Phosphatidylcholine is a major source of phosphatidic acid and diacylglycerol in angiotensin II-stimulated vascular smooth-muscle cells

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In cultured vascular smooth-muscle cells, angiotensin II produces a sustained formation of diacylglycerol (DG) and phosphatidic acid (PtdOH). Since the fatty acid composition of these molecules is likely to determine their efficacy as second messengers, it is important to ascertain the phospholipid precursors and the biochemical pathways from which they are produced. Our experiments suggest that phospholipase D (PLD)-mediated phosphatidylcholine (PtdCho) hydrolysis is the major source of both DG and PtdOH during the late signalling phase. First, in cells labelled with [³H]myristate, which preferentially labels PtdCho, formation of [³H]PtdOH precedes formation of [³H]DG. Second, in contrast with phospholipase C (PLC) activation, DG mass accumulation is dependent on extracellular Ca²⁺. Similarly, DG mass accumulation is not attenuated by protein kinase C activation, which we have previously shown to inhibit the phosphoinositide-specific PLC. Third, the fatty acid composition

of late-phase DG and PtdOH more closely resembles that of PtdCho than that of phosphatidylinositol. Finally, in cells labelled for a short time with [³H]glycerol, the radioactivity incorporated into [³H]DG and PtdOH was greater than that incorporated into PtdIns, but not into PtdCho. We found no evidence that synthesis *de novo* or phosphatidylethanolamine breakdown contributes to sustained DG and PtdOH formation. Thus, in angiotensin II-stimulated cultured vascular smooth-muscle cells, PLD-mediated PtdCho hydrolysis is the major source of sustained DG and PtdOH, whereas phosphoinositide breakdown is a minor contributor. Furthermore, PtdOH phosphohydrolase, which determines the relative levels of DG and PtdOH, appears to be regulated by protein kinase C. These results have important implications for the role of these second messengers in growth and contraction.

INTRODUCTION

Angiotensin II (AngII) stimulation of cultured vascular smooth-muscle cells (VSMC) results in a biphasic biochemical response. The initial phase of signalling comprises phospholipase C (PLC)-mediated breakdown of the inositol polyphospholipids to generate Ins(1,4,5)P₃ [1] and mobilize intracellular Ca²⁺ [2]. The second phase is characterized by a massive, sustained, accumulation of diacylglycerol (DG) [3], activation of protein kinase C [4], phospholipase D (PLD)-mediated hydrolysis of phosphatidylcholine (PtdCho) [5] and intracellular alkalinization [6]. These two phases are not only temporally distinct, but are also differentially regulated. Early signalling events are independent of Ca²⁺ [1] and are attenuated by protein kinase C activation [7]. The sustained response is dependent on intracellular alkalinization [6] and continuing Ca²⁺ influx [5], is independent of protein kinase C [3,5] and is consequent upon cellular processing of the AngII-receptor complex [8].

The complexity of phospholipid hydrolysis raises questions concerning the source of the DG and phosphatidic acid (PtdOH) that accumulate during the two phases of signalling. DG could derive directly from PLC-mediated phospholipid hydrolysis, or indirectly by sequential activation of PLD and PtdOH phosphohydrolase (PPH). PtdOH, on the other hand, could come directly from PLD stimulation or from sequential activation of PLC and DG kinase. Information concerning the phospholipid precursors of these signalling molecules is important, because it will provide insight into the sequence of biochemical events initiated by AngII, and also because lipid second messengers

with different fatty acid compositions may play different roles within the cell. In GH₃ cells, the saturated DGs that accumulate during the sustained phase of the response are unable to activate protein kinase C [9]. Furthermore, different PtdOHs have differential effects on Ca²⁺ influx [10,11] and mitogenesis [12,13]. Clearly, production of these molecules must be tightly regulated by the cell in order to preserve physiological integrity.

We have previously demonstrated that AngII activates both PLC-mediated phosphoinositide hydrolysis [1] and PLD-mediated PtdCho breakdown [5]. Both of these pathways result in the accumulation of PtdOH and DG, but the extent to which each contributes to the composition of these second-messenger pools after stimulation is unclear. The interconversion of PtdOH and DG must also be under hormonal control, although little is known about the regulation of PPH activity. The present study was designed to determine the sources of the PtdOH and DG that are formed in response to AngII, and to identify potential factors that regulate their interconversion. Our results provide insight into the mechanisms utilized by vascular smooth muscle to control the levels of these two important second messengers.

MATERIALS AND METHODS

Cell culture

VSMC were isolated from rat thoracic aorta by enzymic digestion as described previously [5]. Cells were grown in Dulbecco's Modified Eagle's Medium supplemented with antibiotics and 10% calf serum, and were passaged twice a week by harvesting with trypsin/EDTA and seeding at a 1:4 ratio in 75 cm² flasks.

Abbreviations used: AngII, angiotensin II; BSS, balanced salt solution; DCF, 4,4'-dichlorofluorescein; DG, diacylglycerol; PtdOH, phosphatidic acid; PPH, phosphatidate phosphohydrolase; PtdCho, phosphatidylcholine; PDBu, 4 β -phorbol 12,13-dibutyrate; PtdEtn, phosphatidylethanolamine; PtdEtOH, phosphatidylethanol; PLC, phospholipase C; PLD, phospholipase D; PtdSer, phosphatidylserine; VSMC, vascular smooth-muscle cells.

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For experiments, cells between passages 5 and 20 were seeded into 35 mm-diam. dishes, fed every other day, and used at confluence or 1–2 days post-confluence.

Radioisotope labelling

Several labelling protocols were used in this study. For preferential labelling of lipids derived from PtdCho, VSMC were incubated with 1 ml of [^3H]myristate (2 $\mu\text{Ci}/\text{ml}$) for 24 h in normal medium. PtdIns-associated lipids were labelled by incubating cells with 1 ml of [^3H]arachidonic acid (1 $\mu\text{Ci}/\text{ml}$) for 2–3 h in serum-free medium. To estimate total PtdOH, cells were prelabelled with 1 ml of [^{32}P]P_i (10 $\mu\text{Ci}/\text{ml}$) for 60–90 min in balanced salt solution (BSS; see below) before exposure to agonist (the phospholipids incorporate enough ^{32}P during this labelling period to account for the observed [^{32}P]PtdOH, which may be either derived from phospholipid hydrolysis or DG phosphorylation). VSMC used for argentation chromatography were incubated with 2 ml of [^3H]glycerol (20 $\mu\text{Ci}/\text{ml}$) for 72 h in normal medium. To measure synthesis *de novo* of DG and PtdOH, VSMC were preincubated for 1 min with 10 $\mu\text{Ci}/\text{ml}$ [^3H]glycerol in BSS, before addition of AngII to the labelling buffer. Finally, to measure phosphatidylethanolamine (PtdEtn) hydrolysis, VSMC were preincubated for 2 h with 2 $\mu\text{Ci}/\text{ml}$ [^3H]ethanolamine in culture medium, before stimulation with AngII.

Measurement of DG

VSMC were incubated for 20 min at 37 °C in BSS of the following composition (mM): 130 NaCl, 5 KCl, 1.5 CaCl_2 , 1 MgCl_2 , 20 Hepes (buffered to pH 7.4 with Tris base). For Ca^{2+} -free experiments, cells were exposed to EGTA (2 mM)-containing buffer (from which CaCl_2 was omitted) for the final 5 min of the incubation period. The buffer was then aspirated and replaced with 1 ml of the same buffer containing the indicated agonist, and incubated for various times. The reaction was terminated by rapid aspiration of the buffer and addition of 1.5 ml of chloroform/methanol (1:2, v/v). Organic and aqueous phases were separated by the method of Bligh and Dyer [14], and the organic phase evaporated to dryness under nitrogen. For experiments with [^3H]myristate- or [^3H]arachidonate-labelled cells, lipids were redissolved in chloroform and DG was isolated by t.l.c. as described previously [3]. For determination of DG mass, DG was phosphorylated to PtdOH with DG kinase in the presence of [^{32}P]ATP [15]. [^{32}P]PtdOH was separated by t.l.c. on heat-activated silica gel G plates developed in the solvent system chloroform/acetone/methanol/acetic acid/water (90:36:27:19:9, by vol.), detected by autoradiography, scraped and quantified by liquid-scintillation spectrophotometry. DG levels were calculated by reference to a standard curve generated from diolein (0–20 nmol). To differentiate DG from 1-*O*-alkyl-2-acylglycerol we selectively digested the radioactive diacyl PtdOH with lipase from *Rhizopus arrhizus* [16] before separation.

Measurement of PtdOH formation and PLD activation

VSMC were prelabelled as described above, washed, and incubated for 20 min at 37 °C in BSS. Exposure to agonist was as described above for DG, except that lipids were extracted with chloroform/methanol/HCl (20:40:1). To assess PLD activation, transphosphatidylation in the presence of ethanol, resulting in the formation of the PLD-specific product phosphatidylethanol

(PtdEtOH), was measured. For this reaction, cells were incubated with 100 mM ethanol for 5 min before exposure to agonist in the continued presence of ethanol. PtdEtOH and PtdOH were isolated by t.l.c. on heat-activated silica gel G plates that had been dipped in 1% potassium oxalate. The mobile phase consisted of ethyl acetate/acetic acid/iso-octane (9:2:5, by vol.). Radioactive PtdOH and PtdEtOH spots were detected by autoradiography and identified with unlabelled standards, and were scraped from the plate and quantified by liquid-scintillation spectrophotometry.

Subspecies analysis of VSMC lipids

VSMC were labelled with 20 $\mu\text{Ci}/\text{ml}$ [^3H]glycerol for 72 h in culture medium, washed with BSS to remove unincorporated radioisotope, incubated with AngII (100 nM) or buffer (control) for 10 min, and total lipids were extracted in chloroform/methanol/HCl (20:40:1, by vol.). Unlabelled lipid carriers (100–200 μg of PtdIns, PtdCho, PtdOH, DG) were added at this step. In this assay, glassware was silicone-treated with dichlorodimethylsilane and butylated hydroxytoluene was added to retard lipid oxidation (final concn. ~ 50 mg/l) in all organic solutions. Organic and aqueous phases were separated by addition of chloroform and water, and the organic phase was dried under nitrogen, redissolved in 50 μl of chloroform/methanol (1:1, v/v), and applied to a heat-activated silica gel H plate. Plates were developed in the solvent system chloroform/methanol/ammonia (60:35:8, by vol.), and lipids were detected with 0.4% 4',5'-dichlorofluorescein (DCF) in 95% methanol. From this plate three bands were scraped and eluted: neutral lipids (including DG) in chloroform/methanol (1:1, v/v); PtdCho in chloroform/methanol/HCl (167:167:1, by vol.), followed by phase separation by addition of 0.6 vol. of water, removal of the aqueous phase, addition of 0.3 vol. of chloroform/methanol/water (3:48:47, by vol.), and separation of the chloroform phase; and a band containing PtdOH, PtdIns and phosphatidylserine (PtdSer) in the same solvent as PtdCho. The three unresolved lipids were dried under nitrogen, redissolved in chloroform/methanol (1:1, v/v), spotted on heat-activated silica gel H plates and separated in chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, by vol.). Bands were made visible with DCF. PtdOH and PtdIns were scraped from the plate and eluted as described above for PtdCho. Cell-derived DG and DG standards of various fatty acid compositions were then acetylated by incubating the dried lipids with 50 μl of pyridine containing butylated hydroxytoluene and 50 μl of acetic anhydride for 30 min at 70 °C. Phospholipids were subjected to acetolysis overnight at 140 °C by incubation with 600 μl of acetic acid and 400 μl of acetic anhydride. This procedure does not lead to significant intermolecular rearrangement in the phospholipids [17]. The acetylated DG resulting from these two reactions was extracted into hexane after addition of water. Hexane layers were in turn washed with 1 vol. of 0.01 M NaHCO_3 , followed by two washes each with 1 vol. of water. The acetylated DGs were then separated from non-acetylated lipids by spotting on to heat-activated silica gel H plates and developing in hexane/ether (16:5, v/v). Lipids were detected with DCF, and eluted with 95% ethanol, followed by phase separation with 1 vol. of water and 2 vol. of hexane. Acetylated DGs and standards were then dried and spotted on to silica gel G plates containing 20% AgNO_3 to separate the acetylated-DG species according to the number of double bonds, and developed in 0.5% methanol in chloroform. Lipids were again detected with DCF, bands were scraped and ^3H label was quantified as d.p.m. by liquid-scintillation spectrophotometry.

To compare the unsaturation profiles of different lipid species, the radioactivity in each degree of unsaturation was expressed as a percentage of the total radioactivity in all degrees of unsaturation assayed. In addition, for DG and PtdOH, the increases in radioactivity induced by AngII in each degree of unsaturation were expressed as percentages of the total increase in all degrees of unsaturation.

If one makes the assumptions that DG and PtdOH derive from PtdCho and PtdIns and that the activities of the enzymes carrying out the conversions do not depend on the degree of unsaturation of the lipids, then one can compute the relative proportions of PtdCho and PtdIns required to generate a particular profile of DG or PtdOH. This can be done, for DG or PtdOH, by computing a grade of similarity to PtdCho versus PtdIns on a scale of 0 (identical with PtdIns) to 100 (identical with PtdCho). To grade the resemblance of the unsaturation profile of a lipid to that of PtdCho versus PtdIns, a partial resemblance was first computed for each degree of unsaturation. Because partial resemblances are of greater significance for the degrees of unsaturation which show larger differences between PtdCho and PtdIns, the overall resemblance was computed as the weighted average of partial resemblances, with weights proportional to the difference between PtdCho and PtdIns at each degree of unsaturation. If the percentage of incorporated label for each degree of unsaturation is $A_0, \dots, A_4$ for PtdCho, $B_0, \dots, B_4$ for PtdIns, and $C_0, \dots, C_4$ for DG, then the partial resemblances (R_i) of DG to PtdCho versus PtdIns are:

$$R_i = \frac{(C_i - B_i)}{(A_i - B_i)} \cdot 100$$

the partial differences between PtdCho and PtdIns are:

$$E_i = A_i - B_i$$

the individual weights used in the average are:

$$W_i = \frac{|E_i|}{\sum |E_i|}$$

and the overall resemblance of DG to PtdCho versus PtdIns is:

$$E = \sum (R_i \cdot W_i)$$

A similar calculation is performed to determine the resemblance of PtdOH to PtdCho versus PtdIns.

Analysis of [^3H]glycerol-DG and phospholipids

To measure DG and phospholipids in cells prelabeled with [^3H]glycerol, two different systems were used [3]. Cells used to measure DG were extracted with chloroform/methanol (1:2, v/v), phases were separated as described previously [14], and chloroform extracts were dried under a stream of nitrogen. Samples were spotted on heat-activated silica gel G t.l.c. plates and separated with benzene/ether/ammonia (500:250:1, by vol.) as the mobile phase. Bands were revealed with iodine vapour, identified by comparison with standards, scraped, and radioactivity was counted by liquid-scintillation spectrophotometry. Cells used to measure phospholipids were extracted with chloroform/methanol/HCl (20:40:1, by vol.), and samples were prepared as above. Samples were spotted on Whatman LK5 plates impregnated with 1.5% boric acid in 50% ethanol, air-dried and heat-activated. The elution mixture was chloroform/

methanol/water/ammonia (120:75:8:4, by vol.). Plates were autoradiographed and bands of interest were identified by comparison with standards, and scraped and counted as above.

Assay of 1-O-alkyl bonds and mass of PtdIns and PtdCho

The phospholipids were extracted from non-labelled VSMC and purified by t.l.c. as described above. The bands of interest were revealed with iodine, scraped, and lipids were extracted from the silica as described previously [14]. The chloroform phase was extracted twice with water to remove acid contaminants. PtdIns and PtdCho were then hydrolysed to DG *in vitro* with *Bacillus cereus* PLC. The reaction was carried out overnight with a large excess of enzyme at room temperature by continuous shaking of a biphasic system composed of ether and 0.5 M Tris buffer at pH 7.5 with 6 mM CaCl_2 . After evaporation of the ether phase and extraction of the DG as in [14], DG mass and 1-O-alkyl bonds were assayed as described above.

Materials

All chemicals were of analytical grade or better. Salts, solvents and common chemicals were purchased from Fisher (Pittsburgh, PA, U.S.A.). Dulbecco's modified Eagle's medium with 25 mM Hepes and 4.5 g/l glucose and calf serum were from Sigma (St. Louis, MO, U.S.A.). Glutamine, penicillin and streptomycin were from Whittaker Bioproducts (Walkersville, MD, U.S.A.). Trypsin/EDTA was from Gibco (Grand Island, NY, U.S.A.). Cell culture flasks and dishes were from Corning (Marietta, GA, U.S.A.). [$1\text{-}^3\text{H}$]Ethan-1-ol-2-amine hydrochloride (3–30 Ci/mmol) and (1-O- ^3H)octadecyl-*sn*-glycero-3-phosphocholine (80–180 Ci/mmol) were from Amersham (Arlington Heights, IL, U.S.A.); all other radiochemicals were from Du Pont (Boston, MA, U.S.A.): [$\gamma\text{-}^{32}\text{P}$]ATP (3000 Ci/mmol), [^{32}P]P_i (8500–9120 Ci/mmol), [5,6,8,9,11,12,14,15- ^3H]arachidonic acid (60–100 Ci/mmol), [9,10- ^3H]myristic acid (10–60 Ci/mmol), [1,2,3- ^3H]glycerol (30–60 Ci/mmol). LK5 t.l.c. plates were from Whatman (Hillsboro, OR, U.S.A.); all other plates were from Analtech (Newark, DE, U.S.A.). DG kinase was from Lipidex (Westfield, NJ, U.S.A.). *Rhizopus* lipase was from Boehringer Mannheim (Indianapolis, IN, U.S.A.). *Bacillus cereus* PLC Type III was from Sigma. Pyridine, acetic anhydride, DCF and dichlorodimethylsilane were from Eastman Kodak (Rochester, NY, U.S.A.). Diolein was from Serdary Research Laboratories (London, Ont., Canada); other lipid standards were from Avanti Polar Lipids (Pelham, AL, U.S.A.) and Sigma. Dioctanoyl-ethylene glycol was from Avanti Polar Lipids; R59022 and R59949 were from Janssen Research Diagnostics (Flanders, NJ, U.S.A.). Scintillation fluor was Liquiscint from National Diagnostics (Somerville, NJ, U.S.A.). Other chemicals were purchased from Sigma.

RESULTS

Time courses of DG and PtdOH accumulations

DG mass accumulation in VSMC stimulated by AngII is rapid and sustained (Figure 1a). [^{32}P]PtdOH accumulation has a time course similar to that of DG (Figure 1b). This massive accumulation of lipid metabolites suggests that they are derived from a large membrane phospholipid pool or that the turnover of the precursors is very fast. Measurements of mass levels for the phospholipids (see the Materials and methods section) reveal that basal PtdCho is about 25 nmol/ 10^6 cells, and PtdIns is present at a concentration of 5 nmol/ 10^6 cells. DG mass increases from about 0.5 nmol/ 10^6 cells to about 4 nmol/ 10^6 cells after

Figure 1 Time courses of DG and PtdOH accumulation in VSMC after AngII stimulation

After a labelling period (except when DG mass was being measured), VSMC were stimulated with 100 nM AngII for the indicated time, and extracted for lipid analysis. Results are expressed as percentages of the effect of AngII at 10 min or as percentage of the control value (in the absence of agonist) at the corresponding time. (a) DG mass was measured by phosphorylation *in vitro* as described in the Materials and methods section. Data points represent means $\pm$ S.E.M. of values from 4 to 12 independent experiments performed in triplicate. The average DG values at 10 min were 0.84 ± 0.25 and 6.30 ± 1.36 nmol per 35 mm dish in control and AngII-stimulated cells respectively. Inset: early time course of DG mass accumulation. (b) [32 P]PtdOH accumulation was measured after a 1 h labelling period with 10 μ Ci of [32 P]P_i per 35 mm dish. Data points represent means $\pm$ S.E.M. of values from 2 to 8 independent experiments performed in triplicate. The average PtdOH values at 10 min were 6539 ± 2499 and 29991 ± 6754 c.p.m. per 35 mm dish in control and AngII-stimulated cells respectively. (c) [3 H]DG (●) and [3 H]PtdOH (▲) accumulations were measured after a 3 h labelling period with 1 μ Ci of [3 H]arachidonic acid per 35 mm dish. Data points represent means $\pm$ S.E.M. of values from 2 to 5 independent experiments performed in triplicate. The average control values in these experiments were 5047 ± 564 d.p.m. for PtdOH and 3392 ± 75 d.p.m. for DG. (d) [3 H]DG (●) and [3 H]PtdOH (▲) accumulations were measured after a 24 h labelling period with 2 μ Ci of [3 H]myristic acid per 35 mm dish. Data points represent means $\pm$ S.E.M. of 2 to 7 independent experiments (DG) or of 3 to 15 independent experiments (PtdOH) performed in triplicate. The average control values in these experiments were 9068 ± 647 d.p.m. for DG and 12414 ± 967 d.p.m. for PtdOH.

Table 1 Incorporation of [3 H]glycerol in DG, PtdOH, PtdIns and PtdCho

VSMC were incubated in BSS with 20 μ Ci/ml [3 H]glycerol for 40 min, with or without 100 nM AngII for the last 10 min of incubation. After extraction, lipids were separated by t.l.c. as described in the Materials and methods section. The data indicate the radioactivity incorporated in each lipid species, expressed as means $\pm$ S.E.M. from a representative experiment performed in triplicate.

	Incorporation (d.p.m.)			
	DG	PtdOH	PtdIns	PtdCho
Control	3317 ± 262	5804 ± 368	917 ± 110	17953 ± 6926
AngII	5994 ± 165	7189 ± 269	981 ± 148	37427 ± 1992

10 min with 100 nM AngII. We have previously shown that at least a portion of the DG formed in response to AngII derives from the phosphoinositides [3]. The relatively low level of PtdIns present in these cells suggests that at least some of the DG formed is likely to be derived from another phospholipid, presumably PtdCho. Furthermore, in VSMC labelled for 40 min with 20 μ Ci/ml [3 H]glycerol with or without 100 nM AngII during the last 10 min of labelling, the radioactivity incorporated into DG and PtdOH was greater than that which was found in PtdIns but not in PtdCho (Table 1), suggesting that the relative contribution of PtdIns to DG and PtdOH generation was not large.

Further evidence for this conclusion arises from cells labelled with [3 H]myristate. In VSMC, [3 H]myristate preferentially labels PtdCho, with $82 \pm 5\%$ of the label associating with this lipid. Accumulation of [3 H]myristate DG thus serves as a marker for that derived from PtdCho. As shown in Figure 1(d), [3 H]myristate-labelled DG formation is delayed and sustained, and is preceded by formation of [3 H]myristate PtdOH. Since we have previously shown [5] that AngII stimulation of PtdCho hydrolysis is mediated by PLC, the precursor-product relationship suggested in Figure 1(d) would be expected.

In contrast, in VSMC labelled with [3 H]arachidonate, DG and PtdOH are formed rapidly and the earliest peak of DG formation is concomitant with PtdOH accumulation (Figure 1c). This is consistent with an early PLC-mediated hydrolysis of the phosphoinositides, which we have previously demonstrated in these cells [1]. Since [3 H]arachidonate is not as specific a label for PtdIns as [3 H]myristate is for PtdCho (PtdIns incorporates 4% and PtdCho 32% of phospholipid-associated arachidonate), it is unclear what part of the sustained accumulations of [3 H]arachidonate DG and PtdOH derive from PLC-mediated PtdIns hydrolysis followed by activation of DG kinase or from PLC-mediated PtdCho hydrolysis followed by activation of PPH.

Concentration-response relationship of AngII-induced DG and PtdOH formation

The concentration-response relationship of AngII-induced DG mass accumulation ($EC_{50} = 46 \pm 5$ nM) is shifted to the right of

Figure 2 Concentration–effect relationships of AngII-induced accumulation of DG and PtdOH in VSMC

VSMC were exposed to the indicated concentrations of AngII for 5 min before extraction for lipid analysis. Each point represents the mean $\pm$ S.E.M. of values from two independent experiments performed in triplicate, expressed as percentage of the maximal value in the experiment. (●) DG mass was measured as described in the Materials and methods section. The average EC_{50} is 46 ± 5 nM. In these experiments the average DG value at $1 \mu\text{M}$ AngII was 5.9 ± 0.8 nmol above control per 35 mm dish. (▲) [^3H]Myristate PtdOH was measured as described in the Materials and methods section. The average EC_{50} is 11 ± 3 nM. In these experiments, the average PtdOH value at $1 \mu\text{M}$ AngII was 8466 ± 1339 d.p.m. above control per 35 mm dish. The shift between the DG and PtdOH curves was statistically significant ($P < 0.01$ by analysis of variance).

Table 2 Inhibition of DG mass accumulation by propranolol

VSMC were preincubated for 30 min in BSS with the indicated concentration of propranolol and stimulated with or without 100 nM AngII for 10 min in the continuous presence of propranolol. DG mass was then measured as described in the Materials and methods section. The data are means $\pm$ S.E.M. in a representative experiment performed in triplicate.

	DG (nmol/35 mm dish)			
[Propranolol] (μM) ...	0	30	100	300
Control	1.0 ± 0.2	1.5 ± 0.2	2.0 ± 0.1	2.4 ± 0.2
AngII	4.3 ± 0.2	4.6 ± 0.1	3.2 ± 0.3	2.7 ± 0.1

that of AngII-induced [^3H]myristate PtdOH accumulation ($EC_{50} = 11 \pm 3$ nM) (Figure 2). We have shown previously that PLC and PLD are coupled to AngII with an EC_{50} of about 10 nM [1,3,5], in good agreement with the value obtained for [^3H]myristate PtdOH production. The shift in sensitivity of AngII coupling to DG mass suggests that the bulk of DG mass is not produced directly by either phospholipase, but through at least one more enzymic step, such as PtdOH conversion into DG by PPH.

Evidence for PPH activity

The dose–response relationships suggest that DG mass might be derived from PLD-mediated PtdCho hydrolysis via PPH. To investigate this issue, we used two experimental procedures.

First, we attempted to inhibit PPH using propranolol (0.001–1 mM), chlorpromazine (0.01–1 mM) or desipramine (0.01–1 mM), all reputed inhibitors of PPH in other cell types [18,19]. In our system, chlorpromazine had no activity. Propranolol and desipramine almost completely abolished DG mass accumulation at $300 \mu\text{M}$. However, we questioned the specificity of these compounds, since about half of the inhibition could be attributed to a dose-dependent increase in basal DG mass (the data for propranolol are shown in Table 2). In addition, we attempted to inhibit the hydrolysis of PtdCho with the reputed PLD inhibitor wortmannin. This compound completely inhibited [^3H]myristate DG accumulation at $10 \mu\text{M}$, but also significantly inhibited inositol phosphate formation (results not shown), suggesting that it is not specific for PLD in VSMC. Therefore, we measured AngII-stimulated PPH activity directly by following the conversion of exogenously added PtdOH into DG. Incubation of VSMC with $100 \mu\text{g/ml}$ PtdOH (from egg yolk PtdCho) in micelles resulted in a progressive increase in DG mass. DG was elevated as soon as 1 min (0.59 ± 0.08 nmol/dish above control) and steadily increased at least until 20 min (3.46 ± 0.03 nmol/dish above control). This observation supports the existence of a pathway for conversion of PtdOH into DG in VSMC.

Ca^{2+} -dependence of DG and PtdOH formation

We have previously shown that PtdIns-PLC activation is independent of extracellular Ca^{2+} [1], whereas hydrolysis of PtdCho by PLD is partially dependent on extracellular Ca^{2+} [5]. This observation can be used to gain insight into the contributions made by each of these pathways to DG and PtdOH formation. As shown in Figures 3(a) and 3(b), removal of Ca^{2+} inhibited [^{32}P]PtdOH formation, and attenuated DG mass accumulation. The first phase of [^3H]arachidonate DG accumulation, which reflects PLC-mediated hydrolysis of phosphoinositides [3] (Figure 1c), was not affected by removal of extracellular Ca^{2+} (Figure 3c). The second phase, however, was sensitive to the removal of Ca^{2+} , suggesting that its origin may be different, possibly PtdCho hydrolysis. The data obtained by [^3H]myristate labelling support this interpretation, as the accumulations of PtdEtOH, PtdOH and DG (Figures 3d, 3e and 3f) were all significantly inhibited by removal of extracellular Ca^{2+} . The similar inhibitions of DG mass and [^3H]myristate-labelled lipid accumulations suggest that PtdCho is a major source of DG.

Effect of protein kinase C on DG accumulation

To obtain additional evidence concerning the relative contributions of the phosphoinositides and PtdCho to DG accumulation, we took advantage of the differential regulation of PLC and PLD by protein kinase C. We have previously shown that protein kinase C activation inhibits AngII-induced phosphoinositide hydrolysis (PLC) and increases basal PtdCho hydrolysis (PLD) [5,7]. Activation of protein kinase C with 100 nM 4β -phorbol 12-myristate 13-acetate (15 min) slightly increased basal DG mass. This effect was additive with AngII-induced DG mass accumulation (results not shown), suggesting that phosphoinositide hydrolysis is not the major pathway through which DG mass is produced. This is confirmed in experiments in which protein kinase C was down-regulated by preincubation with 200 nM 4β -phorbol 12,13-dibutyrate (PDBu) for 24 h [5,7]. Under these conditions, AngII-stimulated DG mass accumulation was partially inhibited (Figure 4a). Since down-regulation of protein kinase C increases AngII-induced phosphoinositide hydrolysis [20], it is unlikely that the DG

Figure 3 Effect of removal of extracellular Ca^{2+} on AngII-induced DG, PtdOH and PtdEtOH accumulation in VSMC

VSMC were exposed for 5 min to BSS with 1.5 mM Ca^{2+} (black symbols), or to BSS with no added Ca^{2+} and 2 mM EGTA (white symbols). The cells were then stimulated with 100 nM AngII for the indicated times in the continuous presence or absence of Ca^{2+} and extracted for lipid analysis. Results are expressed as percentages of the effect of AngII with Ca^{2+} at 5 or 10 min. (a) DG mass was measured as described in the Materials and methods section. The data points represent means $\pm$ S.E.M. of values from four independent experiments performed in triplicate. The average DG values after 10 min with AngII were 3.7 ± 1.9 and 1.0 ± 0.6 nmol above control per 35 mm dish in the presence and absence of Ca^{2+} respectively. (b) $[^{32}\text{P}]\text{PtdOH}$ was measured as described in the Materials and methods section. The data points represent means $\pm$ S.E.M. of values from three independent experiments performed in triplicate. The average PtdOH values after 10 min with AngII were 34881 ± 2119 and 22233 ± 2869 d.p.m. above control per 35 mm dish in the presence and absence of Ca^{2+} respectively. (c) $[^3\text{H}]\text{DG}$ was measured in cells prelabelled with $[^3\text{H}]\text{arachidonic acid}$, as described in the Materials and methods section. The data points represent means $\pm$ S.E.M. of values from three independent experiments performed in triplicate. The average DG values after 10 min with AngII were 6458 ± 2446 and 564 ± 202 d.p.m. above control per 35 mm dish in the presence and absence of Ca^{2+} respectively. (d) $[^3\text{H}]\text{PtdEtOH}$ was measured in cells prelabelled with $[^3\text{H}]\text{myristic acid}$ and exposed to 100 mM ethanol as described in the Materials and methods section. The data points represent means $\pm$ S.E.M. of two independent experiments performed in triplicate. The average PtdEtOH values after 5 min with AngII were 10988 ± 1428 and 4279 ± 1372 d.p.m. above control per 35 mm dish in the presence and absence of Ca^{2+} respectively. (e) $[^3\text{H}]\text{PtdOH}$ was measured in cells prelabelled with $[^3\text{H}]\text{myristic acid}$ as described in the Materials and methods section. The data points represent means $\pm$ S.E.M. of 3–7 independent experiments performed in triplicate. The average PtdOH values after 5 min with AngII were 10260 ± 2274 and 8527 ± 2295 d.p.m. above control per 35 mm dish in the presence and absence of Ca^{2+} respectively. (f) $[^3\text{H}]\text{DG}$ was measured in cells prelabelled with $[^3\text{H}]\text{myristic acid}$ as described in the Materials and methods section. The data points represent means $\pm$ S.E.M. of 4–5 independent experiments performed in triplicate. The average DG values after 5 min with AngII were 11732 ± 1837 and 6680 ± 527 d.p.m. above control per 35 mm dish in the presence and absence of Ca^{2+} respectively.

measured in this assay is derived from activation of PLC. However, as protein kinase C down-regulation does not affect AngII-induced PtdCho hydrolysis [5], the partial inhibition of DG mass is likely to be due to a decrease in the conversion of PtdOH into DG by PPH after protein kinase C down-regulation. The results in Figure 4(b) suggest that this is the case, since the down-regulation of protein kinase C increased AngII-induced accumulation of $[^3\text{H}]\text{myristate PtdOH}$. Basal levels of PtdOH were also markedly increased (+87%; see legend to Figure 4), supporting the conclusion that PPH activity depends on protein kinase C integrity.

Analysis of fatty acid composition of AngII-stimulated DG and PtdOH

To corroborate the evidence presented so far that PtdCho is a major source of DG and PtdOH in AngII-stimulated VSMC, we analysed the double-bond composition of the fatty acids present in DG, PtdOH, PtdIns and PtdCho before and after stimulation with AngII. As shown in Figure 5(a), under basal conditions the fatty acid compositions of PtdIns and PtdCho are quite different.

Nearly 80% of the PtdIns present in VSMC contains 4 or more double bonds, whereas PtdCho molecular species are almost evenly distributed between 0, 1, 3 and 4 and more double bonds. These proportions do not significantly change upon stimulation with AngII (results not shown). The composition of AngII-induced increases in DG and PtdOH is depicted in Figure 5(b). For both of these lipids, AngII caused an increase in the formation of all molecular species resolved by this technique. However, the distribution of these increases most likely reflects the composition of their phospholipid precursors. This is quantified in relation to PtdIns and PtdCho in Table 3. Under control conditions, 62% of the DG in the cell has a molecular composition similar to that of PtdIns. In contrast, the molecular composition of PtdOH bears an 89% similarity to that of PtdCho. Upon stimulation, the likeness of DG to PtdCho increases, whereas that of PtdOH to PtdCho decreases. The partial nature of these similarities suggests that, although PtdCho hydrolysis is the major source of DG and PtdOH, another phospholipid might contribute to stimulated lipid formation. Analysis of the newly formed lipid supports this conclusion: 78% of PtdOH would appear to derive from PtdCho, whereas 68% of DG can be attributed to this

Figure 4 Effect of protein kinase C down-regulation on AngII-induced DG mass and [^3H]myristate PtdOH accumulations

VSMC were preincubated with (white symbols) or without (black symbols) 200 nM PDBu in serum-free culture medium for 24 h before stimulation with 100 nM AngII for the indicated times. (a) DG mass was measured as described in the Materials and methods section. Data points represent means $\pm$ S.E.M. of values from 5–6 independent experiments performed in triplicate, expressed as percentages of the effect of AngII at 10 min in non-PDBu-treated cells. At 10 min the average DG mass values were 7 ± 0.9 and 4 ± 0.6 nmol above control per 35 mm dish in AngII-stimulated cells in the absence and presence of PDBu respectively. (b) VSMC were labelled for 24 h with [^3H]myristate in normal culture medium before the 24 h exposure to PDBu as above, in the continuous presence of label. Data points represent means $\pm$ S.E.M. of values from 2–4 independent experiments performed in triplicate. The average PtdOH values at 10 min were $21\,293 \pm 6\,715$ and $26\,696 \pm 7\,886$ d.p.m. above control per 35 mm dish in AngII-stimulated cells in the absence and presence of PDBu respectively. The basal values at 10 min were $23\,053 \pm 8\,147$ and $43\,067 \pm 14\,050$ d.p.m. in the absence and presence of PDBu respectively.

Figure 5 Distribution as a function of unsaturation of PtdIns, PtdCho and AngII-induced increases in DG and PtdOH

VSMC were grown for 3 days in the presence of 20 μCi of [^3H]glycerol per 35 mm dish. Cells were then exposed for 10 min to BSS with or without 100 nM AngII and extracted for lipid analysis. Purified PtdIns, PtdCho, DG and PtdOH were fractionated according to the total number of double bonds (0–4 and more) in their fatty acids as described in the Materials and methods section. For PtdIns and PtdCho, the radioactivity measured at each degree of unsaturation is expressed as a percentage of the total radioactivity in PtdIns or PtdCho respectively. For DG and PtdOH, the AngII-induced increase in radioactivity at each degree of unsaturation is expressed as a percentage of the total increase in DG or PtdOH respectively. Bars represent means $\pm$ S.E.M. of triplicate values from a single experiment representative of two that gave similar results. The total radioactivity incorporated in PtdCho and PtdIns was $872\,900 \pm 38\,400$ and $89\,300 \pm 7\,700$ d.p.m. per 35 mm dish respectively. The AngII-stimulated increases in DG and PtdOH were $17\,867 \pm 1\,656$ and $13\,967 \pm 2\,662$ d.p.m. per 35 mm dish respectively.

source after AngII stimulation. This analysis would thus indicate that about 25–30 % of the DG and PtdOH present after a 10 min stimulation with AngII derive from PtdIns.

Lack of AngII-induced synthesis *de novo* of DG and PtdOH

Another possible source for DG and PtdOH after exposure to AngII is synthesis *de novo* from glycerol. To investigate this possibility, we incubated VSMC in BSS with 10 $\mu\text{Ci}/\text{ml}$ [^3H]glycerol with or without 100 nM AngII and monitored the incorporation of label in DG and the phospholipids. With this labelling procedure, radioactivity incorporated into PtdIns and PtdCho was minimal and AngII did not increase the amount of label found in DG and PtdOH above that found in control cells (547 ± 109 d.p.m./dish for DG and 158 ± 18 d.p.m./dish for PtdOH after 1 min). This suggests that synthesis *de novo* does not account for AngII-induced DG and PtdOH accumulations.

Ether-linked lipids

In neutrophils, 48 % of PtdCho is present in the 1-*O*-alkyl form [19], a molecular species hydrolysed by PLD which significantly contributes to the generation of PtdOH and diradylglycerol [21]. Unlike neutrophils, VSMC do not readily incorporate [^3H]1-*O*-alkyl-2-lyso-PtdCho, so that this compound cannot be used to demonstrate the contribution of PLD-mediated PtdCho hydrolysis to the DG pool (results not shown). However, since

Table 3 Resemblance of DG and PtdOH unsaturation profiles to PtdCho versus PtdIns

Data from the experiment described in Figure 5 were used to compute the resemblance of the unsaturation profiles of DG or PtdOH to those of PtdIns and PtdCho, as detailed in the Materials and methods section. The numbers represent a resemblance grade on a scale from 0 (identical with PtdIns) to 100% (identical with PtdCho).

	Resemblance to PtdCho (%)		
	Control	AngII	AngII – control
DG	38	55	68
PtdOH	89	81	78

PtdCho usually contains a significantly higher proportion of ether-linked molecules than PtdIns, comparing the composition of these phospholipids with that of DG may indicate their relative contribution to DG mass. Because the standard DG mass assay does not differentiate between diacyl- and 1-*O*-alkyl-2-acyl-glycerol, we used the modification of Tyagi et al. [16]. To measure the composition of PtdIns and PtdCho, these lipids were first converted *in vitro* into DG with PLC. In non-stimulated VSMC, 7 % of PtdIns and 13 % of PtdCho had the 1-*O*-alkyl structure. The proportion of 1-*O*-alkyl-DG in AngII-induced

DG mass was 8% at 15 s and increased to 13% and 24% at 10 and 20 min respectively. This result is consistent with our previous observations and suggests that the early phase of DG mass may derive mostly from PtdIns, whereas PtdCho may then become a major contributor. After a 20 min exposure to AngII, a pool of PtdCho enriched in 1-*O*-alkyl bonds may become preferentially hydrolysed.

Other possible sources of DG and PtdOH

To investigate whether cellular DG and PtdOH could also be significantly increased by hydrolysis of PtdEtn, a major membrane phospholipid, we labelled VSMC with 2 μ Ci of [3 H]ethanolamine per dish for 2 h before stimulation with AngII. Under these conditions, 97% of the label incorporated in phospholipids was present in PtdEtn. After AngII stimulation there was a transient (complete at 2 min) release ($232 \pm 12\%$ control) of radioactive ethanolamine metabolites from the cells. The short duration of this response suggests that PtdEtn may not contribute significantly to the long-term accumulation of PtdOH and DG.

DISCUSSION

AngII stimulation of vascular smooth muscle induces a cascade of biochemical events, including rapid PLC-mediated hydrolysis of PtdIns(4,5) P_2 leading to Ins(1,4,5) P_3 and DG formation, and a delayed, sustained, breakdown of PtdCho by PLD [9]. The sustained signals generated by AngII are likely to be important in the regulation of long-term events such as growth and tonic contraction. Both DG and PtdOH have been linked to these responses, and are vital second messengers whose metabolism is closely regulated by the cell. In addition, the fatty acid composition of both DG and PtdOH has been shown to affect their ability to activate protein kinase C [9] or growth-related enzymes [12,13], suggesting that the source from which these lipids are derived is regulated as well.

The fatty acid composition of precursor phospholipids varies notably with phospholipid type. In fibroblasts, for example, 16% of PtdIns, but only 1% of PtdCho, contains arachidonic acid [22]. The source from which DG and PtdOH are generated affects their biochemical efficacy and physiological potency, and also influences the metabolic pathways utilized by the cell for their degradation. Therefore, it is of more than anecdotal importance to know which phospholipids are involved in second-messenger generation, in which proportions and with what time sequence. We have previously shown that PtdIns(4,5) P_2 is hydrolysed by PLC rapidly and transiently after AngII stimulation of VSMC, and that PtdCho undergoes sustained hydrolysis by PLD after a delay of 2 min. DG and PtdOH are formed as a consequence of activation of both of these pathways. In the present study, we investigated two possible additional sources of PtdOH and DG: PtdEtn and synthesis *de novo*. We found no evidence for appreciable synthesis *de novo* after AngII stimulation of these cells. In VSMC in which PtdEtn was specifically labelled with [3 H]ethanolamine, AngII induced only a relatively small and transient release of 3 H-labelled metabolites, suggesting that PtdEtn is not a major source for sustained generation of second messengers. Therefore, of the major membrane phospholipids, only the phosphoinositides and PtdCho seem to be good candidates as major sources of DG and PtdOH.

The weight of the evidence presented here suggests that PtdCho is the major source of sustained DG formation in AngII-stimulated VSMC. The amount of DG formed indicates that it must derive either from a large membrane pool such as PtdCho

or from the extraordinarily rapid turnover of a smaller phospholipid pool (the phosphoinositides). Since PLC-mediated phosphoinositide hydrolysis and PLD-mediated PtdCho breakdown are differentially regulated in these cells, analysis of the biochemical mechanisms regulating DG mass formation provides insight into the phospholipid source and pathway utilized. Thus the observation that DG mass accumulation is dependent on extracellular Ca^{2+} argues against a major contribution of phosphoinositide hydrolysis, since PLC-mediated breakdown of these lipids is Ca^{2+} -independent [1], whereas PtdCho hydrolysis by PLD is Ca^{2+} -dependent. Similarly, DG mass is not decreased by protein kinase C activation, one of the primary inhibitors of PLC activity [7]. Finally, the fatty acid composition of late-phase PtdOH and DG is more similar to that of PtdCho than to that of PtdIns, suggesting that the bulk of these newly formed lipids comes from PtdCho.

There are two pathways by which DG could be generated from PtdCho: directly by PLC-mediated hydrolysis or indirectly by the sequential activation of PLD and PPH. The latter pathway appears to be the significant one in VSMC, since (1) in AngII-stimulated VSMC preincubated with [3 H]myristate to label PtdCho, [3 H]PtdOH precedes [3 H]DG generation, and (2) incubation of VSMC with unlabelled PtdOH results in DG mass accumulation. As discussed in more detail below, the differences in concentration-response relationships for AngII-stimulated DG mass, PtdOH accumulation and Ins(1,4,5) P_3 formation also support this hypothesis.

Since DG and PtdOH may be readily interconverted by DG kinase and PPH, the activity of these two enzymes is also important in regulating the levels of these two second messengers. The nearly simultaneous time courses of AngII-induced DG mass and [32 P]PtdOH accumulations supports the interchangeability of these compounds. We evaluated the physiological importance of these enzymes in VSMC, using available inhibitors. Inhibitors of PPH such as propranolol decreased DG accumulation (and increased basal DG levels). In contrast, the DG kinase inhibitors R59022, R59949 and dioctanoyl ethylene glycol slightly decreased DG mass in our system (B. Lassègue and K. K. Griendling, unpublished work), suggesting that DG kinase does not contribute importantly to the metabolism of DG. This is consistent with results from the literature indicating that, in vascular smooth muscle, DG lipase is much more active than DG kinase [23,24]. Additionally, although DG kinase appears to be Ca^{2+} -dependent [25], we saw no accumulation of DG mass in the absence of Ca^{2+} (Figure 3), as would be expected if DG was primarily metabolized by this enzyme.

The concentration-effect relationships of AngII-stimulated lipid second-messenger formation also support a role for PPH in DG mass accumulation. AngII stimulates [3 H]myristate PtdOH formation (Figure 2) and [3 H]choline release [5], both products of PLD-mediated PtdCho hydrolysis in VSMC, with EC_{50} values of 10 nM. The EC_{50} of PLD activation is also identical with that previously presented for AngII-induced PLC activation as measured by [3 H]arachidonate DG accumulation [3] and [3 H]Ins P_3 accumulation [1]. These observations imply that both phospholipases are coupled to a single AngII receptor with the same efficacy. In contrast, the concentration-effect relationship for AngII-induced DG mass accumulation was slightly, but significantly, shifted to the right ($EC_{50} = 46$ nM), suggesting that an additional metabolic step is involved. The most likely candidate for this biochemical step is PPH.

We have shown previously that activation of protein kinase C stimulates basal PLD activity, but does not mediate AngII stimulation of PLD [5]. However, since protein kinase C down-regulation altered the AngII-induced increase in DG mass (partial

inhibition; Figure 4a), DG mass accumulation cannot be the direct result of PtdCho PLD activation alone, as deduced above from the concentration–response relationships. The apparent contradiction between the effect of protein kinase C on [^3H]choline release and DG mass accumulation can be explained by the observation that protein kinase C appears to increase the conversion of PtdOH into DG. Down-regulation of protein kinase C increases [^3H]myristate PtdOH and decreases DG mass accumulation, suggesting an inhibition of PPH under these conditions (Figure 4b). Thus, in addition to supporting the conclusion that PtdCho is the major source of DG, our protein kinase C down-regulation experiments also suggest that PPH activity is regulated by protein kinase C in VSMC. This latter observation adds a new dimension to the regulatory functions of protein kinase C.

The data presented here confirm and extend our previous investigations of AngII signalling mechanisms in VSMC. We have shown that AngII induces a rapid, transient, PLC-mediated hydrolysis of the polyphosphoinositides to yield $\text{Ins}(1,4,5)\text{P}_3$ and arachidonate-containing DG. These second messengers serve to mobilize intracellular Ca^{2+} and to activate protein kinase C, which then attenuates PLC activity. At this time PLD activity is turned on, leading to a sustained accumulation of PtdOH and DG. This sequence of signal generation results in the accumulation of very specific types of PtdOH and DG in the absence of a continued elevation of intracellular Ca^{2+} . Remarkably, this sequence may be specific to AngII, since other agonists such as vasopressin have been shown to activate PLD only transiently in an established VSMC line [26].

Further studies will be necessary to ascertain the full spectrum of physiological events mediated by these lipid second messengers. However, it is already clear that their efficacy in stimulating cellular enzymes is directly related to their fatty acid composition. DGs derived from PtdCho are apparently less effective than those derived from PtdIns in activating protein kinase C [9,27]. However, AngII-stimulated protein kinase C activation is sustained in VSMC [28], suggesting that: either (1) there is enough residual PtdIns-derived DG to activate protein kinase C; or (2) the protein kinase C isoenzyme present in VSMC is responsive to PtdCho-derived DG [29]. Arachidonate-containing PtdOHs appear to be more effective at increasing intracellular Ca^{2+} concentration [10], whereas PtdOHs with fatty acid compositions closer to that found in PtdCho are mitogenic. These latter PtdOHs increase thymidine incorporation in 3T3 cells [30] and human renal mesangial cells [31], induce *c-fos* and *c-myc* mRNA expression in A431 cells [32], increase cell number in mammary epithelial cells [33], inhibit activation of GTPase-activating protein [34] and induce the expression of platelet-derived growth factor mRNA in mesangial cells [31]. In VSMC, the combination of sustained DG and PtdOH formation seen in response to AngII could reflect the fact that the enzymic machinery necessary for growth is up-regulated in culture, conferring hormonal sensitivity and a regulatory function to these two important second messengers.

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