Correlation of Receptor Sequestration with Sustained Diacylglycerol Accumulation in Angiotensin II-stimulated Cultured Vascular Smooth Muscle Cells*

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Angiotensin II stimulates sequential phospholipase C-mediated hydrolysis of initially the polyphosphoinositides and subsequently phosphatidylinositol 4-phosphate (PIP) in cultured rat aortic smooth muscle cells resulting in biphasic, sustained formation of diacylglycerol (DG). The mechanisms underlying this delayed induction of sustained DG accumulation are unknown but may be related to cellular events including processing of the angiotensin II receptor-ligand complex. In the present study, we characterized the kinetics of angiotensin II receptor sequestration and studied the effects of interventions which interfere with receptor processing on the pattern of angiotensin II-induced DG formation and phosphoinositide hydrolysis. Conversion of the angiotensin II receptor to an acid-resistant form was temperature-dependent, with half-times of 1.5 min at 37 °C and 7 min at 19 °C. Reducing the temperature to 25 or 19 °C caused a marked temporal separation between the two phases of DG accumulation. There was a close temporal correlation between the effect of temperature on receptor sequestration and on sustained DG accumulation. Furthermore, phenylarsine oxide (5 min, 10 μM), which inhibited angiotensin II receptor internalization, also selectively inhibited the sustained phase of DG accumulation (81 ± 6% inhibition). Membrane and chloroquin, which interfere with receptor processing through the lysosomal-degradative pathway, had no effect on angiotensin II-induced DG formation in these cells, suggesting that the processing event important to hormonally induced sustained DG accumulation occurs early in the internalization pathway, probably at the level of the plasma membrane. Moreover, the acid-resistant state of the angiotensin II receptor-ligand complex retained its ability to signal, since removal of the surface signal by competitive antagonism with Sar1-Ile3-angiotensin II or acid-wash only slowly reversed accumulation of DG and depression of total cell calcium. These experiments support our previous observation that the initial and sustained phases of angiotensin II-induced diacylglycerol formation in vascular smooth muscle cells are differentially controlled and suggest that an early event in the cellular processing of the angiotensin II-receptor complex is essential to maintenance of DG accumulation.

Angiotensin II (ang II)1 has been shown to stimulate phosphoinositide metabolism in cultured vascular smooth muscle cells (VSMC) (1-5). Although ang II-stimulated inositol trisphosphate formation is transient (1), diacylglycerol (DG) production is biphasic and sustained (3). We have previously demonstrated that the initial, transient phase of DG formation results from polyphosphoinositide hydrolysis, while the sustained phase of DG accumulation results, at least in part, from delayed phospholipase C-mediated hydrolysis of phosphatidylinositol (3). In VSMC, the two phases of ang II-induced DG formation seem to be differentially controlled (3). Early phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP2) hydrolysis, DG and inositol trisphosphate formation, and calcium mobilization are attenuated by activation of the Ca2+/phospholipid-dependent enzyme protein kinase C with phorbol esters or 1-octadecyl-2-acetylgluceral, suggesting that endogenous DG formation may attenuate these initial biochemical responses (2, 3). However, the second, sustained phase of DG accumulation is not attenuated by phorbol esters, and the mechanisms underlying the delay in hormonal induction of this second phase remain unknown.

In other membrane receptor systems, binding of an agonist to the receptor initiates movement of the receptor-agonist complex first within the plane of the membrane and subsequently into intracellular compartments (6). The half-time of receptor internalization at 37 °C ranges from 2.2 min for the asialoglycoprotein receptor in hepatoma cells (7) to 10 min for ang II in bovine adrenal cortical cells (8). Movement or sequestration of the receptor-complex within the plane of the membrane occurs even more rapidly and is measurable within 30 s for insulin in hepatocytes (9) and within 2 min in ang II-stimulated adrenal glomerulosa cells (10). These events occur well within the time frame observed for induction of sustained DG accumulation in VSMC (3). In vascular smooth muscle, binding of angiotensin has been shown to initiate aggregation and subsequent internalization of the ang II-receptor complex (11), but no systematic studies of the kinetics

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1 The abbreviations used are: ang II, angiotensin II; VSMC, vascular smooth muscle cells; DG, diacylglycerol; PIP, phosphatidylinositol; PIP2, phosphatidylinositol 4,5-bisphosphate; HEPPS, 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid; PAO, phenylarsine oxide; DTT, dithiothreitol.
Phospholipids were performed as described previously with a warm, balanced salt solution was terminated by rapid aspiration of the buffer and addition of temperature. Cultures were then maximally stimulated with the angiotensin II receptor-ligand complex is essential to initiating accumulation. Thus, there appears to be a close correlation with this process also preferentially inhibit sustained DG accumulation. Therefore, the angiotensin II receptor is rapidly converted to a seques-
tered, acid-resistant form, and that agents which interfere with receptor processing on the of angiotensin II-receptor internalization and studied the effects of interventions which interfere with receptor processing on the pattern of ang II-induced DG formation and PI and polyphosphoinositide hydrolysis in VSMC. Our data indicate that the angiotensin II receptor is rapidly converted to a seques-
tered, acid-resistant form, and that agents which interfere with this process also preferentially inhibit sustained DG accumulation. Thus, there appears to be a close correlation between receptor sequestration and DG accumulation in ang II-stimulated VSMC, suggesting that cellular processing of the ang II receptor-ligand complex is essential to initiating and sustaining accumulation of DG, and therefore possibly to tonic signal generation.

EXPERIMENTAL PROCEDURES

**Cell Culture**—VSMC were isolated from rat thoracic aorta by enzymatic dissociation as described previously (12, 13). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, glutamine, and antibiotics and were passed twice a week by harvesting with trypsin-verseae and seeding at a 1:4 ratio in 50-cm² flasks. For experiments, cells between passage levels 4–5 were seeded into 22–35– or 100-mm dishes (2 × 10⁵ cells/cm²), fed every other day, and used at confluence (2–6 days).

**Phospholipid Labeling and Extraction**—Labeling, extraction, and separation of the phospholipids, neutral lipids, and inositol phosphates were performed as described previously (3). Briefly, VSMC cultures were incubated with either [1°H]myoinositol (15 Ci/mmol) for 24 h or [3°H]arachidonic acid (1 μCi/ml) for 3 h. Cells were washed with a warm, balanced salt solution (130 mM NaCl, 5 mM KCl, 1.1 mM MgCl₂, 1.5 mM CaCl₂, 20 mM HEPES, buffered to pH 7.4 with Tris base) and incubated for 20 min in this same solution at the indicated temperature. Cultures were then maximally stimulated with 100 nM ang II for various times. In acid-wash experiments, exposure to ang II was followed by a 10-min incubation at 4°C with 0.05 M acetic acid in 150 mM NaCl (pH = 3.0), thorough washing, and transferring to 37°C for 5 min in balanced salt solution. In all cases the reaction was terminated by aspirating the buffer and addition of 1 ml of chloroform/methanol/HCl (1:2:0.05) for phospholipid extraction or chloroform/methanol (1:2) for neutral lipid extraction. Organic and aqueous phases were separated by addition of chloroform and distilled water, centrifugation, and two chloroform washes. The organic phase was immediately concentrated under nitrogen, and lipids were resolved by thin layer chromatography (3, 14). The aqueous phase was also concentrated under nitrogen, and the inositol phosphates were resolved by the column chromatography method of Downes and Michell (15) as described previously (1). All lipids and inositol phosphates were quantitated by liquid scintillation spectro-

**Measurement of 1H-Angiotensin II Binding and Internalization**—Measurement of surface-bound and internalized 1H-Ang II was performed on replicate-plated 22-mm cultures of attached cells. For experiments utilizing phenylarsine oxide (PAO), cells were preincubated with 0.1–100 μM PAO prior to addition of 1H-Ang II. To determine internalization, samples were incubated for 90–120 min at 4°C with 0.5 nM 1H-Ang II (2200 Ci/mmol) in a binding buffer consisting of 50 mM Tris, 5 mM MgCl₂, 100 mM NaCl, pH 7.4. Unbound activity was removed by washing the cells 4 times with ice-cold binding buffer containing 0.25% bovine serum albumin. Cells were then rewarmed for various times at the indicated temperatures, rapidly cooled, washed four times with ice-cold buffer, and exposed to 0.05 M acetic acid in 150 NaCl for 10 min at 4°C. This wash, plus two 0.1–0.2 M HCl washes, removed surface-bound 1H-Ang II activity. In some experiments, the incubation medium was collected and analyzed for radioactivity. The cells were then solubilized with 1% sodium dodecyl sulfate, 0.03% NaOH to determine cell-associated, or internalized, radioactivity. Nonspecific binding of 1H-Ang II was determined at each time point and was defined as that not displaced by 1 μM unlabeled ang II. Such binding was routinely less than 5–10% of the total binding.

For binding experiments where surface and sequestered binding were not differentiated, measurement of bound 1H-Ang II was performed on control cell suspensions. Cells, suspensions of cultured VSMC (final concentration 10⁶ cells/ml) were prepared from replicate-plated 100-mm culture dishes using collagenase, soybean trypsin inhibitor, and BSA (13) and incubated with increasing concentrations of 1H-Ang II for 35 min at 25°C. The reaction was terminated with cold buffer, and samples were filtered over a Whatman GF/C glass fiber filter and counted in a gamma

**Degradation of 1H-Angiotensin II**—Degradation of 1H-Ang II was assayed by thin layer chromatography as described previously (12). Aliquots (50 μl) of surface-associated, medium or cell-associated radioactivity extracted as described above were spotted on thin layer cellulose plates (Eastman 12555), using authentic 1H-Ang II as a standard. Plates were developed with tert-butyl alcohol/3% NH₄ (105:35) as solvent. Developed chromatograms were cut into 0.75-cm strips and counted in a gamma counter.

**Measurement of 4Ca²⁺ Content**—Cells cultures (35 mm) were equilibrated for 24 h at 37°C in 2 ml of fresh culture medium containing 4CaCl₂ (4 mM). On the day of the experiment, ang II (10 nm) was added directly to the culture medium. After 5 min, Sar⁻¹Ile⁻ang II (100 nm) was added to some dishes. The reaction was terminated after various intervals by harvesting the cells at times with ice-cold calcium-free balanced salt solution containing 10 mM LaCl₃. Radioactivity was extracted with 1 ml of 0.1 N HNO₃. Cell 4Ca²⁺ content is expressed as nanomoles per milligram of protein and was calculated from the specific activity of 4Ca²⁺ in the medium. Protein was determined by the method of Lowry et al. (16).

**Materials**—The supplies and vendors used in this study were as follows: [1°H]myoinositol (17.1 Ci/mmol), [3°H]arachidonic acid (83.6 Ci/mmol), [1H]-angiotensin II (2200 Ci/mmol), Du Pont-New England Nuclear, [1H]-angiotensin II (2200 Ci/mmol), Me-

**RESULTS**

**Ang II Receptor Binding and Temperature Dependence of Internalization**—We have previously demonstrated that ang II binds to a single class of high affinity binding sites in rat aortic VSMC (2). The binding is temperature-dependent and is saturable at 5°C in 60 min (17). Using the acid-wash technique described above, we found that after incubation of VSMC cultures with 1H-Ang at 4°C for 90–120 min, 97.0 ± 0.7% of specifically bound radioactivity was associated with the cell surface (acid-releasable). Rewarming the cells resulted in a rapid, temperature-dependent sequestration of the receptor-agonist complex to an acid-resistant form (Fig. 1), with half-times of 1.5 min at 37°C, 2.8 min at 25°C, and 7 min at 19°C. The ang II remaining on the cell surface comigrated with authentic ang II following thin layer chromatography, indicating an absence of degradation.

**Effect of Temperature on Diglyceride Formation and Phosphoinositide Hydrolysis**—To provide insight as to whether receptor sequestration is important in ang II-induced DG formation in VSMC, we studied DG accumulation at three temperatures (37, 25, and 19°C) which exhibited marked differences in rates of receptor internalization (Fig. 1), at 30°C, and at 4°C where internalization did not occur. As we have previously demonstrated, DG accumulation at 37°C is biphasic and sustained (3) (Fig. 2). Reducing the temperature to 30°C did not alter the time course or amplitude of ang II-induced DG production (data not shown). A further decrease in temperature to 25°C caused a marked temporal separation between the early phase of DG formation and development of the second peak of DG accumulation. Reducing the tempera-

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phosphatidycholine in VSMC which is detectable after about 1 min and completely abolished ang II-induced PI breakdown and inositol monophosphate formation (data not shown). However, phospholipids (18), of which PI is a major component of arachidonic acid (1 pCi/ml) for 5 h, washed, and incubated at 37 °C, 25 °C ( ), 19 °C (△), or 4 °C (□) for 20 min. Cells were then exposed to angiotensin II (100 nM) for various periods. Temperature had no effect on basal DG levels (average control, 2191 ± 111 cpm). Each point represents the mean ± S.E. of duplicate determinations from at least three experiments.

FIG. 2. Effect of temperature on angiotensin II-induced diacylglyceride formation. Cultured VSMC were prelabeled with [3H]arachidonic acid (1 μCi/ml) for 5 h, washed, and incubated at 37 °C ( ), 25 °C ( ), 19 °C (△), or 4 °C (□) for 20 min. Cells were then exposed to angiotensin II (100 nM) for various periods. Temperature had no effect on basal DG levels (average control, 2191 ± 111 cpm). Each point represents the mean ± S.E. of duplicate determinations from at least three experiments.

ture to 19 °C delayed the early peak of DG formation from 15 s to 1 min and shifted formation of the second DG peak to 20–30 min (Fig. 2). At 4 °C, DG accumulation was monophasic, peaking at 10 min (Fig. 2) and returning to base line by 80 min (data not shown).

The second or delayed phase of accumulation of DG most likely results from PLC-mediated hydrolysis of several phospholipids (18), of which PI is a major component (3). Preliminary evidence indicates that ang II also induces hydrolysis of phosphatidycholine in VSMC which is detectable after about 1 min. Thus, the second phase of DG accumulation is probably the result of hydrolysis of a combination of phospholipids. However, since we have previously demonstrated that phosphoinositide hydrolysis makes a major contribution to ang II-induced DG formation in VSMC (3), we particularly examined the effect of altering temperature on inositol phospholipid hydrolysis and inositol phosphate formation following ang II stimulation. Reducing temperature to 4 °C completely abolished ang II-induced PI breakdown and inositol monophosphate formation (data not shown). However, hydrolysis of the polyphosphoinositides and formation of inositol bisphosphate and inositol trisphosphate at this low temperature followed a time course identical to that seen for DG, suggesting that the DG formed at 4 °C resulted exclusively from PIP2 and PIP breakdown. Decreasing temperature to 19 or 25 °C slightly reduced and slowed breakdown of PIP2 (Fig. 3B) and PIP (data not shown), but polyphosphoinositide levels still rapidly began to return toward base line, especially at 25 °C. PI hydrolysis was significantly inhibited and delayed at both 19 and 25 °C (Fig. 3A). The PI levels measured here represent a combination of direct hydrolysis by PLC (3), phosphorylation by PI kinase to replenish the polyphosphoinositides (19), and reformation of PI from myoinositol and CDP-DG (19), all of which may have a temperature dependence. Thus, although the decrease in PI cannot be quantitatively related to increased DG formation at temperatures below 37 °C, it is likely significant that temperatures which inhibit DG accumulation also inhibit PI hydrolysis (Figs. 2 and 3A). Furthermore, formation of inositol monophosphate, the other product of PLC-mediated breakdown of PI, is also attenuated at 19 and 25 °C (data not shown).

The fact that early DG formation and phospholipid hydrolysis remain relatively intact during drastic reductions in temperature suggests that the major effect of lowering temperature on the late response is not to decrease the activity of PLC. Rather, the differential effect of temperature on the late DG response correlates well temporally with the effect of temperature on receptor sequestration (Fig. 4). At all temperatures, receptor sequestration precedes or occurs simultaneously with delayed DG formation, and peak DG accumulation occurs only after about 70% of the receptors have become resistant to acid wash. Although the relationship between receptor internalization and sustained DG accumulation could not clearly be analyzed at 37 °C because of overlap between the two phases of DG formation, the clear separation between the early and late peaks of DG accumulation at 25 and 19 °C permitted regression analysis of this relationship. The corre-

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exposed to angiotensin II. That remaining associated with the cell following acid wash.

Internalization and inhibition of ang II-induced phos- phoinositide metabolism. At 100 nM, PAO was more effectively inhibited by PAO than by the same duration of exposure to PAO. For measurements of internalization, cells were washed, exposed to PAO (0.1-100 μM) for 5 min, and stimulated with ang II (100 nM) for 5 min in the continued presence of PAO. For measurements of internalization, cells were exposed to PAO at 37 °C for 5 min prior to 125I-ang II labeling, and then warmed to 37 °C for 5 min in the presence of PAO after 125I-ang II labeling. Results are expressed as a percentage of the ang II-induced DG or internalization response in the absence of PAO. Each point corresponds to a different dose of PAO.

However, DG formation is more sensitive to inhibition by PAO than is internalization. To determine whether the ability of PAO to inhibit sustained DG accumulation is specifically related to its ability to inhibit receptor sequestration, we incubated VSMC with ang II (100 nM) for 5 min at 37 °C to allow internalization to occur, and then exposed cells to PAO (10 μM) in the continued presence of ang II. As shown in Fig. 7, preincubating cells with PAO resulted in an 81 ± 8% inhibition of subsequent ang II-induced DG accumulation (Fig. 7A), but the same duration of exposure to PAO inhibited DG formation by only 29 ± 3% after the receptor was internalized (Fig. 7B). Thus, the 35% rightward shift of the relationship between PAO inhibition of internalization and DG formation coefficients at 25 and 19 °C were 0.95 and 0.84, respectively, indicating a linear relation between receptor internalization and sustained DG accumulation at both temperatures.

Effect of Phenylarsine Oxide on Angiotensin II Receptor Internalization and DG Formation—PAO has been shown to inhibit internalization of a variety of receptors including β-adrenergic, epidermal growth factor, and insulin receptors (20, 21). Sequestration of the ang II receptor in VSMC was also effectively inhibited by PAO in a dose-dependent manner. The threshold for this inhibition was about 1 μM PAO, the IC50 was about 10 μM PAO, and maximal inhibition occurred at 100 μM PAO.

To test further the correlation between receptor sequestration and DG accumulation, we preincubated cells with PAO to block internalization and measured ang II-induced phosphoinositide metabolism. PAO selectively inhibited the late (5 min) sustained phase of ang II-stimulated DG formation in VSMC (Fig. 5). Inhibition was dose-dependent with a threshold of about 0.1 μM PAO, and a half-maximal effect occurring at about 6 μM PAO (data not shown). The correlation between PAO inhibition of receptor sequestration and inhibition of sustained DG formation is linear (Fig. 6).
accretion depicted in Fig. 6 most likely reflects a nonspecific effect of PA0 on DG accumulation.

PA0 has also been shown to have other effects on cell function, such as reducing binding to certain receptors (20). However, in VSMC, PA0 had no effect on either ang II receptor number or affinity as determined by Scatchard analysis (data not shown). Nonspecific and specific effects of PA0 with respect to internalization can be experimentally separated since the former can be abolished by thorough washing, while the specific effects of PA0 on internalization can be reversed using bifunctional sulfhydryl compounds such as dithiothreitol (DTT) (20). In ang II-stimulated VSMC, the inhibitory effect of PA0 on DG was not reversed by extensive washing and removal of PA0. DTT (1 mM, 5 min), however, partially reversed the PA0 inhibition of DG accumulation (PA0: 86% inhibition; PA0 + DTT: 36% inhibition). This reversal was likely incomplete because DTT (5 mM) alone causes a 45% decrease in 125I-ang II binding to VSMC (19). These observations suggest that the major effect of PA0 on sustained DG accumulation is specifically related to its ability to inhibit receptor internalization. Because all concentrations of PA0 tested had some effect on basal levels of DG, 10 µM PA0 was routinely used for phospholipid experiments to insure near-maximal inhibition of DG formation with minimal base-line changes. A 5-min incubation with PA0 (10 µM) caused a 24 ± 5% increase in basal DG and decreased base-line PIP to 74 ± 9% control. Other basal phospholipid levels were not significantly altered. As expected from its effects on ang II-stimulated DG accumulation, PA0 selectively inhibited late, but not early, changes in phospholipid metabolism (Table I). Ang II-induced PI breakdown (5 min) was significantly inhibited, as was phosphatidic acid formation, suggesting that the reduction in DG accumulation by PA0 occurred by inhibition of PLC-mediated PI breakdown, rather than by acceleration of DG phosphorylation.

**Figure 7.** Nonspecific effect of PA0 on DG accumulation. Cultured VSMC were prelabeled with [3H]arachidonic acid (1 µCi/ml) for 3 h and then either preincubated with PA0 (10 µM, 5 min) and exposed to ang II (100 nM) for 5 min (panel A) or exposed to ang II (100 nM, 5 min) and incubated with PA0 (10 µM) in the continued presence of ang II for an additional 5 min (panel B). Each bar represents the mean ± S.E. of duplicate determinations from at least three experiments.

### Table I

**Effect of PA0 on ang II-induced phospholipid metabolism**

Cultured VSMC were labeled with [3H]arachidonic acid (1 µCi/ml) for 3 h or [3H]myoinositol (15 µCi/ml) for 24 h, and were then either exposed to ang II (100 nM) alone or preincubated with PA0 (10 µM, 5 min) and exposed to ang II in the continued presence of PA0. Values are expressed as the mean ± S.E. of duplicate determinations from at least four experiments. Each condition was calculated as a percentage of its own control. PA, phosphatidic acid.

| Lipid | Ang II (15 s) | Ang II (5 min) |
|-------|--------------|----------------|
| % control | −PA0 ±PA0 | −PA0 ±PA0 |
| PA | 166 ± 24 | 138 ± 20 | 451 ± 63 | 189 ± 20* |
| PI | 94 ± 2 | 88 ± 3 | 81 ± 2 | 90 ± 3* |
| PIP | 67 ± 5 | 72 ± 7 | 95 ± 7 | 73 ± 4* |
| PIP₂ | 55 ± 7 | 60 ± 3 | 76 ± 6 | 85 ± 16 |

* Indicates significant difference (p < 0.05) in the presence of PAO.
Ile<sup>8</sup>-ang II, a potent ang II antagonist, to compete off surface-bound ang II, and measured the temporal decline in DG levels and increase in total cell calcium content (Fig. 9). Addition of Sar<sup>1</sup>-Ile<sup>8</sup>-ang II (10 μM) prior to ang II (100 nM) completely abolished subsequent DG formation (data not shown). Addition of Sar<sup>1</sup>-Ile<sup>8</sup>-ang II to VSMC after a 5-min incubation with ang II (100 nM) only slowly reversed the accumulation of DG, requiring at least 15 min for DG levels to return to baseline (Fig. 9, top). In both acid-wash and antagonist experiments, following removal of the surface signal, there is significant residual DG long after the time necessary for metabolism of previously formed DG (see time course of early DG peak, Fig. 2) Another manifestation of the cell response to ang II, a decrease in 45Ca<sup>2+</sup> content, which results from initial release of intracellular calcium and stimulation of 45Ca<sup>2+</sup> efflux, is depressed for up to 2 h in the continued presence of the hormone (Fig. 9, bottom). This persistent decrease in 45Ca<sup>2+</sup> content reflects an inability to refill intracellular calcium stores in the presence of ang II. Upon addition of antagonist, there is a lag time of 15 min in the return of 45Ca<sup>2+</sup> levels to baseline (Fig. 9, bottom). The gradual reversal of changes in DG and calcium reflect a gradual decrease in signal generation by a receptor that, by virtue of its sequestered state, is only slowly accessible to the antagonist.

**Discussion**

The results of the present study confirm and extend our previous observations which suggested that the two phases of DG formation measured after stimulation of VSMC with ang II are biochemically distinct, with different sources and mechanisms of control (3). The two phases can be clearly separated by step-wise reduction in temperature. Furthermore, PAO markedly inhibits sustained DG accumulation while only minimally affecting the early DG peak. The effects of these two interventions on the sustained phase of DG accumulation correlate closely with their effects on conversion of the ang II receptor-ligand complex to an acid-resistant form, suggesting that cellular processing of the ang II receptor is essential to sustained DG accumulation.

There have been relatively few studies concerning ang II receptor internalization. A recent report has described movement of the ang II receptor in adrenal glomerulosa cells from the cell surface to the lysosome within 20 min of [35S]-ang II binding (10). By 5 min, the majority of bound ang II was located in coated pits and coated vesicles (10). In our cells, the half-time for development of an acid-resistant form of the agonist-receptor complex at 37°C was 1.5 min, suggesting that development of acid resistance occurs during movement of the receptor on the cell surface but prior to removal of the receptor-agonist complex from the plasma membrane.

The close correlation between the rate of development of acid resistance of the ang II receptor-ligand complex and the development of the late phase of DG accumulation at various temperatures suggests that the two phenomena are related. This concept embraces two possibilities: either DG accumulation plays a role in the initiation of internalization or receptor sequestration/movement is essential to the development of sustained DG accumulation. In other systems, phorbol ester has been shown to stimulate receptor internalization (23). In VSMC, however, 4β-phorbol 12-myristate 13-acetate (100 nM), an exogenous activator of protein kinase C, had no effect on internalization at 37°C. This suggests that DG does not initiate internalization via a protein kinase C-dependent mechanism, and makes the second possibility considered above more likely.

The actual relationship of receptor sequestration to development of sustained DG formation encompasses several possible mechanisms: different signaling domains on the cell
surface, movement of the receptor to an intracellular compartment from which signal generation then occurs, or movement of the receptor or substrate to the cell surface following internalization, resulting in secondary signaling. Signaling from an intracellular compartment has been suggested by the work of Richards et al. (24), who demonstrated direct breakdown of PI by PLC and accumulation of DG in rat liver lysosomes. Additionally, based on his observation that 80% of acetylcholine-stimulated 32P incorporation into PI in pigeon pancreas slices occurs in the endoplasmic reticulum, Hokin (25) has suggested that the agonist-receptor complex may move through various endocytic vesicles to a PI-rich source, or alternatively, initiate transfer of PI from endoplasmic reticulum to the plasma membrane. However, in VSMC, the inability of chloroquin and monensin, two well-characterized lysosomotropic amines, to inhibit DG formation suggests that the lysosomal degradative pathway has no role in the coupling of receptor activation to the phosphoinositide response. Furthermore, there appears to be very little retroendocytosis in VSMC stimulated with angiotensin II (Fig. 8). Thus, in VSMC, the receptor-processing event integral to sustained DG formation does not appear to be related to either the lysosomal degradative or the retroendocytotic pathway.

Although reducing temperature has effects on cell function other than receptor processing, our experiments with low temperature provide evidence suggestive of distinct signaling domains on the cell surface. One important effect of reducing temperature on cell metabolism is to decrease enzyme activity. However, this does not appear to be the explanation for alteration in sustained DG formation. PLC activity, as evidenced by the early DG response at the various temperatures (19–37°C, Fig. 2), remains intact. The apparent enhancement of the early DG peak at 19°C may be related to removal of the inhibitory effect of protein kinase C stimulation normally converted to a sequestered acid-resistant form, and that dephosphorylation has shown that PAO (100 μM) completely abolishes the ang II-induced fusion of different vesicular fractions of VSMC and prevents the appearance of 125I-ang II in the light vesicular fraction. This suggests that PAO exerts its effect at the level of the plasma membrane. Moreover, in related studies, we have shown that potassium depletion selectively inhibits sustained DG accumulation and concomitantly inhibits receptor internalization (28). The effects of PAO and potassium depletion on DG accumulation are strikingly similar. In human fibroblasts, potassium depletion has been shown to decrease endocytosis of receptor-bound low density lipoprotein (29), and in particular, to decrease coated-pit formation (29) and assembly of the clathrin lattice (30). These observations suggest the intriguing possibility that the coated pit plays an important role in DG formation. Campbell et al. (31) have demonstrated that coated vesicles isolated from bovine brain are enriched in active PI kinase and concluded that coated vesicles may be involved in cellular PI metabolism. Our data suggest the additional possibility that the coated pit/vesicle, or some other distinct domain on the cell surface, may be important in hormonal signaling.

In summary, we have provided evidence that upon ang II stimulation of VSMC, the angiotensin II receptor is rapidly converted to a sequestered acid-resistant form, and that development of this state is essential to sustained accumulation of DG. Low temperature and phenylarsine oxide, both of which inhibit ang II receptor internalization, preferentially interfere with the late phase of DG accumulation, while having little effect on the initial transient phase of DG formation from polyphosphoinositide breakdown. The processing event important to hormonally induced sustained DG accumulation appears to occur very early in the internalization pathway, almost certainly at the level of the plasma membrane.

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REFERENCES

1. Alexander, R. W., Brock, T. A., Gimbrone, M. A., Jr., and Rittenhouse, S. E. (1985) Hypertension 7, 447–451
2. Brock, T. A., Rittenhouse, S. E., Powers, C. W., Ekstein, L. S., Gimbrone, M. A., Jr., and Alexander, R. W. (1985) J. Biol. Chem. 260, 14158–14162
3. Griendling, K. K., Rittenhouse, S. E., Brock, T. A., Ekstein, L. S., Gimbrone, M. A., Jr., and Alexander, R. W. (1986) J. Biol. Chem. 261, 5901–5906
4. Nahita, T., Velletri, P. A., Lovenberg, W., and, Beaver, M. A. (1985) J. Biol. Chem. 260, 4661–4670
5. Smith, J. B., Smith, L., Brown, E. R., Barnes, D., Sabir, M. A., Davis, J. S., and Farese, R. V. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 7812–7816
6. Steinman, R. M., Mellman, I. S., Muller, W. A., and Cohen, Z. A. (1983) J. Cell Biol. 96, 1–27
7. Schwartz, A. L., Fridovitch, E., and Lodish, H. F. (1982) J. Biol. Chem. 257, 4230–4237
8. Croazat, A., Penhoat, A., and Saez, J. M. (1986) Endocrinology 118, 2312–2318
9. Pilch, P. F., Shin, M. A., Benson, R. J. J., and Fine, R. E. (1983) J. Cell Biol. 93, 133–138
10. Bianchi, C., Gutkowska, J., Delean, A., Ballak, M., Anand-Srivastava, M. B., Genest, J., and Cantin, M. (1986) Endocrinology 118, 2605–2607
11. Peach, M. J. (1981) Biochem. Pharmacol. 30, 2745–2751

* K. K. Griendling, F. Bicknell, and R. W. Alexander, unpublished observations.
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12. Gunther, S., Alexander, R. W., Atkinson, W. J., and Gimbrone, M. A., Jr. (1982) J. Cell Biol. 92, 289-298
13. Brock, T. A., Alexander, R. W., Ekstein, L. S., Atkinson, W. J., and Gimbrone, M. A., Jr. (1985) Hypertension 7 (Suppl I), 105-109
14. Rittenhouse, S. E. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 5417-5420
15. Downes, C. P., and Michell, R. H. (1981) Biochem. J. 188, 133-140
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
17. Gunther, S., Gimbrone, M. A., Jr., and Alexander, R. W. (1980) Circ. Res. 47, 278-286
18. Bocckino, S. B., Blackmore, P. F., and Exton, J. H. (1985) J. Biol. Chem. 260, 14201-14207
19. Berridge, M. J. (1984) Biochem. J. 220, 345-360
20. Hertel, C., Coulter, S. J., and Perkins, J. P. (1985) J. Biol. Chem. 260, 12547-12553
21. Knutson, V. P., Ronnett, G. V., and Lane, M. D. (1983) J. Biol. Chem. 258, 12139-12142
22. Marshall, S. (1985) J. Biol. Chem. 260, 13524-13531
23. Iacopetta, B., Carpenter, J., Pozz, T., Lew, D. P., Gordon, P., and Orci, L. (1986) J. Cell Biol. 103, 851-866
24. Richards, D. E., Irvine, R. F., and Dawson, R. M. C. (1979) Biochem. J. 182, 599-606
25. Hokin, L. E. (1985) Annu. Rev. Biochem. 54, 205-235
26. Dunn, W. A., Hubbard, A. L., and Aronson, N. N., Jr. (1980) J. Biol. Chem. 255, 5971-5978
27. He, N. B., and Hui, S. W. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 7304-7308
28. Delafontaine, P., Griendling, K. K., Gimbrone, M. A., Jr., and Alexander, R. W. (1987) J. Biol. Chem. 262, 14549-14554
29. Larkin, J. M., Brown, M. S., Goldstein, J. L., and Anderson, R. G. W. (1983) Cell 33, 273-285
30. Larkin, J. M., Donzell, W. C., and Anderson, R. G. W. (1986) J. Cell Biol. 103, 2619-2627
31. Campbell, C. R., Fishman, J. B., and Fine, R. E. (1985) J. Biol. Chem. 260, 10948-10951