Angiotensin Increases Inositol Trisphosphate and Calcium in Vascular Smooth Muscle

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AND SUSAN E. RITTENHOUSE

SUMMARY Angiotensin II stimulated the breakdown of phosphatidylinositol-4,5-bisphosphate (PIP2) and the generation of inositol trisphosphate (IP3) in cultured rat aortic smooth muscle cells. The decrease in PIP2 and increase in IP3 levels were rapid (measurable at 5 seconds; maximum IP3 levels at 15 seconds). The time course of these changes was comparable to that of angiotensin II-induced increases in cytosolic free calcium, as measured by the calcium-sensitive fluorescent indicator quin 2. The IP3 formation was not stimulated by the calcium ionophore A23187 (5 μM), nor were angiotensin II-induced changes in IP3 formation inhibited by the removal of extracellular calcium with EGTA. Angiotensin II appears to be capable of generating more IP3 than is required for maximal release of intracellular calcium. These data are consistent with the hypothesis that generation of IP3 plays a role in the angiotensin II-induced mobilization of calcium from intracellular storage sites in vascular smooth muscle cells. (Hypertension 7: 447-451, 1985)

KEY WORDS • intracellular calcium • rat aorta • cell culture • inositol phospholipids • polyphosphoinositides

ANGIOTENSIN II contracts vascular smooth muscle, at least in part, by mobilizing calcium from intracellular storage sites. The biochemical events involved in this angiotensin II-induced mobilization of intracellular calcium in vascular smooth muscle have not been defined. Earlier studies on the mechanism of angiotensin II action in nonvascular tissue (adrenal cortex) showed enhanced turnover of phosphatidylinositol (PI) and polyphosphoinositides after 15 to 30 minutes. The potential relationship to calcium mobilization is unclear because angiotensin II-induced calcium translocation occurs within seconds.

New insights have been gained from recent studies with nonvascular tissues that show that hormone-mediated mobilization of internal calcium is related to the breakdown of phosphatidylinositol-4,5-bisphosphate (PIP2) to inositol trisphosphate (IP3), a water-soluble product capable of inducing calcium release from nonmitochondrial intracellular sites. The formation of IP3 in response to hormones that stimulate calcium release is rapid (occurring within seconds) and has been observed in several cell types. In addition, angiotensin II has been shown to induce rapid PIP2 breakdown in hepatocytes and P3 incorporation into PIP2 in kidney slices.

The role of polyphosphoinositide breakdown in angiotensin II-induced mobilization of calcium from intracellular stores has not been defined in vascular smooth muscle. Gaining insight into the biochemical mechanisms by which angiotensin II mediates its effect in vascular smooth muscle has been difficult, in part, because of the structural and cellular heterogeneity of blood vessels. As an approach to this problem, we and others have used cultured vascular smooth muscle cells (VSMC), which express functional angiotensin II receptors to provide a homogeneous population of intact cells in sufficient numbers for biochemical analyses. In the present studies, we found that angiotensin II stimulates a rapid, concentration-dependent breakdown in PIP2 and formation of IP3 that is temporally comparable to increases in cytosolic free calcium.
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The cultured cells of VSMC (35 mm) were incubated for 48 to 72 hours in growth medium, which contained either \(^{3}H\)myoinositol (25-50 \(\mu\)Ci/ml) or \(^{14}C\)glycerol (21 \(\mu\)Ci/ml), or incubated for 1 hour in phosphate-free DME containing \(^{32}P\), (30 \(\mu\)Ci/ml). Unincorporated isotope was removed by washing the cultures with a warm balanced salt solution (BSS; 130 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), 1.5 mM CaCl\(_2\), 20 mM HEPES buffered to pH 7.4 with Tris base) and incubating in 2 ml of BSS containing 10 mM glucose and 1 mg/ml BSA for 5 minutes at 37°C. In some experiments, 2 mM EGTA was substituted for calcium. One milliliter of fresh buffer containing angiotensin II or A23187 was then added for various times, and the reaction was terminated by rapidly aspirating the buffer and adding 1 ml of chloroform/methanol/HCl (1:2:0.05). Organic and aqueous phases of the transferred extracts plus a 1-ml rinse were separated by adding 400 \(\mu\)L of chloroform and 400 \(\mu\)L of distilled H\(_2\)O followed by centrifugation. The upper phase was washed with 800 \(\mu\)L of chloroform, and the chloroform phases were pooled and concentrated under N\(_2\) flow. The PI, phosphatidylinositol-4-phosphate (PIP), and PIP\(_2\) were resolved by thin-layer chromatography and quantitated by liquid scintillation spectrophotometry as described previously.\(^{22}\) Upper phase extracts containing methanol and H\(_2\)O from \(^{3}H\)myoinositol-labeled or \(^{14}C\)glycerol-labeled cells were evaporated by vacuum centrifugation (Speed Vac, Savant Instruments, Hicksville, NY). Two milliliters of distilled H\(_2\)O was added to each sample, and the solutions were titrated to pH 7 with 10 N KOH. The samples were diluted to 10 ml with 5 mM sodium tetraborate and resolved by column chromatography with the use of 0.5 ml of Bio-Rad AG1-X8 resin (Bio-Rad Laboratories, Richmond, CA) as described by Downes and Michell.\(^{23}\) Five-milliliter fractions were collected and counted in 10 ml of Monofluor (National Diagnostics, Somerville, NJ). Recoveries of IP\(_3\) were calculated to be 85% by monitoring recoveries of standard \(^{32}P\)-IP\(_3\) prepared from labeled erythrocyte ghosts by the method of Downes and Michell.\(^{23}\)

**Measurement of \(^{4}Ca^{2+}\) Efflux**

Replicate-plated cultures (35 mm) were equilibrated for 24 hours at 37°C in growth medium containing 2 \(\mu\)Ci/ml \(^{44}CaCl_2\). Efflux was initiated by quickly washing the cultures three times with BSS and adding 1 ml of BSS containing 10 mM glucose and 1 mg/ml of BSA. The efflux reaction was terminated at 3 minutes with ice-cold Ca\(^{2+}\)-free BSS containing 10 mM LaCl\(_3\). The amount of \(^{4}Ca^{2+}\) remaining in the culture was extracted with 0.1 N H\(_2\)NO\(_3\) and counted in 10 ml of Liquisint (National Diagnostics).

**Results and Discussion**

Angiotensin II (100 nM) induced a rapid breakdown of PIP\(_2\) and accumulation of inositol bisphosphate (IP\(_3\)) and IP\(_2\), (Figure 1). The decrease in PIP\(_2\) was detectable at 5 seconds and maximal between 10 to 15 seconds (24 ± 4% at 15 seconds; \(p < 0.02\) versus control). The time course of angiotensin II-induced IP\(_3\) generation was similar. At 5 seconds an increase of about 50% above basal levels was detected. The increase was maximal at 15 seconds, and began to decline at 30 seconds. Decreases in PIP of 15 to 20% were observed...
Angiotensin II increased IP3 formation in a linear fashion between 1 and 100 nM, apparently without achieving maximal stimulation even at this high concentration of the peptide. The EC50 (effective concentration, 50%) for this response could not be determined from these data, but they suggest that it must be greater than 5 nM. Angiotensin II-stimulated IP3 formation was blocked by the antagonist (Sar1, Ile8)-angiotensin II (data not shown). Figure 2 also shows the concentration-response curve for angiotensin II-induced 45Ca2+ efflux from preloaded cultured VSMC. The threshold, half-maximal, and maximal concentrations of angiotensin II that stimulated efflux were 0.01, 0.5, and 10 nM respectively. Inspection of the two curves in Figure 2 suggests that angiotensin II-mediated calcium mobilization occurred at concentrations of the peptide that result in the formation of relatively small amounts of IP3. Furthermore, maximal angiotensin II mobilization of 45Ca2+ occurred at concentrations (10 nM) stimulating considerably less than maximal IP3 generation.

To provide further insights into the temporal and concentration-response relationship between angiotensin II and the rise in cytosolic free calcium concentration ([Ca2+]i) in VSMC, we performed studies using cultured VSMC that were loaded with the calcium-sensitive dye quin 2.19, 25 As shown in Figure 3, angiotensin II stimulated a rapid concentration-dependent increase in [Ca2+]i. At a concentration of 1 nM angiotensin II, for example, the stimulated [Ca2+]i levels peaked within 30 seconds. The threshold and half-maximal responses occurred at about 0.01 and 0.5 nM and were similar to those for angiotensin II-induced 45Ca2+ efflux. The increase in basal fluorescence over the course of the experiment (see Figure 3) may not represent a true increase in basal [Ca2+]i, but may reflect a time-dependent leakage of quin 2 out of the cells, as we previously have observed.19 In six experiments, the maximal increases in [Ca2+]i at 10 and 100 nM angiotensin II were 5.2 ± 0.5-fold and 5.7 ± 0.7-fold above basal levels respectively. This difference was not significant (p = 0.25). These data provide...
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Effects of Angiotensin II and Calcium Ionophore A23187 on Inositol Bisphosphate and Inositol Trisphosphate Generation in Cultured Vascular Smooth Muscle Cells

<table>
<thead>
<tr>
<th></th>
<th>IP3 (cpm)</th>
<th>IP2 (cpm)</th>
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<tbody>
<tr>
<td>Control</td>
<td>815 ± 28</td>
<td>1912 ± 223</td>
</tr>
<tr>
<td>Angiotensin II (100 nM)</td>
<td>1396 ± 55</td>
<td>10754 ± 770</td>
</tr>
<tr>
<td>A23187 (5 µM)</td>
<td>854 ± 9</td>
<td>2222 ± 118</td>
</tr>
</tbody>
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Data are expressed mean ± se for three identically treated cultures. The incubation time was 30 seconds. cpm = counts per minute; IP2 = inositol bisphosphate; IP3 = inositol trisphosphate.

Figure 3. Effects of angiotensin II on cytosolic free calcium determined with quin 2. Angiotensin II at the indicated concentrations was added at the arrow, and [Ca2+]i was calculated. This experiment was replicated six times.

evidence that 45Ca2+ efflux reflects an increase in [Ca2+]i. Thus, angiotensin II-stimulated increases in calcium mobilization and IP3 generation appear to be kinetically similar but differ in their concentration-response relationship to angiotensin II.

The implications of the apparent differences in the potency of angiotensin II in stimulating IP3 formation and intracellular calcium mobilization in vascular smooth muscle are not completely understood. Similar observations have been made recently regarding vasopressin in hepatocytes. The data suggest that in each instance the hormone at high concentration can induce more IP3 from than is needed for maximal calcium mobilization. An alternative explanation is that all of the counts in the IP3 fraction do not represent inositol-1,4,5-trisphosphate but contain an as yet undetermined portion of an isomer such as inositol-1,3,4-trisphosphate. This latter compound has recently been reported to be the predominant form of IP3 in the rat parotid. The implications of these findings are unknown because the potency of inositol-1,3,4-trisphosphate in releasing intracellular calcium has not been reported.

Angiotensin II is known to release calcium from intracellular storage sites in vascular smooth muscle, presumably the endoplasmic reticulum. Furthermore, IP3 has been shown to release calcium from intracellular storage sites in saponin-permeabilized pig coronary arteries. In other cell types, IP3, among phosphorylated inositol derivatives, has been shown to be uniquely potent in releasing Ca2+. Nonetheless, to provide insight into the possibility that IP3 generation may be a consequence of, rather than a cause for, calcium mobilization, studies with the calcium ionophore A23187 were performed. As shown in Table 1, A23187 (5 µM, 30 seconds) had no significant effect on IP2 and IP3 generation. In contrast, angiotensin II (100 nM, 30 seconds) resulted in approximately 60% and five-fold increases in IP3 and IP2, respectively. Furthermore, substituting EGTA for extracellular calcium (2 mM for 5 minutes) did not affect angiotensin II stimulation of IP3 formation (data not shown). The implications of the larger increases in IP3 in comparison with IP2 are not known. Similar observations recently have been reported for thrombin-induced increases in IP3 and IP2 in human platelets.

Thus, the calcium-independent stimulation of IP3 production by angiotensin II and its temporal relationship to angiotensin II-mediated increases in cytosolic free calcium are consistent with the hypothesis that the contractile effects of angiotensin II on vascular smooth muscle may be initiated by the phospholipase C-mediated breakdown of PIP2 and formation of IP3. Although the initial contraction of vascular smooth muscle in response to angiotensin II results from the mobilization of intracellular calcium, the sustained contractile effects require the influx of extracellular calcium. Whether angiotensin II-mediated effects on polyphosphoinositide metabolism are involved in modulating smooth muscle cell plasma membrane permeability to calcium requires further investigation.

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References


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