Regulation of Cytosolic Calcium by Angiotensins in Vascular Smooth Muscle

David E. Dostal, Tetsuya Murahashi, and Michael J. Peach

The carboxy terminal homologue of angiotensin II (Ang II), Ang-(3-8) or hexapeptide, was used as a model peptide to examine the types of receptor mechanisms involved in calcium mobilization in cultured vascular smooth muscle cells. Hexapeptide did not produce tachyphylaxis but did produce a sustained increase in intracellular calcium. Differences in the increase in intracellular calcium ([Ca\textsuperscript{2+}]) and the pattern of inositol phosphate production indicate that Ang-(3-8) and maximal concentrations of Ang II mobilize calcium through different mechanisms. The calcium-mobilizing mechanisms that predominate appear to depend on the concentration of angiotensin. Concentrations of Ang II greater than 10\textsuperscript{-8} M produce sharp calcium transients in which the [Ca\textsuperscript{2+}], returns close to baseline within 1 minute after stimulation, but concentrations of Ang II equal to or less than 3\times10\textsuperscript{-9} M result in a plateau increase in calcium. Pretreatment with Bordetella pertussis toxin does not abolish either the calcium transient induced by Ang II or the plateau phase induced by Ang-(3-8), indicating that the GTP-transducing protein that couples the receptor to phospholipase C or, possibly, a receptor-operated calcium channel is not Bordetella pertussis toxin sensitive. (Hypertension 1990;15:815-822)

Angiotensin II (Ang II) is a potent vasoconstrictor agent that normally contributes to the maintenance of blood pressure\textsuperscript{1} and might play an important role in the pathogenesis of hypertension.\textsuperscript{2} Angiotensin is believed to induce contraction of vascular smooth muscle by a series of events. Binding of Ang II to its receptor results in activation of phospholipase C, which breaks down phosphatidylinositol 4,5-bisphosphate to give diacylglycerol and inositol 1,4,5-trisphosphate (IP\textsubscript{3}). The IP\textsubscript{3} in return, releases Ca\textsuperscript{2+} from intracellular stores, presumably the endoplasmic reticulum, resulting in an increase in cytoplasmic Ca\textsuperscript{2+} levels. The increased cytosolic Ca\textsuperscript{2+} then binds to calmodulin and activates the calmodulin-dependent myosin light chain kinase.\textsuperscript{3} Activation of the myosin light chain kinase produces phosphorylation of myosin light chain resulting in actin and myosin cross-bridge formation.\textsuperscript{4}

Although the mechanism or mechanisms by which the Ang II receptor activates phospholipase C remain to be elucidated, indirect evidence suggests that this interaction involves a guanine nucleotide-binding protein because guanine nucleotides affect Ang II binding.\textsuperscript{5} Studies in liver\textsuperscript{6} have shown that ADP-ribosylation of all the G, with Bordetella pertussis toxin (BPT) completely blocks the inhibitory actions of Ang II on adenylate cyclase but has no effect on Ang II-induced breakdown of polyphosphoinositides. GTP exerts a pronounced effect on Ang II-binding in toxin-treated liver membranes, indicating that the Ang II receptor in liver is coupled by two or more guanine nucleotide-binding (G) proteins, one of which is G\textsubscript{i}. Very little is known, however, concerning the coupling mechanisms associated with Ang II and activation of phospholipase C in vascular smooth muscle.\textsuperscript{7}

Studies in rabbit aorta have shown that contractions induced by Ang II and carboxy terminal homologues such as angiotensin III (Ang III) have a different dependence on extracellular Ca\textsuperscript{2+}.\textsuperscript{8} Because [Ca\textsuperscript{2+}], is required for contractile responses in vascular smooth muscle, regulation of this intracellular messenger is important in the maintenance of vascular tone. In the present study, a carboxy terminal fragment of Ang II, Ang-(3-8), was used as an agonist to characterize Ang II receptor mechanisms in vascular smooth muscle cells cultured from rat aorta. The increase in cytosolic Ca\textsuperscript{2+}, phosphatidylinositides, and G-protein coupling mechanisms were compared between Ang II and the hexapeptide Ang-(3-8).

Methods

Reagents

Tissue culture medium-199 (M199), Dulbecco's modified Eagle's medium (DMEM), Ham's F-12, and fetal bovine serum (FBS) were obtained from GIBCO Laboratories (Grand Island, New York). The reagents insulin, transferrin, ascorbate, penicil-
lin-streptomycin, bovine serum albumin, GTPyS, and trypsin-EDTA were obtained from Sigma Chemical Co. (St. Louis, Missouri). Ang II and Ang-(3-8) were obtained from Bachem Inc. (Torrance, California). Arginine vasopressin was obtained from Peptide Institute, Inc. (Osaka, Japan).

Culturing Vascular Smooth Muscle Cells

Vascular smooth muscle cells were isolated from male Sprague-Dawley rats and cultured by a modification of the procedures as described by Owens et al. Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air with media changes three times weekly. Cells that were 2–4 days postconfluent were used for experiments. In some cases where indicated, cells were transferred from this growth medium to serum-free media that contained equal parts of DMEM and Ham's F-12 supplemented with insulin (10⁻⁶ M), transferrin (5 µg/ml), ascorbate (0.2 mM), and antibiotics.

Measurement of Indo-1 Fluorescence

Cells were grown to confluency in 75-cm² culture flasks (Corning, Wexford, Pennsylvania) in the presence of M199 supplemented with 10% FBS as previously described. The [Ca²⁺] concentration was measured in the cultured rat aortic vascular smooth muscle in suspension by means of the fluorescent Ca²⁺ indicator indo-1. Briefly, monolayers of confluent cells were incubated (95% air, 5% CO₂, and 50% humidity) at 37°C for 1 hour with 4×10⁻⁶ M of the pentaacetoxymethyl ester of indo-1 (5 mM stock indo-1 acetoxymethyl ester in 100% dimethylsulfoxide, Molecular Probes, Eugene, Oregon) in HEPES–Krebs-Ringer bicarbonate solution (HKRB) (20 mM HEPES, 103 mM NaCl, 4.77 mM KCl, 0.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, and 15 mM glucose, pH 7.39), after which media were removed and cells were incubated for an additional 30 minutes. Cells were removed from the dishes by using 6 ml trypsin-EDTA (trypsin 0.01%:EDTA 0.02%) solution and were transferred to HKRB containing soybean trypsin inhibitor (0.6 mg/ml), counted, centrifuged for 2 minutes at 200g, resuspended to a density of 10⁶ cells/ml in warmed (37°C) oxygenated (95% O₂ and 5% CO₂) HKRB, and transferred into 3-ml cuvettes. Fluorescence was measured in a thermostatically controlled fluo-
Figure 3. Recordings showing dependence of Ang-(3-8) responses on extracellular calcium. Smooth muscle cells loaded with the fluorescent dye indo-1 were stimulated with 10^{-6} M Ang-(3-8) in the presence (0.5 mM, n=4) and absence (2 mM EGTA added, n=4) of extracellular calcium. Maximal changes in intracellular calcium concentration ([Ca^{2+}]_i) (nM) were 62.3±5.4 and 55.8±4.7 in the presence and absence of extracellular calcium, respectively. Baseline and [Ca^{2+}]_j concentrations (nM) were 123±3.0 and 84.3±10 in the presence and absence of extracellular calcium, respectively.

Inositol Trisphosphate Measurements

Rat aortic smooth muscle cells were grown in M199 containing 10% heat-inactivated FBS as previously described. After the cells had become 2 days postconfluent, the medium was changed to serum-free medium containing 1.33 μCi/ml of myo-[1,2-3H(N)]inositol (New England Nuclear Research Products, Boston, Massachusetts). After 40 hours, the medium was aspirated and the cells were rinsed three times with unlabeled serum-free medium. Two milliliters serum-free medium was added to each flask, and the cells were allowed to incubate for 20 minutes before hormone was added. Cells were stimulated with either 3×10^{-4} M Ang-(3-8) or 10^{-6} M Ang II for various lengths of time (10–600 seconds) at 37° C. The phosphatidylinositol isomers 1,4,5 IP_3, 1,3,4 IP_3, and 1,3,4,5 IP_4 were separated by high-pressure liquid chromatography as previously described.12

Treatment of Cells With Bordetella Pertussis Toxin

Confluent smooth muscle cells in growth media were treated with 400 ng BPT for 30 hours. Treated cells were either used for [Ca^{2+}]_i fluorescence measurements, as previously described, or assayed for extent of ADP-ribosylation. In vitro ADP-ribosylation of membrane preparations was performed as described by Bokoch and colleagues.13 The samples were processed for sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography.6

Photolabeling of Membrane Proteins With 8-Azido GTP [γ-32P]

Membranes from postconfluent cultured rat aortic smooth muscle cells were incubated for 15 minutes at 4°C in 0.5-ml microcentrifuge tubes containing 100 μg protein, 10 mM MgCl_2, and 4.83 μM (9.66 μCi) 8-azido guanosine-5′-triphosphate (GTP) [γ-32P], triethylammonium salt (specific activity, 5–25 Ci/mmol,
ICN Biomedicals Inc., Costa Mesa, California) with and without 400 μM GTPγS. The samples were photolyzed by a 10-minute exposure to an ultraviolet mineral light (UVSL-54, Ultra-violet Products, Inc., San Gabriel, California). The membrane proteins were separated using discontinuous 12% polyacrylamide gel electrophoresis according to the procedure of Laemmli. The gels were silver stained (Rapid-Ag-Stain, ICN Biomedicals Inc.) and sealed in cellophane (Olin Ecusta Paper and Film Group, Pisgah Forest, North Carolina), after which the labeled protein bands were visualized by autoradiography (XRP 5, Eastman Kodak Co.-Lab. and Res. Prods. Div., Rochester, New York).

Statistical Analysis

Curve-fitting analysis for dose–response curves was performed by use of GRAPHPAD (GRAPHPAD Software, San Diego, California). Data are expressed as the mean±SEM.

Results

Carboxy terminal homologues of Ang II were screened for their ability to increase [Ca2+]i in rat aortic vascular smooth muscle cells loaded with the fluorescent dye indo-1. Using 10−6 M concentrations, Ang II [(Ang-(1-8)] and the two Ang II homologues, angiotensin III [Ang III [Ang-(2-8)] and hexapeptide [Ang-(3-8)], produced increases in [Ca2+]. (Figure 1), whereas the carboxy terminal homologues, Ang-(4-8) and Ang-(5-8), had no effect. Ang II and Ang III produced Ca2+ transients in which the [Ca2+]i returned to near baseline levels within 40–60 seconds after stimulation. Ang-(3-8) produced an increase in [Ca2+], that could be maintained for several minutes in the presence of the ligand. Additionally, Ang-(3-8) did not produce tachyphylaxis to itself or to Ang II. Because of these large differences, the Ca2+-mobilizing effects of Ang II and Ang-(3-8) were compared in this study. When dose–response experiments were performed, it was found that the ED50 for Ang II was (2.24±0.045)×10−8 M (n=7) as compared with (4.47±0.09)×10−6 M (n=7) for Ang-(3-8), indicating that the potency of Ang-(3-8) was approximately 200-fold less than Ang II (Figure 2). Ang II was found to have a threshold dose at 10−10 M and produced a maximal response of 311±35.3 nM at 10−6 M. Ang-(3-8) was found to have a threshold dose at 3×10−8 M and produced a maximal response of 281±17.9 nM at 3×10−8 M. It was also noted that Ang-(3-8) at 10−6 M did not prevent subsequent Ca2+ transients to maximal concentrations of Ang II, indicating that Ang-(3-8) did not desensitize the cells to Ang II.

The contribution of Ca2+ influx to the maintenance of the plateau increase produced by 10−6 M Ang-(3-8) was assessed by removal of extracellular Ca2+ by addition of 2 mM EGTA (Figure 3). Under Ca2+-free conditions, Ca2+ transients were observed in which [Ca2+] levels returned to baseline within 150–200 seconds after stimulation. To gain further insight into the mechanisms responsible for maintaining the plateau phase of the Ang-(3-8)–induced [Ca2+]i response, increases in 1,4,5 IP3, 1,3,4,5 IP4, and 1,3,4 IP3 were measured at various times after stimulation with 10−6 M Ang-(3-8) or 10−6 M Ang II (Figure 4). The pattern of inositol phosphate production was different in cells stimulated with 10−6 M Ang-(3-8) as compared with 10−6 M Ang II. Ang II produced a brief increase in 1,4,5 IP3, which returned to below the original baseline after 2 minutes, whereas Ang-(3-8) produced a rapid increase in 1,4,5 IP3, which was maintained at a plateau level even at 10 minutes. Similar differences between Ang II–stimulated and Ang-(3-8)–stimulated production of 1,3,4 IP3 and 1,3,4,5 IP4 were also observed. It was determined whether the Ca2+-mobilizing effects of Ang-(3-8) were unique to this Ang II homologue.
Dose–response experiments were performed (n=7) in which cultured smooth muscle cells were stimulated with an initial concentration of Ang II that was varied from 10^{-10} M to 10^{-6} M. The cells were stimulated with a maximal dose of Ang II (10^{-6} M) 80 seconds later. After the second dose of Ang II, the cells were stimulated with a maximal dose of angiotensin vasopressin (10^{-7} M) to ensure that cells were still capable of mobilizing Ca^{2+} by a phospholipase C-coupled system. A typical set of responses from an experiment is shown in Figure 5. The increase in [Ca^{2+}], decreased as the initial concentration of Ang II was decreased; however, cells only responded to the second maximal concentration of Ang II when the initial concentration of Ang II was less than or equal to 3 \times 10^{-9} M. When an initial concentration less than or equal to 3 \times 10^{-9} M Ang II was given, 1) a plateau increase in [Ca^{2+}], occurred and 2) a Ca^{2+} transient to a subsequent concentration of Ang II was elicited.

Assuming that G proteins were involved in receptor-dependent IP{sub}$_3$–mediated release of Ca^{2+} from intracellular stores, we investigated the effect of Bordetella pertussis toxin on the Ang II–induced and Ang-(3-8)–induced increase in [Ca^{2+}]. As shown in Figure 6, pretreatment of cultured smooth muscle cells with 400 ng/ml Bordetella pertussis toxin for 30 hours at 37° C had no effect on increases of intracellular free [Ca^{2+}], induced by either Ang-(3-8) (88±23 nM) or Ang II (344±67 nM) as compared with control responses to Ang-(3-8) (62±5 nM) and Ang II (218±10 nM). Based on results of in vitro ribosylation assays, the toxin treatment was sufficient to completely ribosylate membrane proteins at approximately 40,000, the reported molecular weight of G, (Figure 7). The smooth muscle cells appear to have other GTP-binding proteins because, after photolysis, the GTP agonist 8-azido GTP [γ-32P] bound to protein bands with molecular weights at 45,000 and greater than 97,000, in addition to a 40,000 band (Figure 8). The bands at 40,000 and 45,000 correspond to GTP-binding proteins $G_1$ and $G_6$, which have been well documented in other tissues. Addition of Ang II to membrane preparations resulted in a decrease in labeling the 45,000 band with small changes in the remaining bands. The GTP agonist GTPγS prevented incorporation of the photolabel into the protein bands.

Discussion

Previous experiments in our laboratory demonstrated differences between Ang II and carboxy terminal homologues. For example, Ang II–induced contractile responses in aortic strips from rabbits are dependent on extracellular Ca^{2+}, whereas contractile responses to Ang III are not. Additionally, others have shown a failure to produce cross-tachyphylaxis
to Ang II and Ang III as well as difficulty in obtaining tachyphylaxis to Ang III in rat uterus and aortic strips.\textsuperscript{15,16} Based on these studies, it was hypothesized that Ca\textsuperscript{2+}-mobilization mechanisms for Ang II are different than those of the carboxy terminal homologues of Ang II. We have found that the hexapeptide homologue of Ang II, when screened for its ability to induce Ca\textsuperscript{2+} transients in cultured smooth muscle cells, is unique because at all concentrations it produces a plateau increase in [Ca\textsuperscript{2+}]\textsubscript{i}. Additionally, it does not produce cross-tachyphylaxis to Ang II.

Many cells respond to appropriate ligands with a rapid increase in cytosolic Ca\textsuperscript{2+} concentration, followed by a return to a steady-state level of [Ca\textsuperscript{2+}]\textsubscript{i} that remains above the basal level throughout the period of stimulation. The mechanism underlying the sustained component of the Ang-(3-8)–stimulated [Ca\textsuperscript{2+}]\textsubscript{i} signal appears to be an influx of extracellular Ca\textsuperscript{2+} because removal of Ca\textsuperscript{2+} from the extracellular medium abolishes the sustained increase in cytosolic Ca\textsuperscript{2+}. The sustained Ca\textsuperscript{2+} influx might be related to phosphatidylinositol bisphosphate (PIP\textsubscript{2}) hydrolysis because Ang-(3-8) produces a plateau increase in 1,4,5 IP\textsubscript{3}, 1,3,4 IP\textsubscript{3}, and 1,3,4,5 IP\textsubscript{4}, whereas Ang II produces transient responses in these inositol phosphate metabolites. The sustained increase in cytosolic Ca\textsuperscript{2+} in response to Ang-(3-8) might be maintained by the secondary increase in 1,4,5 IP\textsubscript{3} or in conjunction with elevated levels of 1,3,4,5 IP\textsubscript{4}. Evidence that 1,4,5 IP\textsubscript{3} binding sites copurify with the plasma membrane as well as microsomes\textsuperscript{17} has given credence to the possibility that 1,4,5 IP\textsubscript{3} also participates in the regulation of Ca\textsuperscript{2+} entry from the extracellular medium.\textsuperscript{18} Additionally, 1,3,4,5 IP\textsubscript{4} has been shown to promote the extracellular Ca\textsuperscript{2+}-dependent activation of sea urchin eggs only in the presence of 1,4,5 IP\textsubscript{3}. In the mouse lacrimal gland, 1,4,5 IP\textsubscript{3} with 1,3,4,5 IP\textsubscript{4} stimulates a Ca\textsuperscript{2+}-activated potassium conductance, indicating that these two agents can act in concert to regulate a Ca\textsuperscript{2+}-entry mechanism.\textsuperscript{19} It is possible that Ca\textsuperscript{2+} channels can be activated by intracellular messengers such as Ca\textsuperscript{2+},\textsuperscript{20} 1,4,5 IP\textsubscript{3},\textsuperscript{21} 1,3,4,5 IP\textsubscript{4},\textsuperscript{22} or both 1,4,5 IP\textsubscript{3} and 1,3,4,5 IP\textsubscript{4}.\textsuperscript{23}

In the present study, the effects observed with Ang-(3-8) are only seen with concentrations of Ang II less than or equal to 3 \times 10^{-8} M. When cells are given Ang II in concentrations greater than 10^{-7} M, a transient increase in [Ca\textsuperscript{2+}] occurs, resulting in a return of [Ca\textsuperscript{2+}] to almost baseline levels. A concentration of Ang II at less than or equal to
Figure 7. Photomicrograph of in vitro ribosylation of Bordetella pertussis toxin-treated cells. Membranes from toxin-treated vascular smooth muscle cells and control cells (nontreated) were ribosylated in vitro using Bordetella pertussis toxin in the presence of $[^{32}\text{P}]-\text{nicotine adenine dinucleotide}$. Membrane proteins were then separated using discontinuous polyacrylamide gel electrophoresis after which autoradiography was performed. Lanes C and PT refer to control membranes and membranes from toxin-pretreated cells, respectively. These procedures were performed twice in triplicate.

Figure 8. Photomicrograph showing photolabeling of GTP-binding proteins in membranes. Membranes from vascular smooth muscle cells were exposed for 15 minutes to 8-azido GTP $[^{32}\text{P}]$, triethylammonium salt at 4°C and photolyzed in absence (C) and presence of $10^{-6}$ M angiotensin II (AII) or 400 μM GTP-$\gamma$S. These procedures were performed twice in triplicate.
3 × 10⁻⁹ M, like 10⁻⁶ M Ang-(3-8), produces a plateau increase in [Ca²⁺], and the cells display a pronounced loss of desensitization to a maximal dose of Ang II. It is possible that the sustained increase in [Ca²⁺] induced by Ang-(3-8) or with low concentrations of Ang II is maintained by slow asynchronous transient responses from the population of cells. Because only a finite number of transient responses appear after an individual cell has been exposed to a particular concentration of agonist, it is not clear if a continuous increase in observed fluorescence could be maintained by this mechanism for several minutes. It would be interesting to extend this technique to the single cell level in which it could be determined whether Ang-(3-8) produces a continuous series of transient increases in aortic smooth muscle cells and whether these same cells also respond for brief periods of time to maximal concentrations of Ang II.

It has been reported that pretreatment of the cultured aortic smooth muscle cells with Bordetella pertussis toxin inhibits Ang II–induced inositol trisphosphate production and the release of Ca²⁺ from intracellular stores. It is presumed that the toxin exerts its action by ADP-ribosylation of GTP-binding protein Gi or Gq, therefore uncoupling Ang II receptors from their effector. In liver, kidney, and fetal heart, one of the Ang II–binding sites appears to be coupled to adenylate cyclase through a Bordetella pertussis toxin–sensitive mechanism, whereas the other receptor site is coupled to phospholipase C and is not BPT sensitive. In the present study, rat aortic smooth muscle cells intoxicated with BPT do not demonstrate any changes in the increase in [Ca²⁺] in response to stimulation with either peptide. It appears that the G proteins Gi or Gq are not coupled to Ca²⁺ mobilization in these cells. It was interesting, however, that membranes incubated with 8-azido GTP [³²P] in the presence of Ang II decrease the degree of labeling of a membrane protein having a molecular weight of 45,000. Differences between the results of toxin treatment in this study and those previously reported might be explained by the length of exposure to the toxin or by some nonspecific action.

We have described differences in Ca²⁺ mobilization between Ang II and the carboxy terminal homologue of Ang II, Ang-(3-8), in cultured rat aortic smooth muscle cells. These differences in Ca²⁺ mobilization might be caused by ligand-receptor interactions, multiple forms of the angiotensin receptor, or differences in levels of second messengers induced by peptide stimulation.

References
15. Morin AP, Gallacher DV, Irvine RF, Peterson OH: Synergism of inositol trisphosphate and tetrakisphosphatc in acti-
Regulation of cytosolic calcium by angiotensins in vascular smooth muscle.
D E Dostal, T Murahashi and M J Peach

Hypertension. 1990;15:815-822
doi: 10.1161/01.HYP.15.6.815

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1990 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/15/6_Pt_2/815

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/