Sustained Diacylglycerol Formation from Inositol Phospholipids in Angiotensin II-stimulated Vascular Smooth Muscle Cells*

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Angiotensin II acts on cultured rat aortic vascular smooth muscle cells to stimulate phospholipase C-mediated hydrolysis of membrane phosphoinositides and subsequent formation of diacylglycerol and inositol phosphates. In intact cells, angiotensin II induces a dose-dependent increase in diglyceride which is detectable after 5 s and sustained for at least 20 min. Angiotensin II (100 am) stimulated diglyceride formation is biphasic, peaking at 15 s (257 ± 19% control) and at 5 min (903 ± 23% control). Simultaneous analysis of labeled inositol phospholipids shows that at 15 s phosphatidylinositol 4,5-bisphosphate (PIP₂) and phosphatidylinositol 4-phosphate (PIP) decline to 52 ± 6% control and 63 ± 5% control, respectively, while phosphatidylinositol (PI) remains unchanged. In contrast, at 5 min, PIP₂ and PIP have returned to control levels (92 ± 2 and 82 ± 4% control, respectively), while PI has decreased substantially (81 ± 2% control). The calcium ionophore ionomycin (15 μM) stimulates diglyceride accumulation but does not cause PI hydrolysis. 4β-Phorbol 12-myristate 13-acetate, an activator of protein kinase C, inhibits early PIP₂ and PIP₂ breakdown and diglyceride formation, without inhibiting late-phase diglyceride accumulation. Thus, angiotensin II induces rapid transient breakdown of PIP₂ and PIP₂ and delayed hydrolysis of PI. The rapid attenuation of polyphosphoinositide breakdown is likely caused by a protein kinase C-mediated inhibition of PIP₂ and PIP₂ hydrolysis. While in vascular smooth muscle stimulated with angiotensin II inositol 1,4,5-trisphosphate formation is transient, diglyceride production is biphasic, suggesting that initial and sustained diglyceride formation from the phosphoinositides results from different biochemical and/or cellular processes.

Polyphosphoinositide metabolism recently has been shown to be important in the generation of intracellular signals for hormones which act by increasing cytosolic free calcium (reviewed in Refs. 1–4). Attention has been focused on the early breakdown of PIP₂, the resultant formation of IP₃, and the release of calcium from non-mitochondrial intracellular storage sites (5). The other product of PIP₂ hydrolysis, diacylglycerol (DG), has been proposed as a second messenger for activation of a calcium- and phospholipid-sensitive protein kinase (protein kinase C) (6), but relatively few systematic investigations of hormonally induced DG formation have been reported.

Rasmussen and Barret (7) have suggested that each of these two messengers may have a unique temporal role: the IP₃/calcium system being responsible for an initial transient cellular response and the DG/protein kinase C system being responsible for a sustained phase of the cellular response to various hormonal stimuli. There is at present, however, some question as to whether DG formation is sustained. In platelets, production of DG in response to thrombin is transient, and DG levels return to control values within 2 min (8). In contrast, the angiotensin II-induced increase in DG formation in adrenal glomerulosa cells is sustained for at least 10 min (9). In hepatocytes, two studies report a sustained increase in DG in response to vasopressin (10, 11), while another reports only a transient increase (12).

While IP₃ presumably can be formed only from PIP₂ breakdown, DG could also be formed from phospholipase C-mediated hydrolysis of PIP or PI. Although Berridge (3) has suggested that phospholipase C acts on PIP₂, rather than on PI, to generate DG and IP₃ in equimolar amounts, in angiotensin II-stimulated adrenal glomerulosa cells, PIP₂ and PIP₂ are returning to control levels at a time when DG levels are still increased (9). Many other cell types which exhibit sustained physiological responses to stimulation by calcium-mobilizing hormones also display only transient breakdown of the polyphosphoinositides (13–15). Wilson et al. (16) have demonstrated that purified phospholipase C can directly hydrolyze PI, PIP, and PIP₂ in unilamellar vesicles at a rate dependent upon ambient calcium concentration and phospholipid composition of the membrane. In intact cells, although a delayed agonist-stimulated PI turnover has been observed for many years (2), decreases in PI are usually attributed to phosphorylation by kinases rather than to hydrolysis by phospholipase C (5). Wilson's observation in vesicles raises the possibility that PI is hydrolyzed in situ.
possibility that PI could be the source of agonist-stimulated DG production in intact cells after polyphosphoinositide breakdown, has decreased.

There are few studies in which the time courses of agonist-induced DG and IP₃ production and of PI, PIP, or PIP₂ hydrolysis have been characterized. The objectives of the experiments reported here were to define in cultured vascular smooth muscle the pattern of DG and IP₃ formation after angiotensin II stimulation, to establish the source of the DG formed, and to gain insight into the mechanisms controlling phospholipase C-mediated hydrolysis of the various potential phosphoinositide substrates.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**VSMC were isolated from rat thoracic aorta by enzymatic dissociation as described previously (17, 18). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, glutamine, and antibiotics and were passaged twice a week by harvesting with trypsin/versenes and seeding at a 1:4 ratio in 75-cm² flasks. For experiments, cells between passage levels 4–25 were seeded into 35- or 100-mm dishes (2 × 10⁶ cells/cm²), fed every other day, and used at confluence (2–6 days).

**Phospholipid Labeling and Extraction—**VSMC cultures were incubated with either [³H]myoinositol (25 μCi/ml) for 48 h in standard growth medium or [³H]arachidonic acid (1 μCi/ml) for 5 h in serum-free Dulbecco's modified Eagle's medium containing 0.2 mg/ml bovine serum albumin. Unincorporated isotope was removed by washing cultures in a warm balanced salt solution of the following composition (mM): 130 NaCl, 5 KCl, 1.5 MgCl₂, 1.5 CaCl₂, 20 HEPES (buffered to pH 7.4 with Tris base). Cells were then incubated for 20 min in 2 ml of balanced salt solution at 37 °C. In some experiments, cells were incubated for an additional 5 min in 100 nM PMA and then exposed to angiotensin II in the continued presence of PMA; otherwise, the balanced salt solution was replaced by 1 ml of fresh buffer containing angiotensin II. Unless otherwise indicated, a maximal concentration of angiotensin II (100 nM) was used as a stimulus. The reaction was terminated after various intervals by rapid aspiration of the buffer and addition of 1 ml of chloroform/methanol (1:2, v/v). Samples were applied to heat-activated Silica Gel 60 plates and developed in the first direction in the solvent system benzenediethy1 ether:ethanol:ammonia (100:80.2, v/v). Separation of neutral lipids was verified using a two-dimensional chromatography method of Dows and Michell (20) as described previously (21). All lipids were quantified by liquid scintillation spectrophotometry.

**Measurement of Cell Calcium—**Fura-2, a calcium-sensitive dye, was used to monitor changes in [Ca²⁺], using procedures described previously for quin-2 in these cells (18). Cell suspensions (5 × 10⁶ cells/ml) were prepared using 8 × 100-mm cultures, incubated for 30 min with 5 μM fura-2/acetoxymethyl ester, washed twice (200 × g, 3 min) with BSS, and resuspended to 4 × 10⁶ cells/ml with BSS containing 10 μM glucose. The cells were kept at room temperature, centrifuged in a Beckman Microfuge (model B) prior to use, and resuspended to 2 × 10⁶ cells/ml. Measurements of fluorescence were made at 37 °C using a Spex fluorolog-2 spectrofluorimeter (excitation 340 nm, slit 2.25 nm; emission 505 nm, slit 4.5 nm) equipped with a thermostated cuvette holder, stirring apparatus, and chart recorder. The calibration of fura-2 fluorescence and calculation of [Ca²⁺], were determined using digitonin (50 μM) and Tris/EGTA (5 mM, pH 10) to obtain maximal (Fₘₐₓ) and minimal (Fₘᵢₙₐᵢ₅) fluorescence signals, respectively. [Ca²⁺], was calculated using the formula: [Ca²⁺] = Kₛ (Fₘ - Fₘᵢₙₐᵢ₅)/(Fₘₐₓ - Fₘᵢₙₐᵢ₅) where Fₘ is the fluorescence intensity of fura-2 within the cell and Kₛ was 224 nm (22).

**Materials—**The supplies and vendors used in this study were as follows: [³H]myoinositol (15 Ci/mmol), Amersham, Chicago, IL; [³H]arachidonic acid (88.3 Ci/mmol), New England Nuclear; Dulbecco's modified Eagle's medium, calf serum, glutamine, penicillin and streptomycin, MA Bioproducts, Walkersville, MD; angiotensin II, Peninsula Laboratories, Belmont, CA; bovine serum albumin (Pentex), Miles Laboratories, Elkhart, IN; lipid standards (phosphatidylinositol, phosphatic acid, phosphotidylycerine), Avanti Polar Lipids, Inc., Birmingham, AL; diolein, Serdary Research Labs, Ontario, Canada; phorbol esters (PMA, 4-O-methyl-PMA), Sigma; fura-2/acetoxymethyl ester, Molecular Probes, Inc., Junction City, OR.

**RESULTS**

**Time Course and Dose Dependence of Diglyceride Formation—**Angiotensin II (100 nM) stimulation of rat aortic VSMC cultures induced a substantial increase in DG which was detectable at 5 s and was sustained for at least 20 min (Fig. 1). DG formation consistently showed two distinct phases. An early peak occurred at 15 s (227 ± 19% control, mean ± S.E., n = 16), while a larger late peak occurred at 5 min (303 ± 23% control, n = 17). Both phases were dose dependent, with the threshold for angiotensin II-induced DG formation occurring at 1 nm angiotensin II and maximal DG production occurring at 100 nM angiotensin II (Fig. 2). This observation agrees well with the dose dependence of angiotensin II-induced IP₃ formation in these cells (21).

**Phosphoinositide Hydrolysis and Inositol Phosphate Formation—**To determine the source(s) of the observed DG, angiotensin II-induced DG formation and hydrolysis of PI, PIP, and PIP₂ were simultaneously measured at both 15 s and 5 min (Fig. 3). Cells labeled with either [³H]myoinositol or [³H]arachidonic acid showed essentially the same results. At the early time point, PIP and PIP₂ decreased to 63 ± 5 and 52 ± 6% control, respectively (n = 7, p < 0.001), while there was little change in the level of PI (98 ± 2% control, n = 15, p > 0.05). By 5 min, however, PIP and PIP₂ had returned toward, but had not reached, control levels (82 ± 4 and 92 ± 2% control, respectively, n = 9, 10, p < 0.01), while PI had decreased substantially (81 ± 2% control, n = 18, p < 0.001). There was no significant hydrolysis of the other major phospholipids after 5 min of angiotensin II stimulation (PC and PE: 100 ± 1% control (n = 6, p > 0.1); PS: 94 ± 3% control).
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**Fig. 2. Dose dependence of angiotensin II-induced diacylglycerol formation.** Cultured VSMC were prelabeled with \(^{3}H\) arachidonic acid (1 \(\mu\)Ci/ml) for 3 h, and then exposed to angiotensin II for 5 min (- - -) or 15 s (O-O-O). Each curve is representative of three experiments which yielded similar results.

\((n = 6, p > 0.11)\) nor was there any change in triglyceride levels \((107 \pm 5\% \text{ control, } n = 14, p > 0.1)\). Lyso phosphati-dylinositol had decreased 5 min after angiotensin II stimulation \((82 \pm 2\% \text{ control, } n = 5, p < 0.001)\), indicating an absence of PI-specific phospholipase \(A_2\) activity.

As shown in Fig. 3B, the radioactivity lost from labeled PIP and PIP\(_2\) at 5 min appears to be insufficient to account for the large increase in DG, especially since there was a concomitant sustained increase in PA, a major metabolite of DG \((8,154 \pm 963 \text{ cpm (control) versus } 14,948 \pm 1,497 \text{ cpm (angiotensin II)}, p < 0.02)\). The large drop in PI at 5 min suggests that PI hydrolysis may contribute to sustained DG formation. In support of this hypothesis, IP\(_3\), the other product of phospholipase C action, on PI, remained elevated in these cells at times \((2-20 \text{ min})\) at which only minor increases in IP\(_3\) and IP\(_4\) were observed (Fig. 4B).

As noted above, there was a small continued hydrolysis of PIP and PIP\(_2\) in the presence of angiotensin II (Fig. 4A). This observation was confirmed by the persistence of a modest elevation in IP\(_3\) \((141 \pm 37\% \text{ increase, } n = 4)\) and IP\(_4\) \((42 \pm 21\% \text{ increase, } n = 3)\) at 5 min (Fig. 4B). At the early timepoint \((15 \text{ s})\), 95\% of the total IP\(_3\) formed by these cells was the physiologically active 1,4,5-IP\(_3\) isomer. By 10 min, fully 77\% of the total IP\(_3\) formed was 1,4,5-IP\(_3\) as measured by high pressure liquid chromatography.

**Effect of Calcium on Phosphoinositide Hydrolysis and DG Formation**—Because of the observation that calcium stimulated PI hydrolysis in an isolated enzyme system \((16)\), the effect of calcium on phospholipase C-mediated hydrolysis of the phosphoinositides was investigated in intact cells in an attempt to determine whether calcium plays a pivotal role in altering the substrate for phospholipase C. The calcium ionophore ionomycin \((\text{free acid, } 15 \mu\text{M})\) caused an immediate sustained increase in \([Ca^{2+}]\), at least as large as that seen transiently with 100 \(nM\) angiotensin II (Fig. 5). At this concentration of ionomycin, cells remained viable, as judged by their ability to exclude trypan blue. At 15 s, the ionomycin-induced increase in \([Ca^{2+}]\) was not associated with changes in DG formation or phosphoinositide hydrolysis. However, after 5 min of sustained elevation of \([Ca^{2+}]\), there was an increase above control in DG \((1,987 \pm 161 (n = 7) \text{ to } 5,209 \pm 707 (n = 8) \text{ cpm, } p < 0.01)\), PA \((3,221 \pm 358 \text{ to } 4,407 \pm 402 \text{ cpm, } n = 7, p < 0.05)\), and PIP \((2,921 \pm 384 (n = 6) \text{ to } 4,996 \pm 413 (n = 5) \text{ cpm, } p < 0.01)\). Changes in other phospholipids at 5 min were not significant: PI \((126,622 \pm 6,917 \text{ to } 114,121 \pm 7,219 \text{ cpm, } n = 6, p > 0.1)\), PIP\(_2\) \((3,564 \pm 336 \text{ to } 3,288 \pm 321 \text{ cpm, } n = 5, p > 0.1)\), PS \((20,139 \pm 2,408 (n = 6) \text{ to } 18,726 \pm 2,093 (n = 7) \text{ cpm, } p > 0.1)\), and PC and PE \((220,460 \pm 18,337 (n = 6) \text{ to } 210,312 \pm 11,159 (n = 7) \text{ cpm, } p > 0.1)\).

Although angiotensin II-induced increases in \([Ca^{2+}]\) return to basal levels by 5 min, there appears to be a persistent increase in calcium permeability in cultured VSMC\(^8\) \((23)\) which is presumably accompanied by an increase in calcium efflux. To test the possibility that a high intracellular calcium environment in the region of the cell membrane could contribute to the breakdown of PI, we abolished sustained hormonally induced calcium influx by preincubating cells with EGTA \((2 \text{ mM, } 1 \text{ min})\). Removing extracellular calcium had no effect on PI hydrolysis at 5 min (angiotensin II, 85 \pm 5\%)

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**FIG. 4. Time course of angiotensin II-stimulated phospholipid breakdown and inositol phosphate production.** Cultured VSMC were prelabeled with [3H]myoinositol (25 μCi/ml) for 48 h and then exposed to angiotensin II (100 nM) for different intervals. A, phospholipid hydrolysis. Data are expressed as per cent control. △—△, PIP₂, control = 3,513,500 cpm; ○—○, PIP, control = 10,423 cpm; •—•, PIP₃, control = 11,757 cpm. B, inositol phosphate production. Data are expressed in cpm. △—△, IP; ○—○, IP₂; •—•, IP₃. Each point represents the mean of duplicate determinations. Two additional experiments with fewer time points yielded similar results.

**FIG. 5. Effect of angiotensin II and ionomycin on [Ca²⁺].** Cultured VSMC were incubated with fura-2/acetoxymethyl ester, washed, and resuspended to a final concentration of 2 × 10⁶ cells/ml. Measurements of [Ca²⁺] were made spectrofluorimetrically. Top, typical fura-2 fluorescence in response to 100 nM angiotensin II (peak: 6.1 ± 0.5-fold increase, n = 9). Bottom, typical fura-2 fluorescence in response to 15 μM ionomycin. Addition of each agent is indicated by the arrow. Calculated values for [Ca²⁺] are indicated at the right.

**TABLE I**

<table>
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<tr>
<th>Condition</th>
<th>DG (15 s)</th>
<th>DG (5 min)</th>
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<tbody>
<tr>
<td>Angiotensin II</td>
<td>117 ± 14</td>
<td>164 ± 28</td>
</tr>
<tr>
<td>Angiotensin II + PMA</td>
<td>72 ± 10</td>
<td>233 ± 42</td>
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94 ± 6% control, n = 5, p < 0.01) but had no effect on PI hydrolysis (angiotensin II, 98 ± 3% control; angiotensin II plus PMA, 98 ± 4% control, n = 7, p > 0.1). In contrast, PMA did not inhibit DG formation at 5 min (Table I). The less potent phorbol ester, 4-O-methyl PMA, had no effect on angiotensin II-induced DG formation. Thus, PMA appears to inhibit selectively the early, but not the late phase, of DG production.

**DISCUSSION**

The results of the present study demonstrate that angiotensin II-induced DG formation is sustained in cultured rat aortic VSMC, irrespective of inhibition of early changes in DG and IP₃ by phorbol ester. The existence of two peaks in DG production and the difference in the profiles of phosphoinositide hydrolysis at early (15 s) and late (5 min) times suggest a shift in the substrate for phospholipase C from the polyphosphoinositides to PI with time. Angiotensin II induces rapid breakdown of PIP₂ and PIP and delayed hydrolysis of PI which is not detectable until after 2 min of hormonal stimulation and does not appear to require a sustained elevation of calcium. PIP₂ and PIP breakdown are attenuated after 2 min while PI hydrolysis is not, suggesting a selective inhibition of polyphosphoinositide hydrolysis during the sustained cellular response.

Although many other studies using different cell types have demonstrated rapid hydrolysis of the polyphosphoinositides (13, 14, 25) and late turnover of PI (14, 26), there are few studies which have measured concurrent changes in DG. Sustained DG formation (10 min) in vasopressin-stimulated hepatocytes was not accompanied by hydrolysis of PIP and PIP₂ (10). However, the authors did not examine late PI.

control; angiotensin II plus EGTA, 82 ± 4% control, n = 3, p > 0.1).

Effect of PMA on Phosphoinositide Hydrolysis and DG Formation—We have previously shown that PMA, an exogenous activator of protein kinase C, inhibited angiotensin II-stimulated polyphosphoinositide hydrolysis and calcium flux in these cells (24). To test whether PMA would also inhibit angiotensin II-induced breakdown of PI, we investigated the effect of PMA on angiotensin II-stimulated hydrolysis of all three phosphoinositides and on DG formation. Basal levels of all the phospholipids were unchanged by a brief incubation with PMA (100 nM, 5 min). However, early (15 s) angiotensin II-stimulated DG formation was inhibited by PMA (43 ± 9% inhibition, n = 4, p < 0.02, Table I), which is consistent with the effect of PMA on angiotensin II-induced IP₃ formation and cytosolic calcium changes at 15 s (24). In agreement with our previous observations (24), PMA also attenuated early breakdown of PIP (angiotensin II, 78 ± 7% control; angiotensin II plus PMA, 103 ± 6% control, n = 5, p < 0.06) and PIP₂ (angiotensin II, 65 ± 4% control; angiotensin II plus PMA,
breakdown and attributed the late DG formation to agonist-stimulated lipogenesis. In adrenal glomerulosa cells stimulated with angiotensin II, DG formation was biphasic and was sustained for at least 10 min, but no data relevant to determining its source were reported (9). In cultured VSMC stimulated with angiotensin II, DG formation previously has not been measured directly. However, Smith et al. (27) found that PA, the product of DG kinase action on DG, was increased 1.5-fold by 100 nM angiotensin II at 5 min, which is consistent with our observations. In addition, Nakiba et al. (28) reported a sustained increase in IP formation after angiotensin II stimulation of VSMC. They also demonstrated an increase in total phosphoinositide hydrolysis over 20 min but did not distinguish among PI, PIP, and PIP2.

Stimulated loss of PI in other systems has been attributed to its phosphorylation by kinases in the plasma membrane to replace the polyphosphoinositides hydrolyzed by phospholipase C (3). Although some PI is shuttled through this pathway, replenishment of the PI and PIP pools could account for only 75% of the PI lost in VSMC. In order for PI to be consumed solely by kinase activity, PIP2 would have to be hydrolyzed and replaced with a much greater rate of turnover than was observed in the initial response. Hydrolysis of IP3 and IP2 would have to accelerate as well, since there was no progressive accumulation of IP2 or IP3. Although we cannot rule out the possibility that increased turnover could account for the late PI hydrolysis, the appearance of a large sustained increase in both IP and DG is consistent with a direct action of phospholipase C on PI. Direct hormonally induced phosphodiesteratic cleavage of PI has recently been demonstrated in mouse pancreatic lobules (29).

The mechanism underlying the delay in induction of PI hydrolysis is at present unknown but might be related to a competition among phosphoinositide substrates for phospholipase C. Wilson et al. (16) showed that in unilamellar phospholipid vesicles of a phosphoinositide composition similar to that found in the cell membrane, PI and the polyphosphoinositides competed for a limiting amount of phospholipase C. It is thus possible that PI is hydrolyzed only after levels of PIP and PIP2 have decreased. It seems unlikely, however, that changes in substrate concentration could alone account for this late PI breakdown in VSMC, since PI continues to be hydrolyzed even when polyphosphoinositides are returned toward base line (Fig. 4A) and when hydrolysis of polyphosphoinositides is blocked by exposure of VSMC to PMA. Other possible explanations for delayed PI hydrolysis include the existence of two distinct phospholipase Cs (16) with temporal differences in activation, a shift in phospholipase C activity due to agonist-induced changes in pH (30), or the existence of different domains within the cell selectively enriched in polyphosphoinositides or PI (2) and sequentially activated by the receptor-agonist II complex.

Alternatively, the late onset of PI hydrolysis might be related to the angiotensin II-induced increase in [Ca2+], since PI breakdown by purified phospholipase C is calcium dependent (16). However, in cultured VSMC, an increase in [Ca2+] does not appear to be a sufficient explanation for the late angiotensin II-induced activation of direct PI hydrolysis by phospholipase C. PI hydrolysis was not observed during the time when [Ca2+] was elevated but became detectable only after the calcium levels had returned to base line (2 min, Fig. 5). Furthermore, abolishing putative high membrane calcium flux during the tonic phase of stimulation (5 min) by removing extracellular calcium with EGTA did not attenuate the late PI breakdown. In addition, rapidly increasing cytosolic calcium with ionomycin (15 s) did not increase PI hydrolysis. A 5-min incubation with calcium ionophore causing a sustained increase in [Ca2+], however, did cause accumulation of both DG and PA, although to a lesser extent than did angiotensin II (combined DG plus PA increase: ionomycin, 238%; angiotensin II, 338%). The source of ionophore-stimulated DG is unclear, since ionomycin induced small but not statistically significant changes in each of the major phospholipids. Bocckino et al. (11) have shown by fatty acid analysis that calcium ionophore-induced DG formation in hepatocytes derives from multiple sources, and such may be the case in our study. Despite the ability of calcium to stimulate DG formation, it does not reproduce the late phase of the hormonal response, since the pattern of phosphoinositide metabolism induced by angiotensin II is markedly different from that seen with calcium ionophore alone. Specifically, angiotensin II induced a highly significant 19% breakdown in PI and an 18% breakdown in PIP at 5 min, while ionophore caused an insignificant breakdown in PI and a 70% increase in PIP. Thus, while we cannot exclude a role for calcium in initiating events leading to the angiotensin II-induced hydrolysis of PI, the late phase of the hormonal response does not appear to require a sustained elevation of cytosolic calcium.

The mechanism for the shift in phospholipase C activity away from the polyphosphoinositides is most likely related to generation of DG itself. The ability of phorbol ester to inhibit the early but not the sustained DG formation is suggestive of a role for DG-activated protein kinase C in the selective inhibition of phospholipase C-mediated breakdown of PIP2 and PIP. In support of this hypothesis, we have previously demonstrated that PMA and 1-oleoyl-2-acetylglycerol inhibit angiotensin II-stimulated IP2 and IP3 formation at 15 s in these cells (24).

The observation that phospholipase C directly hydrolyzes all the phosphoinositides in intact VSMC following angiotensin II stimulation has several implications. The existence of multiple sources for DG provides a mechanism for selectively maintaining DG formation, while permitting a transient peak in 1,4,5-IP3 formation. Maintenance of increased DG levels may ensure that those cellular responses which are controlled by protein kinase C can be sustained throughout agonist stimulation. Transient large changes in IP3 resulting from attenuation of early PIP2 hydrolysis suggest a mechanism for the observed transience of the increase in [Ca2+] (21) and of the calcium-calmodulin-dependent phosphorylation of the myosin light chain (31). The small persistent elevation in 1,4,5-IP3 may function to inhibit refilling of intracellular storage sites during tonic agonist stimulation. In support of this hypothesis, we have observed that total cell calcium remains depressed for up to 2 h in the presence of angiotensin II.3 The physiological significance of the residual IP3 remains to be determined.

Although phosphoinositide hydrolysis by phospholipase C would correlate well with the phospholipid changes measured here, there are other possible explanations for the observed decrease in PI and the late increase in DG. If isotopic equilibrium is not reached before the agonist is added, the measured changes can be partially due to changes in specific activity of relevant phospholipid pools. However, equivalent results were obtained for those lipids which were labeled with either [3H]arachidonic acid or [3H]myoinositol (PI, PIP, PIP2), and each isotope labeled these three constituents in the same proportion (94:2:63:34). Therefore, changes in radioactivity most likely reflect actual changes in mass. PI, however, could also be metabolized by phospholipase A2. This enzyme does not appear to be activated by angiotensin II in VSMC, since we observed a small drop, rather than a rise, in lysophosphati-
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dylinositol. There are also other possible sources for DG, particularly other phospholipids (PC, PE, PS) or triglyceride. Although we found no difference in any of these parameters in cells stimulated with angiotensin II for 5 min, we cannot rule out the possibility that small changes, particularly in the phospholipids, might contribute to the observed increase in DG.

In summary, we have shown that angiotensin II-induced DG formation in cultured VSMC is sustained for as long as 20 min after initial exposure to the agonist. Our data strongly suggest a time-dependent variation in the rate of hydrolysis of all three phosphoinositides by phospholipase C. Initially, phospholipase C is that DG formation is not necessarily coupled to IP$_2$ generation, suggesting a possible role for DG in the mediation of the sustained VSMC response to angiotensin II.

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