Angiotensin II Stimulation of Vascular Smooth Muscle Phosphoinositide Metabolism
State of the Art Lecture

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SUMMARY Phosphoinositide hydrolysis is an integral step in the activation of vascular smooth muscle by angiotensin II. Sequential phospholipase C-mediated hydrolysis of the polyphosphoinositides and phosphatidylinositol in cultured vascular smooth muscle cells stimulated with angiotensin II results in a coordinated series of biochemical events: a transient formation of inositol trisphosphate associated with calcium mobilization, and a biphasic, sustained formation of diacylglycerol associated with activation of protein kinase C and cytosolic alkalinization. The initial, rapid phase and the sustained phase of the angiotensin II response appear to be differentially controlled. Formation of inositol trisphosphate and mobilization of calcium are attenuated by activation of protein kinase C. Sustained diacylglycerol formation is promoted by cytosolic alkalinization, and appears to require cellular processing of the angiotensin II-receptor complex. Calcium and cyclic guanosine 3',5'-monophosphate do not appear to regulate phospholipase C-mediated phosphoinositide hydrolysis in vascular smooth muscle. Thus, regulation of angiotensin II–stimulated second messenger generation in vascular smooth muscle is complex, perhaps involving protein kinase C activation, changes in intracellular pH, and processing of the angiotensin II–receptor complex.

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KEY WORDS • diacylglycerol • inositol trisphosphate • sodium-hydrogen exchange

Calcium-mobilizing hormones have been shown to stimulate phosphoinositide metabolism in a variety of cell types. This agonist-induced, phospholipase C (PLC)–mediated hydrolysis of the phosphoinositides results in the generation of two putative second messengers, inositol trisphosphate (IP₃) and diacylglycerol (DG). Each of these molecules serves a different function within the cell: IP₃ mediates calcium mobilization from the endoplasmic reticulum, and DG activates the calcium- and phospholipid-sensitive enzyme, protein kinase C.

In cultured vascular smooth muscle cells (VSMCs) stimulated with angiotensin II (ANG II), formation of IP₃ and DG have been clearly demonstrated. The mechanisms controlling generation of these second messengers have only been partially elucidated, and their separate and synergistic biochemical and physiological consequences are not fully understood. We review here recent data on the role of phosphoinositide metabolism in ANG II activation of vascular smooth muscle, and discuss the various possible mechanisms controlling the agonist-mediated phosphoinositide response in VSMCs.

Phosphoinositides and ANG II Activation of Vascular Smooth Muscle

ANG II causes a rapid contraction of rat aorta that attenuates significantly after several minutes even in the continued presence of agonist (Figure 1A). Attenuation of the contractile response is temporarily associated with a fall in phosphatidic acid, a metabolite of DG, suggesting that sustained contraction might be dependent upon continued formation of DG. To facilitate the study of cellular and biochemical events associated with ANG II–mediated activation of vascular smooth muscle, we have performed experiments using
a cultured rat aortic VSMC system.\textsuperscript{1,6,11} In this cultured VSMC system, ANG II stimulates a rapid increase in [Ca\textsuperscript{2+}] and IP\textsubscript{3} formation (Figure 1C),\textsuperscript{6} which temporally correlates with initiation of contraction in rat aorta (see Figure 1A).\textsuperscript{6} Furthermore, ANG II–stimulated production of DG is biphasic and sustained (see Figure 1C).\textsuperscript{6} We have utilized protein kinase C–mediated activation of Na\textsuperscript{+}-H\textsuperscript{+} exchange\textsuperscript{22} in cultured VSMC as a physiological correlate of sustained DG production. As shown in Figure 1D, ANG II stimulates intracellular alkalinization of cultured VSMCs that temporally correlates with sustained DG formation (see Figure 1C).

On the basis of observations about ANG II–stimulated secretion of aldosterone in adrenal glomerulosa cells, Rasmussen and Barrett\textsuperscript{12} have proposed a general model in which IP\textsubscript{3} formation and Ca\textsuperscript{2+} release are responsible for the initial, phasic cellular response, and DG formation and presumably protein kinase C activation contribute to the tonic, sustained response. Observations on the stimulation of phosphoinositide breakdown in cultured VSMCs are consistent with this general model as outlined above (see Figure 1 A–D).

The observation that formation of IP\textsubscript{3} is transient, while production of DG is biphasic and sustained, suggests not only that each messenger has a different physiological function but also that formation of each is differentially controlled. IP\textsubscript{3} can only be formed from PLC–mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}), while DG can be formed from PLC–mediated hydrolysis of PIP\textsubscript{2}, phosphatidylinositol 4-phosphate (PIP\textsubscript{3}), phosphatidylinositol (PI), or other membrane phospholipids. In VSMCs stimulated with ANG II, the biphasic formation of DG (see Figure 1B) appears to result from sequential hydrolysis initially of PIP\textsubscript{3} and PIP and subsequently of PI.\textsuperscript{6} Significant hydrolysis of other phospholipids to form DG does not seem to occur in these cells.\textsuperscript{6} Thus, when VSMCs are exposed to ANG II, there is a rapid (peak, 15 seconds) initial hydrolysis of PIP\textsubscript{3} and PIP that is quickly attenuated,\textsuperscript{5} accounting for the transient formation of IP\textsubscript{3} and the early phase of DG formation. Continued stimulation with ANG II (> 2 minutes) results in a direct hydrolysis of PI to form DG (peak, 5 minutes),\textsuperscript{5} which is sustained for the duration of the stimulus (up to at least 30 minutes). Hormonally induced direct hydrolysis of PI has also been demonstrated in other cell types.\textsuperscript{14,15} The mechanisms responsible for the attenuation of the early response and the delay in hormonal induction of PI hydrolysis are areas of active investigation. The two phases of DG formation and their biochemical and physiological correlates can be conveniently studied by utilizing interventions that selectively affect one phase or the other. Some of the most likely candidates for control mechanisms are reviewed below.

**Potential Mechanisms Controlling Phosphoinositide Hydrolysis Sequence**

**Protein Kinase C**

In a number of cell types, phorbol esters, which are potent activators of protein kinase C, have been shown to inhibit polyphosphoinositide hydrolysis,\textsuperscript{16–18} suggesting a possible role for protein kinase C in attenuation of the early phosphoinositide response in VSMCs. In ANG II–stimulated VSMCs, 4\beta-phorbol 12-myristate 13-acetate (PMA) inhibits early (15 seconds) IP\textsubscript{3} formation and PIP\textsubscript{3} hydrolysis\textsuperscript{18} but has no effect on late (5 minutes) DG formation and PI hydrolysis.\textsuperscript{6} The ANG II–induced increase in [Ca\textsuperscript{2+}] is also inhibited by PMA.\textsuperscript{18} This suggests that the DG produced from rapid breakdown of PIP\textsubscript{3}, probably through activation of protein kinase C, feeds back to attenuate further hydrolysis of PIP\textsubscript{3}, in effect terminating the early calcium-mobilizing IP\textsubscript{3} signal. DG forma-
tion then would continue, with PI as the source. Activation of protein kinase C may selectively inhibit the early events in ANG II stimulation of VSMCs, and therefore serve as a control mechanism to modulate the early phosphoinositide response.

**Calcium**

Since the increase in [Ca$^{2+}$], is one of the earliest events resulting from activation of many cells by non-adenylylate cyclase–coupled hormones, it has been suggested that calcium itself may serve to regulate the course of phosphoinositide metabolism. In other systems, several forms of PLC have been isolated with different pH and calcium concentration optima for enzyme activity. Furthermore, in isolated unilamellar vesicles, PI hydrolysis by purified PLC has been shown to be calcium-dependent. These observations suggest that the increase in [Ca$^{2+}$] resulting from ANG II stimulation of IP, formation may serve to shift dominant PLC activity from the polyphosphoinositides to PI. However, this does not appear to be the case in VSMCs, since the calcium ionophore ionomycin (15 μM) does not cause breakdown of PI. Thus, in VSMCs, calcium alone cannot mimic the hormonal response and therefore is not likely to be responsible for induction of the delayed phosphatidylinositol hydrolysis. It remains possible, however, that calcium modulates the activity of other enzymes involved in phosphoinositide metabolism.

**Intracellular pH**

The intracellular concentration of hydrogen ion (pH) has also been postulated to be important to the function of enzymes involved in the phosphoinositide pathway. Activity of purified PLC has recently been shown to have a strong pH dependency. Nakinishi et al. have isolated two forms of PLC with different activities at alkaline or acidic pH, depending upon calcium concentration. This putative control mechanism may be particularly relevant in VSMCs, since ANG II alters pH, in VSMC grown on cover slips. Starting from a resting pH, of 7.15 to 7.35, ANG II increases rapidly within the cell in response to ANG II, that the majority of acetylcholine-stimulated 32 P-ATP incorporation into PI in pigeon pancreatic slices occurs in the endoplasmic reticulum, suggesting that the surface signal must somehow be translocated for PI hydrolysis to occur. There is some evidence to support this hypothesis in VSMCs. Two interventions that interfere with receptor processing, low temperature and phenylarsine oxide, also disrupt development of susceptible PLC activity from the polyphosphoinositides to PI hydrolisis. There is some evidence to support the view that the temporal delay in the onset of PLC-mediated PI hydrolysis appears to inhibit PLC-mediated PI/Pi hydrolysis.

**Cyclic GMP**

Cyclic guanosine 3',5'-monophosphate (cGMP), a mediator of vascular smooth muscle relaxation and an inhibitor of vessel contraction, has been proposed as an inhibitor of PI hydrolysis in platelets. It is thus possible that cGMP mediates vascular smooth muscle relaxation by attenuating hormonally induced phosphoinositide metabolism. Rapoport has shown that 8-bromo-cGMP inhibits both noradrenaline-induced contraction and inositol monophosphate generation in rat thoracic aorta. Furthermore, Ganz et al. have demonstrated that cGMP moderately attenuates both early and late ANG II--induced DG formation in cultured rat aortic VSMCs. However, this inhibition is probably not due to inhibition of PLC, since PiP hydrolysis and IP formation are not similarly attenuated (unpublished observations). Thus, although cGMP may modulate DG accumulation in VSMC, it does not appear to inhibit PLC-mediated PI/Pi hydrolysis.

**Cellular Processing of the ANG II–Receptor Complex**

Recent work has shown that 125I-ANG II bound to adrenal glomerulosa and adrenocortical cells is internalized via endocytosis, and can be demonstrated in lysosomes in less than 20 minutes. In vascular smooth muscle, binding of ANG II has been shown to initiate aggregation and subsequent internalization of the ANG II receptors. Since these cellular events generally occur over a period of minutes, it seems possible that the temporal delay in the onset of PLC-mediated PI hydrolysis (2–2 minutes) observed in ANG II–stimulated VSMCs may be related to some obligatory cellular events involving processing of the agonist-receptor complex. Hokin has demonstrated that the majority of acetylsalicylic-acid-stimulated 32P-ATP incorporation into PI in pigeon pancreatic slices occurs in the endoplasmic reticulum, suggesting that the surface signal must somehow be translocated for PI hydrolysis to occur. There is some evidence to support this hypothesis in VSMCs. Two interventions that interfere with receptor processing, low temperature and phenylarsine oxide, also disrupt development of sustained ANG II–induced DG formation in VSMCs, while only minimally affecting the early phosphoinositide response. Decreasing the temperature from 37°C to 19°C, which prevents fusion of endocytic vesicles with lysosomes, slightly delays the early ANG II–stimulated DG peak and dramatically attenuates devel-
**Conclusions**

Activation of VSMCs by ANG II results in the PLC-mediated hydrolysis of PIP$_2$/PIP to form IP$_3$ and DG, and in the subsequent hydrolysis of PI to form DG. These second messengers are then responsible for calcium mobilization, protein kinase C activation, alterations in intracellular pH (perhaps indirectly), and ultimately, contraction. The mechanisms controlling the initial and more prolonged cellular responses to ANG II are at present incompletely understood. The mechanisms that we consider most likely to be important are depicted in Figure 2. Figure 2A describes the biochemical events that occur after ANG II stimulation and depicts possible biochemical processes controlling phosphoinositide metabolism, calcium mobilization, and intracellular alkalization. We suggest that early formation of DG from PIP$_2$ activates protein kinase C, which then feeds back to attenuate further PIP$_2$ hydrolysis, IP$_3$ formation, and calcium mobilization. Activation of protein kinase C may also stimulate Na$^+$/H$^+$ exchange, resulting in intracellular alkalization. The secondary increases in pH$_i$ may regulate the subsequent phosphoinositide response by promoting sustained DG formation. Figure 2B depicts the cellular events that may be involved in regulation of DG formation. Preliminary evidence strongly suggests a role for cellular processing of the ANG II-receptor complex in the sustained DG response. The biochemical events discussed earlier, particularly changes in pH$_i$, may also modulate these cellular events by some as yet undescribed mechanism. It is clear that regulation of ANG II-stimulated second messenger generation in VSMCs is complex. Much work remains to be done before our understanding of the mechanisms controlling phosphoinositide metabolism is complete.

**Note added in proof:** The unpublished observations of Berk et al. mentioned in the text have now been published.

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