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Angiotensin II-induced phosphatidylcholine hydrolysis in cultured vascular smooth-muscle cells

Regulation and localization

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In cultured vascular smooth-muscle cells (VSMC), angiotensin II (AngII) induces a biphasic, sustained increase in diacylglycerol (DG) of unclear origin. To determine whether hydrolysis of phosphatidylcholine (PC) is a possible source of DG, we labelled cellular PC with [^{3}H]choline, and measured the formation of intra- and extra-cellular [^{3}H]choline and [^{3}H]phosphocholine after stimulation with AngII. AngII induced a concentration-dependent release of choline from VSMC that was significant at 2 min and was sustained over 20 min. In contrast, accumulation of choline inside the cells was very slight. AngII also increased the formation of [^{3}H]myristate-labelled phosphatidic acid, and, in the presence of ethanol, of [^{3}H]phosphatidylethanol, characteristic of a phospholipase D (PLD) activity. Extracellular release of choline was partially inhibited by removal of extracellular Ca^{2+} (54 ± 9 % inhibition at 10 min) or inhibition of receptor processing by phenylarsine oxide (79 ± 8 % inhibition at 20 min). The protein kinase C activator phorbol myristate acetate also stimulated a large release of choline after a 5 min lag, which was unaffected by the Ca^{2+} ionophore ionomycin, but was additive with AngII stimulation. Down-regulation of protein kinase C by a 24 h incubation with phorbol dibutyrate (200 nM) decreased basal choline release, but had no effect on AngII stimulation. We conclude that AngII induces a major PC hydrolysis, probably mainly via PLD activation. This reaction is partially dependent on Ca^{2+} and is independent of protein kinase C, and appears to be mediated by cellular processing of the receptor–agonist complex. Our results are consistent with a preferential hydrolysis of PC from the external leaflet of the plasmalemma, and raise the possibility that PC hydrolysis occurs in specialized 'signalling domains' in VSMC.

INTRODUCTION

The response of vascular smooth-muscle cells (VSMC) to angiotensin II (AngII) comprises both transient and sustained biochemical signals. Among the earliest events following receptor stimulation is hydrolysis of phosphatidylinositol 4,5-bisphosphate to form diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP_3), with consequent mobilization of intracellular Ca^{2+} [1–3]. Both IP_3 formation and the increase in intracellular Ca^{2+} are transient, and have returned to near-basal levels by 2 min. In contrast, a second, sustained, phase of DG formation becomes apparent after 2 min, and is accompanied by activation of protein kinase C [4,5]. These two phases of the AngII response are differentially regulated by extracellular Ca^{2+}, pH and protein kinase C [5–10]. Additionally, there appears to be a requirement for movement of the receptor–agonist complex within the plane of the membrane before the sustained phase of DG accumulation can develop [11].

The clear temporal difference in AngII-induced IP_3 formation and generation of DG suggests that the two second messengers are derived from different phospholipid precursors. We have previously provided evidence suggesting that phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol might contribute to the sustained phase of DG formation [4]. However, recent studies in other systems have shown that phosphatidylcholine (PC) also undergoes agonist-induced hydrolysis (for reviews see [12,13]). In these cells, PC breakdown was often accompanied by prolonged accumulation of DG, suggesting that PC is an alternative source for DG.

One unresolved issue which may be important in the sequence of signalling events coupling agonists to phospholipid hydrolysis is the spatial relationship of the phospholipids and the activated receptor. In red-cell plasma membranes, the system in which lipid distribution has been most carefully studied, 75 % of PC is located in the outer membrane leaflet, whereas the inner leaflet is enriched in phosphatidylinositol [14]. In addition, there is some evidence that the phospholipids are inhomogeneously distributed in the plane of the membrane, and thus may potentially be viewed as existing in distinct membrane domains [15–17]. We have also presented inferential evidence that there might be 'signalling domains', i.e. areas of the membrane enriched in the lipids, proteins or enzymes essential to signal generation [11].

To gain insight into these issues, we investigated whether AngII is coupled to PC hydrolysis in cultured VSMC, and focused on the localization of the PC hydrolysis products. In addition, we addressed the issues of whether agonist-receptor processing events were involved in the coupling to PC metabolism, as well as whether PC breakdown is controlled by some of the same regulatory events that we have previously shown to modulate phosphoinositide hydrolysis and the biphasic formation of DG. We conclude that AngII causes a marked phospholipase D (PLD)-mediated breakdown of PC, which primarily releases choline outside the cell. This signalling event is independent of protein kinase C activation and is partially dependent on Ca^{2+}. In addition, we provide evidence that PC hydrolysis is, at least in part, dependent on cellular processing of the AngII receptor, raising the possibility that PC hydrolysis occurs in specialized 'signalling domains'.

Abbreviations used: AngII, angiotensin II; DG, 1,2-diacyl-*sn*-glycerol; IP_3, inositol 1,4,5-trisphosphate; PA, phosphatidic acid; PAO, phenylarsine oxide; PC, phosphatidylcholine; PDBu, 4β-phorbol 12,13-dibutyrate; PEt, phosphatidylethanol; PLC, phospholipase C; PLD, phospholipase D; PMA, 4β-phorbol 12-myristate 13-acetate; VSMC, vascular smooth-muscle cells.

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MATERIALS AND METHODS

Culture of VSMC

Primary cultures of VSMC were obtained by enzymic digestion of male Sprague–Dawley rat thoracic aortas as described elsewhere [18]. Cells were passaged in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) calf serum, as previously described [4]. Myocytes from passages 5–20 were seeded on 35 mm-diam. dishes (at 2×10^4 cells/cm^2), fed every other day, and used at confluence.

[^{3}H]Choline labelling and measurement of PC metabolites

VSMC were labelled for 3 h with 0.5 μCi of [^{3}H]choline (80 Ci/mmol) per dish in 1 ml of serum-free culture medium. Cells were then washed 3 times and incubated at 37 °C for 20 min in a buffer of the following composition (mM): 130 NaCl, 5 KCl, 1 $MgCl_2$, 1.5 $CaCl_2$, 1 choline chloride, 20 Hepes (buffered to pH 7.4 with Tris base). Incubation buffer was removed and replaced with 1 ml of buffer with or without agonist for various times. Just before extraction with 1.5 ml of chloroform/methanol/12.5 M-HCl (20:40:1, by vol.), the extracellular medium was removed from each dish for determination of extracellular PC metabolites. After addition of 900 μl of distilled water and 500 μl of chloroform to the cell extract, phases were separated by centrifugation (500 *g*, 5 min). The aqueous phase was washed with 2 × 1 ml of chloroform and then processed for separation of intracellular PC metabolites. The pooled organic phases were evaporated under a stream of nitrogen and used to determine the label incorporated into lipids. PC metabolites were initially separated by t.l.c. [19]. These experiments demonstrated that choline and phosphocholine were the only metabolites labelled to a significant extent in these cells. Thereafter we routinely separated [^{3}H]choline from [^{3}H]phosphocholine with tetraphenylboron in heptanone [20]. Briefly, 400 μl of aqueous extract was vortex-mixed with 200 μl of water and 600 μl of tetraphenylboron (75 mg/ml in heptan-3-one). After centrifugation, 300 μl samples of each phase were removed and transferred to scintillation vials. The organic phases were evaporated. To decrease chemiluminescence in organic phases of intracellular origin, a drop of 12.5 M-HCl was added to each vial. Radioactivity was quantified by liquid-scintillation spectrophotometry. Cross-contamination between phases was 6–7%, as determined by extraction of known amounts of labelled standards.

[^{3}H]Myristate labelling for phosphatidic acid (PA) and phosphatidylethanol (PEt) measurements

VSMC were preincubated for 24 h with 2 μCi of myristic acid (53 Ci/mmol)/dish in 1 ml of culture medium with 10% calf serum. Before the experiment, cells were incubated for 15 min at 37 °C in the balanced salt solution described above, and for an additional 5 min in the absence or in the presence of 100 mM-ethanol. Cells were then exposed to the agonist in the absence or continued presence of ethanol and extracted for lipid analysis as described above.

Lipid analysis

Lipids from the organic extracts were separated by t.l.c. [21], detected by autoradiography, identified by co-migration with unlabelled standards revealed with iodine vapour and quantified by liquid-scintillation spectrophotometry. [^{3}H]Myristate-labelled PA and PEt were separated from other phospholipids by t.l.c. ([22]; solvent system I) on Whatman K6 silica-gel plates. [^{3}H]-Choline was incorporated into PC, lysophosphatidylcholine and sphingomyelin. After [^{3}H]myristate incubation, 82% of the total label in the phospholipids was incorporated into PC.

Materials

All chemicals were of analytical grade or better. Salts and solvents were purchased from Fisher (Pittsburgh, PA, U.S.A.), [^{3}H]choline, [^{32}P]ATP and [^{32}P]P_i from Du Pont (Boston, MA, U.S.A.), phospho[^{14}C]choline and [^{3}H]myristate from Amersham (Arlington Heights, IL, U.S.A.), and t.l.c. plates from Whatman (Clifton, NJ, U.S.A.). Scintillation fluor was Liquiscint from National Diagnostics (Somerville, NJ, U.S.A.). Other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

RESULTS

Time course of the formation of PC metabolites after AngII stimulation

To determine whether AngII induces PC hydrolysis in VSMC, we labelled cells with [^{3}H]choline and measured the appearance of the water-soluble products of PC hydrolysis in the extracellular medium as well as in the intracellular extract. The time course of PC-metabolite production induced by 100 nM-AngII is presented in Fig. 1. The agonist stimulated the appearance of [^{3}H]choline in the extracellular buffer as early as 1 min. [^{3}H]Choline increased rapidly during the first 5 min of stimulation and slowly reached a plateau at 15 min (400% of control); the response was maintained for at least 20 min. AngII also induced a small increase in extracellular [^{3}H]phosphocholine, which began at 2 min and was sustained over 20 min (180% of control). In contrast, AngII caused only a slow and comparatively modest accumulation of [^{3}H]choline in the cytosol (150% of control after a 10 min exposure), and had a small transient effect on intracellular accumulation of phosphocholine (Fig. 1*b*). We were unable to measure a decrease in cellular [^{3}H]PC during stimulation, most likely because the hormone-sensitive phospholipid only represents a minute fraction of the total pool.

The production of [^{3}H]choline in the extracellular medium could be due to a direct release, or to a release of phosphocholine

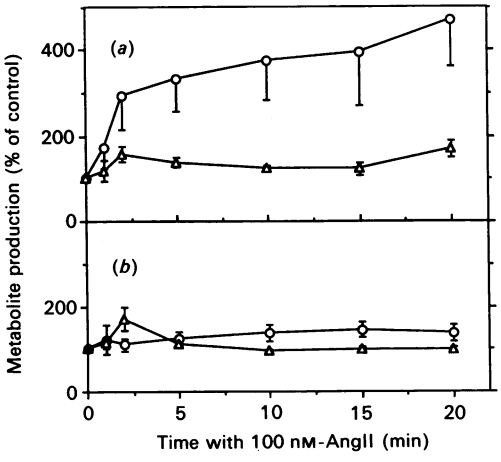

Fig. 1. Time course of the accumulation of PC metabolites in response to AngII

VSMC labelled for 3 h with [^{3}H]choline were exposed to 100 nM-AngII for the indicated time. [^{3}H]Choline (○) and [^{3}H]phosphocholine (△) were measured in the extracellular buffer (*a*) and in the cellular extract (*b*). Each point is the mean ± S.E.M. of measurements from 4 to 11 independent experiments performed in triplicate, expressed as percentage of control. Control values at 20 min for extracellular [^{3}H]Choline and [^{3}H]phosphocholine were 1700 and 850 d.p.m./dish respectively. Intracellular control values were 4400 and 21000 d.p.m./dish for [^{3}H]choline and [^{3}H]phosphocholine respectively.

Table 1. Absence of extracellular transformation of phospho[^{14}C]choline or [^{3}H]choline

VSMC were preincubated for 10 min with either 9000 c.p.m. of phospho[^{14}C]choline in a saline solution containing 1 mM-phosphocholine (PCholine), or 15000 d.p.m. of [^{3}H]choline in a saline solution containing 1 mM-choline. The extracellular buffer was then assayed for labelled choline and phosphocholine as described in the Materials and methods section. Values are means ± S.E.M. of triplicate measurements from one representative experiment.

	Phospho[^{14}C]choline (c.p.m.)		[^{3}H]Choline (d.p.m.)	
	Choline	PCholine	Choline	PCholine
Control	298± 7	8002±32	12230±474	1502±39
AngII	317±12	7723±90	12800± 75	1507± 4

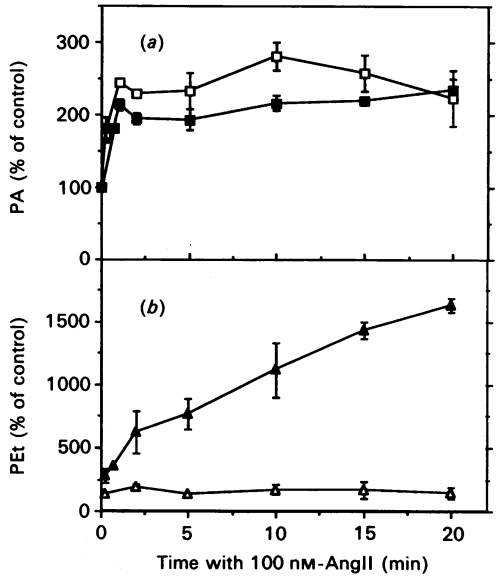

Fig. 2. Time course of AngII-induced PA and PEt accumulation

VSMC labelled for 24 h with [^{3}H]myristate were preincubated for 5 min with or without 100 mM-ethanol and exposed to 100 nM-AngII for the indicated time, in the continued absence (□, △) or presence (■, ▲) of 100 mM-ethanol. The formation of [^{3}H]PA (*a*) and [^{3}H]PEt (*b*) were determined as described in the Materials and methods section. Each point is the mean ± S.E.M. of measurements from two to three independent experiments, expressed as percentage of control. Control values at 20 min for [^{3}H]PA and [^{3}H]PEt were 4200 and 900 d.p.m./dish respectively, and were not affected by the presence of ethanol.

and a subsequent conversion into choline. To distinguish between these possibilities, we incubated myocytes with phospho-[^{14}C]choline for 10 min in the absence or presence of AngII. As shown in Table 1, there was no conversion of phospho[^{14}C]choline into [^{14}C]choline, indicating that choline is directly released by the cells. Additionally, in cells labelled for 20 min with [^{3}H]choline, a time period insufficient to incorporate choline into PC, we were unable to measure any agonist-stimulated release of either choline metabolite (results not shown). These results are consistent with AngII induction of a substantial PLD-mediated PC hydrolysis, and suggest that the formation of choline metabolites is primarily localized in the outer leaflet of the plasma membranes in VSMC.

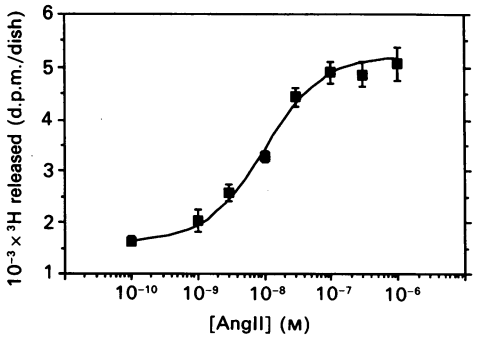

Fig. 3. Concentration–response curve of AngII-induced PC hydrolysis

[^{3}H]choline-labelled VSMC were exposed for 10 min to AngII at the indicated concentrations. The total tritiated metabolites released in the extracellular buffer were measured. Each point represents the mean ± S.E.M. of triplicate determinations in a single representative experiment. Similar results were obtained in two additional experiments.

Time course of AngII-induced PA and PEt formation

To determine whether AngII-induced choline release can be correlated with the activation of a PLD in our system, cells were incubated with [^{3}H]myristate, which preferentially labels PC, and preincubated for 5 min with or without 100 mM-ethanol. Cells were then exposed to the buffer or to the agonist in the continued presence or absence of 100 mM-ethanol. It has previously been shown that PLD hydrolyses PC in the presence of water to generate PA and, in the presence of ethanol, catalyses a unique transphosphatidylation reaction to generate PEt in addition to PA [22]. Preincubation with either AngII or ethanol alone induced no formation of PEt. However, in the presence of ethanol, AngII induced a large accumulation of PEt (Fig. 2), indicating that this agonist is coupled to activation of a PLD. As expected, PA formation is incompletely inhibited by incubation with ethanol, probably reflecting the preferential hydrolysis of PC by PLD with water rather than ethanol, as well as the generation of PA by the sequential activation of phosphoinositide-specific PLC and DG kinase [4]. Together with the data in Fig. 1, these results suggest that AngII-stimulated PC hydrolysis is most likely mediated by PLD.

Dose-dependency of AngII-induced release of PC metabolites

The ability of AngII to induce the appearance of the metabolic products of PC in the extracellular buffer was concentration-dependent. AngII stimulation was significant in the 1–100 nM range, with an EC_{50} (50% effective concn.) of 10 nM (Fig. 3). This is the same concentration range that induces the formation of the second messengers IP_3 and DG [2,4]. In all subsequent experiments, 100 nM was used as a near-maximally active AngII concentration.

Effect of phenylarsine oxide (PAO)

We have previously shown that treatments which interfere with the cellular processing of the AngII-receptor–agonist complex also inhibit the long-term production of arachidonoyl-labelled DG and phosphoinositide metabolism [11], and have suggested the possibility that there are 'signalling domains' on the cell surface. Since polyphosphoinositide hydrolysis begins within seconds and PC breakdown appears to be a relatively late event, we examined the dependence of PC hydrolysis on receptor processing. Pretreatment of VSMC with 10 μM-PAO for 5 min before addition of AngII, a manoeuvre that we have previously shown to inhibit receptor sequestration specifically and to attenuate markedly the late phase of arachidonoyl-DG formation

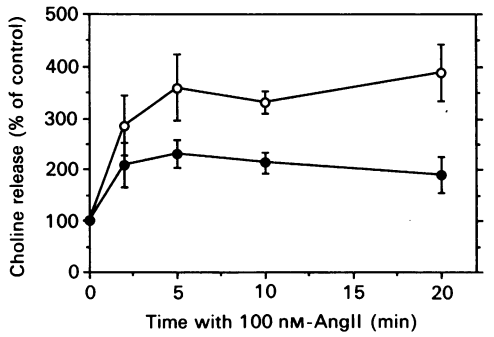

Fig. 4. Effect of PAO on AngII stimulation of extracellular choline release

VSMC labelled for 3 h with [^{3}H]choline were preincubated without (○) or with (●) 10 μM-PAO for 5 min, before exposure to 100 nM-AngII for the indicated time. Released [^{3}H]choline was measured in the extracellular buffer. Each point represents the mean ± S.E.M. of measurements from three independent experiments performed in triplicate, expressed as percentage of control. Control values at 20 min were 4500 and 3100 d.p.m./dish in the absence and in the presence of PAO respectively.

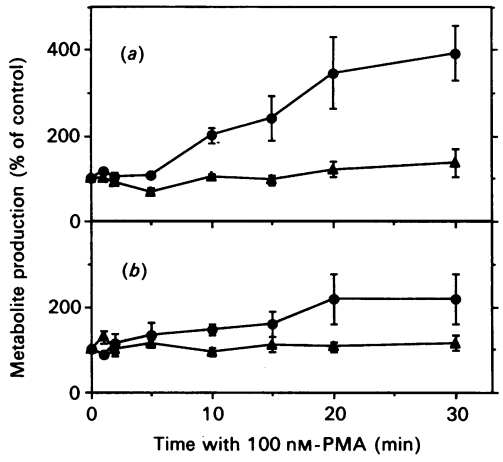

Fig. 5. Time course of the production of PC metabolites in response to PMA

VSMC labelled for 3 h with [^{3}H]choline were exposed to 100 nM-PMA for the indicated time. [^{3}H]Choline (●) and [^{3}H]phosphocholine (▲) were measured in the extracellular buffer (*a*) and in the cellular extract (*b*). Each point is the mean ± S.E.M. of measurements from 4 to 11 independent experiments performed in triplicate, expressed as percentage of control. Control values at 20 min for extracellular [^{3}H]choline and [^{3}H]phosphocholine were 3300 and 2000 d.p.m./dish respectively. Intracellular control values were 8000 and 40000 d.p.m./dish for [^{3}H]choline and [^{3}H]phosphocholine respectively.

[11], inhibited the increase in extracellular choline (79 ± 8 % inhibition at 20 min; Fig. 4). In addition, exposure to nigericin in the presence of low K^+, which also inhibits receptor processing [23], inhibited extracellular choline formation induced by AngII (100 nM, 10 min) by 81 %. These results suggest that a significant portion of PC hydrolysis is also dependent on the processing of, or conformational changes in, the AngII-receptor–agonist complex in the plasma membrane.

Effect of protein kinase C activation on the production of PC metabolites

Our previous measurements of AngII-induced arachidonoyl-DG formation showed that the initial, but not the sustained, phase of DG formation is inhibited by protein kinase C [4]. To determine whether protein kinase C mediates AngII-induced hydrolysis of PC, we incubated [^{3}H]choline-labelled VSMC with phorbol myristate acetate (PMA), an exogenous activator of protein kinase C. In marked contrast with its effect on phosphoinositide metabolism [7–9], after a lag of 5 min PMA (100 nM) induced a quasi-linear increase in [^{3}H]choline in the extracellular phase, which lasted at least 30 min (Fig. 5). PMA had no effect on [^{3}H]phosphocholine levels in the extracellular buffer, but induced a small and sustained increase in intracellular choline accumulation (200 % of control). The effect of PMA was concentration-dependent, with a threshold of 10 nM and an EC_{50} of 300 nM.

Table 2. Simultaneous challenge with AngII and PMA

[^{3}H]choline-labelled VSMC were exposed to either 100 nM-AngII or 100 nM-PMA or to both compounds simultaneously for 10 min. [^{3}H]choline and [^{3}H]phosphocholine (PCholine) were measured in the extracellular buffer and in the cellular extract. The values are means ± S.E.M. from two to three experiments performed in triplicate, expressed as percentage of controls. Extracellular controls were 1750 and 600 d.p.m./dish for [^{3}H]choline and [^{3}H]phosphocholine respectively. Intracellular controls were respectively 2300 and 30000 d.p.m./dish.

	Extracellular (% of control)		Intracellular (% of control)	
	Choline	PCholine	Choline	PCholine
AngII	215 ± 47	118 ± 4	117 ± 12	93 ± 4
PMA	222 ± 31	100 ± 11	188 ± 12	97 ± 2
AngII + PMA	353 ± 39	174 ± 23	202 ± 8	93 ± 8

The delayed effect of PMA as compared with AngII on the formation of PC metabolites (Figs. 1 and 5) suggests that AngII-induced PC hydrolysis is not initially mediated by protein kinase C, and raises the possibility that protein kinase C may be involved only in the prolonged production of PC metabolites. To test this hypothesis, VSMC were exposed for 10 min to 100 nM-PMA or -AngII or to both compounds simultaneously. As shown in Table 2, the effects of PMA and AngII were approximately additive on extracellular [^{3}H]choline (simultaneous addition 4657, sum of independent stimulations 4262 d.p.m./dish above control), suggesting that the hydrolysis of PC induced by AngII and protein kinase C activators occurs by different mechanisms.

Effect of protein kinase C down-regulation

To confirm the apparent lack of involvement of protein kinase C in AngII-induced PC breakdown, the enzyme was down-regulated by preincubation of VSMC for 24 h with 200 nM-4β-phorbol 12,13-dibutyrate (PDBu). In our system this protocol decreases protein kinase C activity by 90 %, as measured by the inhibition of phorbol-ester-induced phosphorylation of a 76 kDa protein [24,25]. PDBu decreased basal extracellular [^{3}H]choline production, but had no significant effect on basal extracellular [^{3}H]phosphocholine or on the production of intracellular metabolites (results not shown). PDBu did not affect the time course of AngII-induced extracellular [^{3}H]choline or [^{3}H]phosphocholine production (Fig. 6). Similarly, PDBu had no effect on AngII-induced accumulation of intracellular metabolites (results not shown). The PDBu-induced decrease in basal PC metabolites is consistent with the PMA-stimulated PC breakdown depicted in Fig. 5. The absence of effect of PDBu on agonist-induced PC breakdown is additional evidence that

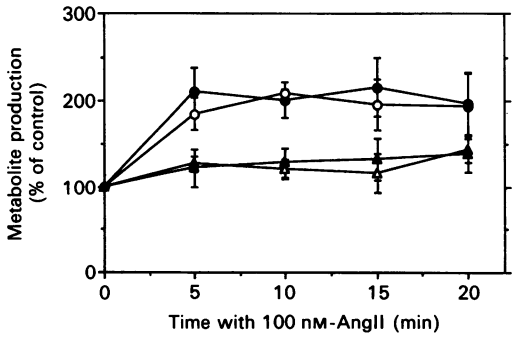

Fig. 6. Effect of protein kinase C down-regulation on AngII-induced production of PC metabolites

VSMC were preincubated for 24 h in serum-free medium without (○, △) or with (●, ▲) 200 nM-PDBu, and labelled for 3 h with [^{3}H]choline. The myocytes were challenged with 100 nM-AngII for the indicated times, before measurement of [^{3}H]choline (○, ●) and [^{3}H]phosphocholine (△, ▲) in the extracellular buffer. Each point represents the mean ± S.E.M. of values from four independent experiments performed in triplicate, expressed as percentage of control. Choline control values at 20 min were 2200 and 1600 d.p.m./dish in the absence and in the presence of PDBu respectively. Phosphocholine control values at 20 min were 1000 and 970 d.p.m./dish in the absence and in the presence of PDBu respectively.

Table 3. Effect of Ca^{2+} on PMA-induced formation of PC metabolites

[^{3}H]choline-labelled VSMC were exposed to either 15 μM-ionomycin or 100 nM-PMA, or to both compounds simultaneously for 10 min. [^{3}H]Choline and [^{3}H]phosphocholine (PCholine) were measured in the extracellular buffer and in the cellular extract. The values are means ± S.E.M. from four to six independent experiments performed in triplicate, expressed as percentage of controls. Extracellular control values were 1900 and 950 d.p.m./dish for [^{3}H]choline and [^{3}H]phosphocholine respectively. Intracellular controls were respectively 7500 and 43000 d.p.m./dish.

	Extracellular (% of control)		Intracellular (% of control)	
	Choline	PCholine	Choline	PCholine
Ionomycin	134±15	107± 6	123±17	110±2
PMA	207±31	110± 8	133±18	96±2
Ionomycin +PMA	229±20	135±17	149±31	109±2

AngII promotes PC hydrolysis through a protein kinase C-independent pathway.

Effect of Ca^{2+}

One obvious protein kinase C-independent AngII-stimulated pathway in VSMC is the increase in cytoplasmic Ca^{2+} concentration, via either IP_3-induced mobilization of intracellular Ca^{2+} [26] or receptor-mediated Ca^{2+} influx [27]. To determine whether AngII-induced PC hydrolysis is mediated by Ca^{2+}, we exposed cells to AngII in the absence of extracellular Ca^{2+} or to the Ca^{2+} ionophore ionomycin. As shown in Table 3, a 10 min incubation with ionomycin (15 μM) had only a small effect on intracellular or extracellular accumulation of choline metabolites. Furthermore, simultaneous addition of ionomycin and PMA did not significantly increase PMA-induced formation of PC metabolites (Table 3). However, removal of extracellular Ca^{2+} by chelation with EGTA (2 mM) inhibited AngII-stimulated choline release by 54±9% at 10 min (Table 4). These results suggest that Ca^{2+} influx is necessary, but not sufficient, for maximal PC hydrolysis.

Table 4. Effect of Ca^{2+} on AngII-induced formation of PC metabolites

[^{3}H]Choline-labelled VSMC were exposed to 100 nM-AngII for 10 min in the presence (1.5 mM-$CaCl_2$) or absence (2 mM-EGTA, no added $CaCl_2$) of Ca^{2+}. [^{3}H]Choline and [^{3}H]phosphocholine (PCholine) were measured in the extracellular buffer and in the cellular extract. The values are means ± S.E.M. from five to eight experiments performed in triplicate, expressed as percentage of controls. Intracellular controls were 2250 and 20800 d.p.m./dish for [^{3}H]choline and [^{3}H]phosphocholine respectively. Extracellular controls were 1130 d.p.m./dish for [^{3}H]choline. EGTA raised extracellular [^{3}H]phosphocholine controls from 700 to 1700 d.p.m./dish.

	Extracellular (% of control)		Intracellular (% of control)	
	Choline	PCholine	Choline	PCholine
AngII	204±13	112±7	108±9	89±2
AngII/EGTA	155±10	98±9	107±7	95±1

DISCUSSION

Our results show that AngII caused a delayed, sustained, increase in the formation of the water-soluble products of PC hydrolysis in VSMC, at concentrations ranging from 1 to 100 nM, and a sustained activation of PLD activity. Phorbol esters also increased extracellular [^{3}H]choline accumulation, but with a slower time course. However, AngII-stimulated PC hydrolysis appeared to be independent of protein kinase C, and was instead partially dependent on extracellular Ca^{2+}. The effect of AngII was also contingent upon cellular processing of the AngII-receptor–agonist complex, and was primarily manifested as an extracellular accumulation of [^{3}H]choline, consistent with a localization of the relevant PC in the extracellular leaflet of the plasma membrane.

Absence of permeability to PC metabolites and asymmetry of PC hydrolysis

One of the most striking features of PC hydrolysis in VSMC is the preferential extracellular increase in PC metabolite products. This is likely to be the result of specific agonist-mediated events, and not merely due to passive diffusion, since both choline and phosphocholine are charged molecules unlikely to cross a lipid bilayer spontaneously. Our own data support this presumption, since cells maintain a 20-fold higher amount of phosphocholine in the cytoplasm as compared with the extracellular space (see legends to Figs. 1 and 5). In AngII-stimulated cells, any choline produced intracellularly is rapidly converted into phosphocholine, thus decreasing the possibility of its release from the cytoplasm. In addition, we included 1 mM-choline in our extracellular buffer to help remove label from non-specific sites, and in so doing created a concentration gradient against which passive diffusion is unlikely to occur. Finally, in cells incubated for 20 min with [^{3}H]choline to label only cytoplasmic choline and phosphocholine and not PC, we were unable to demonstrate agonist-induced release of choline. Taken together, these data suggest that the choline metabolites are directly produced in the intra- or extra-cellular compartment in which they are measured. Although there may be a small intracellular formation of choline, masked by rapid phosphorylation to phosphocholine (Fig. 1), our results suggest

that a major portion of PC hydrolysis occurs in the external leaflet of the plasma membrane.

An external localization of PC metabolites has also been observed in several other systems [20,28–35]. However, in fibroblasts, some reports describe an intracellular accumulation of PC metabolites [36,37], whereas others describe formation outside these cells [30,35,38]. Conflicting reports also exist in endothelial cells, where extracellular PC metabolites were found in bovine aortic endothelial cells [28], but not in bovine pulmonary-artery endothelial cells [39]. Thus, although some cells exhibit agonist-stimulated accumulation of PC hydrolysis products intracellularly, in a majority of cases PC metabolism is primarily manifested by accumulation of hydrolysis products in the extracellular space, raising the possibility that PC located in the outer leaflet of the plasma membrane is being preferentially metabolized upon agonist stimulation.

This formulation is consistent with the notably higher proportion of PC found in the outer leaflet in several cell types [14], and further suggests that the PA generated simultaneously with choline may also be localized in the outer leaflet of the sarcolemma. This asymmetry in the accumulation of metabolites may be a simple consequence of PC localization (which may serve other functions); alternatively, it may have important functional implications. Although the release of extracellular choline probably has little significance, owing to its high extracellular concentration (15 μM in plasma [40]), the formation of PA in the external rather than the internal leaflet may be significant. It has been suggested for many years that diphosphatidate complexed with Ca^{2+} could migrate through the plasma membrane, thus playing the role of a Ca^{2+} conduit [41–44], or could mobilize intracellular Ca^{2+} via IP_3 generation [45–48]. These possibilities remain to be explored in VSMC.

Mechanism of agonist-induced PC hydrolysis

As noted above, the present data indicate that AngII and PMA induce PC hydrolysis in the external leaflet of the VSMC plasma membrane. It is likely that there is also some phospholipase activity on the PC found in the intracellular leaflet, since there was a transient formation of phosphocholine inside the cells. However, the sustained response consists almost entirely of accumulation of extracellular choline (Fig. 1). As phosphocholine was not converted into choline outside the cells (Table 1), choline seems to be the primary product of PC hydrolysis, which is consistent with an initial generation of choline via PLD activation. Direct evidence for the existence of a PLD that is coupled to the AngII receptor derives from the ethanol experiments. The large accumulation of PEt seen following stimulation of VSMC with AngII in the presence of ethanol (Fig. 5) is indicative of a transphosphatidylation reaction catalysed uniquely by a PLD [22]. In conjunction with the simultaneous generation of choline by AngII in these cells, these data strongly suggest that a major portion of AngII-induced PC hydrolysis is mediated by PLD. The minor stimulation of extracellular phosphocholine induced by AngII alone or AngII with PMA could be due to an additional PLC activity, since no extracellular conversion of choline into phosphocholine was observed (Table 1). The existence of a PLD-mediated PC hydrolysis [20,22,28,33,36,39,49,50] and PLC-stimulated PC breakdown [29,38,51] have been reported in other systems. In VSMC, different agonists may be linked to different phospholipases, as vasopressin was found primarily to induce a PLC activity [32].

Regulation of PC hydrolysis

The present data suggest that agonist-stimulated PC hydrolysis has several distinct regulatory mechanisms in VSMC. AngII and PMA appear to induce PC hydrolysis by different pathways, as indicated by the additivity of the stimulations by PMA and a maximally effective concentration of AngII at 10 min (Table 2), and the fact that AngII-stimulated PC hydrolysis remains intact after down-regulation of protein kinase C (Fig. 6). This observation was somewhat unexpected, since we have previously shown that AngII fully activates protein kinase C by 2 min, as determined by the phosphorylation of a 76 kDa protein [5]. This apparent discrepancy could reflect either a direct stimulation of PC hydrolysis by phorbol esters or a differential activation of protein kinase C isoenzymes by the two treatments. It is possible that PMA activated PLD independently of protein kinase C in this system, since 4α-phorbol dibutyrate, a derivative inactive on the kinase, partially stimulated PLD activity (B. Lassègue, R. W. Alexander & K. K. Griendling, unpublished work). Alternatively, AngII-induced PC hydrolysis might be totally independent of protein kinase C, or may be mediated by an isoenzyme insensitive to down-regulation [52–55] and insensitive to phorbol ester activation [55–57]. We cannot distinguish between these possibilities at present.

Our results also suggest that Ca^{2+} influx is an integral regulatory step in AngII activation of PC hydrolysis. Formation of PA and DG are also Ca^{2+}-dependent (B. Lassègue, R. W. Alexander & K. K. Griendling, unpublished work) in VSMC, and PC breakdown has been shown to require Ca^{2+} in other systems [22,28,49,58]. However, removal of extracellular Ca^{2+} inhibited the formation of choline metabolites only partially, implying that AngII has an additional effect on VSMC which is independent of Ca^{2+} influx and protein kinase C. This may be related to the physical location of the agonist–receptor complex, as suggested by the PAO experiments.

In the context of our previous observations on signal transduction in AngII-stimulated VSMC, it seems likely that PC is a primary source for sustained DG formation via PLD and PA phosphatase. Furthermore, activation of the phosphoinositide and PC pathways appears to have distinct regulatory mechanisms and functional consequences. Polyphosphoinositide hydrolysis is an early event (15 s–1 min), which is inhibited by protein kinase C-activating phorbol esters [7–9] and is independent of receptor–agonist movement [11]. PC breakdown, in contrast, only appears after 1 min, is stimulated by phorbol esters, and is markedly dependent on cellular processing of the receptor–agonist complex. The lipophilic and water-soluble metabolites of these two phospholipids probably accumulate in distinct regions of the plasma membrane and aqueous compartments, supporting our previous suggestion [11] that there may be discrete cellular signalling domains in VSMC.

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