7-9,31]. Ang II causes an immediate activation of PLC, producing an initial rapid and transient generation of DAG. In contrast, our time course for the activation of PLD by Ang II showing a more delayed and sustained activity may account for the more sustained phase of DAG. Furthermore, we find that both the initial and secondary peaks of DAG formation induced by Ang II are inhibited by the AT₁ receptor antagonist losartan. Since losartan inhibits PLD and has previously been shown to inhibit PLC in rat aortic VSMC [13], these results further agree with the idea that the initial peak of DAG accumulation results from phosphoinositide hydrolysis, while the secondary, more sustained, peak of DAG formation is due to hydrolysis of PC or PI by PLD. A role for the DAG produced by the sequential activation of PLD and PP has not been clearly

established. Although the DAG produced from phosphoinositide hydrolysis activates PKC, some, but not all, of the DAG produced from PLD hydrolysis has been shown to activate PKC [32–34]. This differential activation may result from differences in the species of DAG produced during the two phases of its formation or due to different isoenzymes of PKC. In addition, increasing evidence suggests that DAG may be involved in mitogenic responses [33,35–37]. Since Ang II is mitogenic in VSMC, activation of PLD by Ang II may participate in mitogenesis.

We previously showed that Ang II and Ang-(2–8) activate an AT₁ receptor on rat VSMC that is coupled to the hydrolysis of phosphoinositides, while Ang II and Ang-(1–7) produce PGs [13,14]. We now report that Ang II and Ang-(2–8), but not Ang-(1–7), also activated PLD in cultured rat VSMC. The ability of the C-terminal heptapeptide Ang-(2–8) to mimic Ang II activation of PLD and the inability of Ang-(1–7) to affect PLD activity suggested that PLD activation was linked to the AT₁ receptor subtype. In addition, the AT₁-selective antagonist losartan, EXP 3174 or L-158,809 potently inhibited Ang II stimulation of PLD activity, whereas the AT₂ receptor antagonist CGP 42112A or PD 123177 was ineffective, indicating that activation of PLD by Ang II is coupled exclusively to an AT₁ receptor. Thus an alteration in the N-terminus of Ang II does not abolish its ability to stimulate PLD activity, while modification in its C-terminus prevents PLD activation. This suggestion is supported by a report showing that neither Ang-(1–7) nor Ang-(1–6) was able to activate PLD in cultured mesangial cells [8]. In addition, Ang I did not activate PLD in VSMC. This was not unexpected, however, since we previously showed that the major metabolite produced when VSMC were incubated with Ang I is Ang-(1–7) [38].

PLD activated by Ang II in VSMC may hydrolyse PC or PI, as the amount of label in both phospholipids was decreased by incubation with Ang II. Griendling et al. [7] previously reported a rapid and transient hydrolysis of radiolabelled polyphosphoinositides in response to Ang II stimulation of VSMC, but a delayed breakdown of PI. Furthermore, Lassegue et al. [9] showed that radiolabelled choline was released both intracellularly and extracellularly from choline-labelled VSMC stimulated with Ang II, and that Ang II stimulated the production of [³H]myristate-labelled PA or [³H]myristate-labelled PEt, in the presence of EtOH. Alternatively, the hydrolysis of PI and/or PC may result from activation of a PI- or PC-selective PLC. Although many agonists have now been reported to activate PLD in a variety of different types of cells [39,40], most of these reports focus on the hydrolysis of PC. However, Huang et al. [41] recently identified two forms of PLD in Madin–Darby canine kidney cells, one which was selective for the hydrolysis of PC and the other for PI. Whether the hydrolysis of PI and/or PC that occurs in VSMC in response to Ang II is a result of activation of PLC or PLD and whether two separate PLD isoenzymes that are selective for PI versus PC are present in VSMC remain to be determined.

Activation of PLD by Ang II could be attenuated by removal of extracellular Ca²⁺ or its chelation by EGTA. In addition, PLD activity was increased by elevating the extracellular concentration of Ca²⁺. Following the hydrolysis of phosphoinositides by PLC in response to Ang II, the intracellular concentration of Ca²⁺ is transiently elevated [2]. Thus PLD may be activated by the Ca²⁺ produced in response to Ang II activation of PLC. However, the removal of extracellular Ca²⁺ by chelation with EGTA only reduced PLD activity by 50%. The remainder of the PLD activity may be produced in response to the mobilization of intracellular Ca²⁺, which is essentially unaffected by the removal

of extracellular Ca²⁺ [2]. Alternatively, PLD in VSMC may also be activated by the DAG produced in response to Ang II activation of PLC, since phorbol esters which mimic endogenous DAG in the stimulation of PKC have also been shown to activate PLD in VSMC [9] as well as in other types of cells [40,41]. Although PLD may be indirectly activated by the increase in intracellular Ca²⁺ concentration or DAG levels that occur in response to Ang II activation of PLC, our recent results suggest that activation of PLD by Ang II in VSMC is dependent on an elevation in cytosolic Ca²⁺ [42]. Furthermore, since both PLC and PLD activities can be inhibited by AT₁ receptor antagonists, PLD may not be directly coupled to an AT₁ receptor (or its GTP-binding component), but may be sequentially activated, through the coupling of PLC to an AT₁ receptor.

We previously showed that Ang II and Ang-(1–7) stimulate the production of vasodilatory prostaglandins E₂ and I₂ in a variety of cultured cell lines, including cultured human astrocytes and rat aortic VSMC [13–16]. Stimulation of PG release by Ang II has previously been reported to occur in rat aortic VSMC [30] and porcine VSMC [29]. Although Leung et al. [29] report that PG release from porcine VSMC in response to Ang II can be antagonized by AT₁ and not AT₂ receptor antagonists, we have found that PG release from cultured human astrocytes in response to stimulation by Ang II could be inhibited by both AT₁ and AT₂ receptor antagonists [15,16]. In contrast, PGs produced in response to Ang-(1–7) in cultured human astrocytes occurred only through activation of AT₂ receptors. We initially proposed that PLD could participate in PG production in response to angiotensin peptides, since DAG formed by the sequential activation of PLD and PP could be hydrolysed by DAG lipase to release free arachidonic acid. However, Ang-(1–7) does not produce DAG or activate PLD. In addition, neither DAG formation nor the activation of PLD is inhibited by AT₂ selective receptor antagonists. Therefore Ang-(1–7)-evoked PG synthesis must occur through an alternative pathway that is yet to be elucidated.

In conclusion, we have demonstrated that activation of AT₁ receptors on VSMC results in the stimulation of both PLC and PLD, since Ang II or the heptapeptide Ang-(2–8) activates both processes, and AT₁ antagonists inhibit them both. Activation of both PLC and PLD by Ang peptides could result from both phospholipases being coupled to the same or to a different AT₁ receptor or to the same or a different GTP-binding protein that is coupled to an AT₁ receptor. Alternatively, PLD may be sequentially activated by Ang II stimulation of an AT₁ receptor coupled to a PLC and the subsequent increase in intracellular Ca²⁺. In contrast, Ang-(1–7) causes neither the formation of DAG nor activates PLD. Thus the two heptapeptides appear to discriminate between different subtypes of angiotensin peptide receptors, suggesting that multiple responses to angiotensins may result from the presence of different subtypes of receptors or different processing enzymes that preferentially generate Ang-(2–8) or Ang-(1–7).

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