Angiotensin Increases Inositol Trisphosphate and Calcium in Vascular Smooth Muscle

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SUMMARY Angiotensin II stimulated the breakdown of phosphatidylinositol-4,5-bisphosphate (PIP$_2$) and the generation of inositol trisphosphate (IP$_3$) in cultured rat aortic smooth muscle cells. The decrease in PIP$_2$ and increase in IP$_3$ levels were rapid (measurable at 5 seconds; maximum IP$_3$ 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 IP$_3$ formation was not stimulated by the calcium ionophore A23187 (5 μM), nor were angiotensin II-induced changes in IP$_3$ formation inhibited by the removal of extracellular calcium with EGTA. Angiotensin II appears to be capable of generating more IP$_3$ than is required for maximal release of intracellular calcium. These data are consistent with the hypothesis that generation of IP$_3$ 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 (PIP$_2$) to inositol trisphosphate (IP$_3$), a water-soluble product capable of inducing calcium release from nonmitochondrial intracellular sites. In addition, angiotensin II has been shown to induce rapid PIP$_2$ breakdown in hepatocytes and $^{32}$P incorporation into PIP$_2$ 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 PIP$_2$ and formation of IP$_3$ that is temporally comparable to increases in cytosolic free
calcium concentration. These angiotensin II-induced changes in polyphosphoinositide metabolism are independent of extracellular calcium and not mimicked by the calcium ionophore A23187.

Materials and Methods

The supplies and vendors in this study were as follows: [3H]myoinositol (15 Ci/mmol), Amersham-Searle, Chicago, IL; [14C]glycerol (8.7 mCi/mmol) and carrier-free 32P, New England Nuclear, Boston, MA; 45CaCl2 (27.2 mCi/g), ICN, Irvine, CA; quin 2/AM (quin-2-tetra[acetoxymethyl]ester), Lancaster Synthesis, Morecambe, Lancaster, UK; Dulbecco’s Modified Eagle’s Medium (DME), calf serum, glutamine, penicillin, and streptomycin, MA Bioproducts, Walkersville, MD; angiotensin II and (Sar1,Ile8)-angiotensin II, Peninsula Laboratories, Belmont, CA; bovine serum albumin (BSA, Pentex), Miles Laboratories, Elkhart, IN.

Cell Culture

The VSMC were isolated from rat thoracic aorta by enzymatic dissociation techniques described previously by this laboratory.15-19 Cells were propagated in DME with 10% calf serum and antibiotics and passaged twice a week by harvesting with trypsin-versene and seeding at a 1:4 ratio in 75-cm2 flasks. For experiments, cells between passage levels 4 to 25 were seeded into 35- or 100-mm dishes (2 x 104 cells/cm2), fed every other day, and used after 4 to 6 days.

Phospholipid Labeling

The cultures of VSMC (35 mm) were incubated for 48 to 72 hours in growth medium, which contained either [3H]myoinositol (25–50 μCi/ml) or [14C]glycerol (21 μCi/ml), or incubated for 1 hour in phosphate-free DME containing 32P (30 μCi/ml). Unincorporated isotope was removed by washing the cultures with a warm balanced salt solution (BSS; 130 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.5 mM CaCl2, 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 of 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 addition of 400 μL of chloroform and 400 μL of distilled H2O followed by centrifugation. The upper phase was washed with 800 μL of chloroform, and the chloroform phases were pooled and concentrated under N2 flow. The PI, phosphatidylinositol-4-phosphate (PIP), and PIP2 were resolved by thin-layer chromatography and quantitated by liquid scintillation spectrophotometry as described previously.22 Upper phase extracts containing methanol and H2O from [3H]myoinositol-labeled or [14C]glycerol-labeled cells were evaporated by vacuum centrifugation (Speed Vac, Savant Instruments, Hicksville, NY). Two milliliters of distilled H2O 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 IP3 were calculated to be 85% by monitoring recoveries of standard [32P]-IP3 prepared from labeled erythrocyte ghosts by the method of Downes and Michell.23

Measurement of 45Ca Efflux

Replicate-plated cultures (35 mm) were equilibrated for 24 hours at 37°C in growth medium containing 2 μCi/ml 45CaCl2. 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 Ca2+-free BSS containing 10 mM LaCl3. The amount of 45Ca2+ remaining in the culture was extracted with 0.1 N HNO3 and counted in 10 ml of Liquisint (National Diagnostics).

Measurement of Quin 2 Fluorescence

Quin 2, a calcium-sensitive tetracarboxylate dye, was used to monitor changes in cytosolic free calcium concentration ([Ca2+]) in cultured suspensions of VSMC as described previously.19 Cell suspensions (5 x 104 cells/ml) were prepared with 8 x 100-mm cultures, incubated for 20 minutes at 37°C with 50 μM quin 2/AM, washed twice (200 g for 3 minutes) with BSS, and resuspended to 4 x 106 cells/ml with BSS containing 10 mM glucose and 1 mg/ml BSA. The cells were kept at room temperature, centrifuged in a Beckman microfuge (Model B, Beckman Instruments, Fullerton, CA) before use, and resuspended to 2 x 106 cells/ml. Measurements of fluorescence were made at 37°C with a Perkin-Elmer 44A spectrofluorometer (excitation, 339 nm, slit, 4 nm; emission, 492 nm, slit, 10 nm; Perkin-Elmer Corp., Norwalk, CT) equipped with a thermostated cuvette holder, stirring apparatus, and chart recorder. The calibration of quin 2 fluorescence and calculation of [Ca2+] were determined as described by Tsien et al.25

Results and Discussion

Angiotensin II (100 nM) induced a rapid breakdown of PIP2 and accumulation of inositol bisphosphate (IP2) and IP3 (Figure 1). The decrease in PIP2 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 IP3 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 (100 nM) increased IP3 formation in a linear fashion (Table I). The increase in IP3 levels at 1 nM angiotensin II was significantly greater than basal values (p < 0.01). 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. 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.

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...
Evidence that 

\[ \text{Ca}^{2+} \] efflux reflects an increase in 

\[ [\text{Ca}^{2+}]_o \]. 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 formation 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 

\[ \text{Ca}^{2+} \]. 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) 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 IP₂ in comparison with IP₃ are not known. Similar observations recently have been reported for thrombin-induced increases in IP₂ and IP₃ 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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