Intracellular Angiotensin II Stimulates Voltage-Operated Ca\(^{2+}\) Channels in Arterial Myocytes

Kimika Eto, Yusuke Ohya, Yoshito Nakamura, Isao Abe, Mitsuo Iida

Abstract—Although the presence of intracellular angiotensin II (Ang II) and of Ang II–binding sites has been reported, their roles in cell function have not been fully clarified. The purpose of the present study was to test the hypothesis that intracellular Ang II modifies voltage-operated Ca\(^{2+}\) channels in vascular smooth muscle. Ca\(^{2+}\) channel currents were recorded in guinea pig mesenteric arterial myocytes with the whole-cell patch-clamp method. Intracellular dialysis of Ang II increased the amplitudes of Ca\(^{2+}\) channel current (133±9% of the control with 10 nmol/L Ang II, n=16). Concomitant dialysis of the Ang II type 1 receptor antagonist, CV-11974 (1 μmol/L, n=11), but not the bath application of this drug, suppressed this Ang II action. In contrast, the dialysis of the Ang II type 2 receptor antagonist, PD123319 (1 μmol/L, n=5), failed to affect the Ang II action. Dialysis of either a phospholipase C inhibitor (U-73122, 10 μmol/L, n=5) or protein kinase C inhibitors (calphostin C, 100 nmol/L, n=5; protein kinase C inhibitor peptide-[19-36], 1 μmol/L, n=5) suppressed the Ang II action. Dialysis of KT5720 (100 nmol/L, n=5), an inhibitor of cAMP-dependent protein kinase, did not affect the Ang II action. Intracellular dialysis of angiotensin I (10 nmol/L) enhanced Ca\(^{2+}\) channel currents (13±8%, n=6), which were sensitive to intracellular enalaprilat (1 μmol/L, n=5) or CV-11974 (n=5). These results suggest that intracellular Ang II has a stimulating action on voltage-operated Ca\(^{2+}\) channels in vascular smooth muscle, possibly through intracellular binding sites similar to the Ang II type 1 receptor, which are associated with phospholipase C and protein kinase C. (Hypertension. 2002;39[part 2]:474-478.)

Key Words: calcium channels ■ muscle, smooth, vascular ■ protein kinases ■ receptors, angiotensin II ■ angiotensin-converting enzyme ■ angiotensin

Local renin-angiotensin systems in the heart, kidneys, and vascular wall play a pivotal role in the pathogenesis of hypertensive organ damage and atherosclerosis.\(^1,2\) Recent evidence suggests that angiotensin II (Ang II) and its generating system are present also inside the cell.\(^1-5\) Most physiological actions of Ang II are mediated through Ang II receptors on the plasma membrane. However, soluble cytoplasmic Ang II–binding protein and nuclear Ang II–binding sites, which have different physicochemical properties from Ang II receptors at the plasma membrane, have been reported.\(^6-11\) The roles of intracellular Ang II and its binding sites in the cardiovascular system are not yet fully understood; however, several reports regarding this subject exist. An activation of nuclear Ang II receptors initiates the transcription of renin and angiotensinogen mRNA in liver cells.\(^11\) An intracellular administration of Ang II reduces Ca\(^{2+}\) currents in rat ventricular myocytes but increases these currents in hamster ventricular myocytes.\(^5,12\) Intracellular Ang II has also been shown to decrease the junctional conductance of cardiac muscles.\(^5\) In cultured aortic muscle cells, microinjection of Ang II increases intracellular Ca\(^{2+}\) concentration, as determined by Ca\(^{2+}\)-sensitive dye.\(^13\) Moreover, intracellular application of Ang II by the liposome method contracts the rat aorta.\(^14\) In the above 2 studies of smooth muscle, the intracellular Ang II actions are abolished by a removal of extracellular Ca\(^{2+}\), suggesting that Ca\(^{2+}\) influx from extracellular spaces could be responsible for these actions. However, the mechanism by which intracellular Ang II modifies intracellular Ca\(^{2+}\) concentration and vascular tone has not been fully understood. In the present study, we dialyzed Ang II, Ang II receptor antagonists, and protein kinase inhibitors into the cell during the whole-cell patch-clamp experiments to assess the action of intracellular Ang II on voltage-operated Ca\(^{2+}\) channels and its signal-transduction pathway in vascular smooth muscle cells.

Methods

Single smooth muscle cells were isolated from the small mesenteric arteries (diameter, <300 μm) of guinea pigs (200 to 300 g) by collagenase treatment, as described previously.\(^15,16\) The study protocol was approved by the Committee of Ethics of Animal Experimentation, Faculty of Medicine, Kyushu University.

Conventional whole-cell patch-clamp methods were performed with a patch pipette through a voltage-clamp amplifier (Axopatch 1-D, Axon Instruments Inc), as previously described.\(^16,17\) Recording electrodes were made from Pyrex glass capillary tubing (resistance, 5 to 6 MD). Test command potentials of 10 mV with a duration of 50 ms were applied every 20 seconds from a holding potential of

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−80 mV, unless otherwise stated. Membrane currents were digitized with a sampling frequency of 5 to 10 kHz and stored in a personal computer system for subsequent analysis. The liquid junction potential of 10 mV was corrected. The leak and residual capacitive currents were subtracted by use of the P/4 protocol. The traces are shown after low-pass filtering at 1 kHz. All experiments were performed at room temperature (22°C to 24°C). Average value of the cell capacitance was 17.8±0.2 pF (n=52).

To isolate Ca²⁺ channel currents, the pipette was filled with a high Ca⁺ solution of the following composition (mmol/L): CsCl 132.5, ATP-Na, 3, GTP 0.1, MgCl₂ 3, EGTA 10, and HEPES 10, pH 7.3 (titrated with CsOH). Ba²⁺ ion was used as a carrier of Ca²⁺ channel currents. The Ba²⁺-containing bath solution consisted of (mmol/L) BaCl₂ 10, NaCl 150, glucose 5.4, and HEPES 5, pH 7.3 (titrated with NaOH).

Ang II and angiotensin I (Ang I) were purchased from Sigma Chemical Co. Nifedipine, protein kinase C (PKC) inhibitor peptide-[19-36], calphostin C, and KT5720 were from Calbiochem. CV-11974, enalaprilat, and PD123319 were gifts from Takeda Pharmaceutical Co (Tokyo, Japan), Merck Co (West Point, Pa), and Parke-Davis Co (Ann Arbor, Mich), respectively.

The very tip of the recording pipette was filled with a drug-free solution (control solution), and the remainder of the pipette was filled with the drug-containing solution (test solution). In our preliminary experiments, a drug in the test solution diffuses and reaches the bath solution (control solution), and the reminder of the pipette was filled with a high Ca²⁺ solution. Without Ang II in the pipette solution, the amplitude of Ca²⁺ channel currents 5 to 6 minutes after the membrane rupture. Because the amplitude of the Ca²⁺/H₁₁₀₀₁ solution inhibits K⁺ channel currents, the pipette tip experiments, a drug in the test solution diffuses and reaches the bath solution (control solution), and the remainder of the pipette was filled with a drug-free solution (control solution). The Ang II action was not affected by the bath solution. The Ang II action, CV-11974 (10 nmol/L) in the pipette solution. CV-11974, but not Ang II, 10 nmol/L Ang II (n=16). Data are mean±SEM. **P<0.01. Illustrations on the right side indicate actual traces at 3 minutes and 10 minutes with absence (control, top) or presence (Ang II, bottom) of intracellular Ang II. B, Current (i)-voltage relationship of Ca²⁺ channel currents obtained at 3 minutes and 10 minutes with Ang II (10 nmol/L) in the pipette test solution. Plotted values are relative to current amplitude at 0 mV after 3 minutes. Data are mean±SEM (n=5). Illustrations on the right side indicate actual traces at −40, −20, 0, 20, and 40 mV at 3 minutes and 10 minutes. Duration of the command potential was 100 ms.

Results

Without Ang II in the pipette solution, the amplitude of Ca²⁺ channel current reached a steady level at ≈3 minutes after achieving the whole-cell condition and did not apparently run down for up to 20 minutes (Figure 1A).²⁻¹ When Ang II (10 nmol/L) was included in the test solution, the amplitudes of Ca²⁺ channel currents continued to increase for 7 to 8 minutes (Figure 1A). This continuous increase in the amplitude of Ca²⁺ channel currents was observed in the presence of Ang II at ≧1 nmol/L; however, the enhancement did not apparently differ among various Ang II concentrations (at 10 minutes: without Ang II, 106±6% [n=14]; at 0.1 nmol/L Ang II, 104±10% [n=13]; at 1 nmol/L Ang II, 126±9% [n=10], at 10 nmol/L Ang II, 133±9% [n=16]; and at 100 nmol/L Ang II, 130±9% [n=5]).

Figure 1B shows the effects of intracellular Ang II on the current-voltage relationship of the Ca²⁺ channel currents. Intracellular Ang II enhanced the amplitude but did not apparently affect the shape of the current-voltage curve, although the enhancement was less evident at negative potentials. The major part of the Ca²⁺ channel current in vascular muscle cells from the mesenteric artery was of the L type, which is sensitive to dihydropyridines, including nifedipine.¹⁷⁻¹⁸ However, nifedipine-insensitive Ca²⁺ channels exist in small branches of mesenteric arteries.¹⁸ To evaluate whether intracellular Ang II modifies nifedipine-insensitive Ca²⁺ channel currents, intracellular Ang II action was evaluated in the presence of 10 μmol/L nifedipine in the bath solution. The nifedipine-insensitive current was not enhanced by the intracellular dialysis of 10 nmol/L Ang II (at 10 minutes, 103±7% of the control [n=7]). These results suggest that administered Ang II enhances primarily L-type Ca²⁺ channels.

To determine which receptor subtype is involved with the Ang II action, CV-11974 (1 μmol/L), an Ang II type 1 (AT₁) receptor antagonist, or PD123319 (1 μmol/L), an Ang II type 2 (AT₂) receptor antagonist, was administered with Ang II (10 nmol/L) in the pipette solution. CV-11974, but not PD123319, inhibited the Ang II action (Figure 2). In the next experiment, to exclude the possibility that intracellular Ang II leaks from the cell and acts on the surface membrane AT₁ receptor, CV-11974 (1 μmol/L) was applied to the bath solution. The Ang II action was not affected by the bath application of CV-11974 (Figure 2). In our preliminary experiments, an intracellular administration of a peptide Ang II receptor antagonist such as [Sar,Val,Ala] Ang II alone had a weak stimulating action on Ca²⁺ channel currents. Thus, we did not use peptide Ang II antagonists as an inhibitor of intracellular Ang II–binding sites.

To evaluate whether intracellular Ang I affects Ca²⁺ channels, Ang I was administered instead of Ang II. Intracellular Ang I enhanced the amplitude of Ca²⁺ channel currents, and the enhancement was less evident at negative potentials. The major part of the Ca²⁺ channel current in vascular muscle cells from the mesenteric artery was of the L type, which is sensitive to dihydropyridines, including nifedipine.¹⁷⁻¹⁸ However, nifedipine-insensitive Ca²⁺ channels exist in small branches of mesenteric arteries.¹⁸ To evaluate whether intracellular Ang I modifies nifedipine-insensitive Ca²⁺ channel currents, intracellular Ang I action was evaluated in the presence of 10 μmol/L nifedipine in the bath solution. The nifedipine-insensitive current was not enhanced by the intracellular dialysis of 10 nmol/L Ang I (at 10 minutes, 103±7% of the control [n=7]). These results suggest that administered Ang I enhances primarily L-type Ca²⁺ channels.
cellular administration of Ang I (10 nmol/L) enhanced Ca\(^{2+}\)/H\(_{11001}\) channel currents. To evaluate whether Ang I is converted to Ang II intracellularly, enalaprilat (1 \(\mu\)mol/L) or CV-11974 (1 \(\mu\)mol/L) was administered to the pipette solution with Ang II. The effect of Ang I was inhibited by both enalaprilat and CV-11974 (Figure 3).

The signal-transduction pathway of intracellular Ang II action was evaluated in the next series of experiments. Intracellular dialysis of 10 \(\mu\)mol/L U-73122 (a phospholipase C inhibitor), 100 nmol/L calphostin C (a PKC inhibitor, \(n=5\)), or 1 \(\mu\)mol/L PKC inhibitor peptide-[19-36] inhibited the intracellular Ang II action. In contrast, intracellular dialysis of KT5720 (an inhibitor of cAMP-dependent protein kinase, 100 nmol/L), did not significantly affect the Ang II action (Figure 4).

**Discussion**

In the present study, we demonstrated for the first time that Ang II administered intracellularly stimulates voltage-operated Ca\(^{2+}\) channels in vascular smooth muscle cells. Our findings are in good agreement with previous observations that intracellular administration of Ang II increases intracellular Ca\(^{2+}\) concentration of smooth muscle and causes contraction via an extracellular Ca\(^{2+}\)-dependent mechanism.\(^\text{13,14}\)

The intracellular AT\(_1\) receptor antagonist inhibited the intracellular Ang II action, but the intracellular AT\(_2\) receptor

**Figure 2.** Effects of CV-11974 and PD123319 on the intracellular Ang II action. A, Relative amplitudes of the currents at various times are plotted. Except for the control condition, Ang II (10 nmol/L) was included in test pipette solutions. Control indicates without any drugs (\(n=14\)); Ang II, with Ang II only (\(n=16\)); CV-11974 (in), intracellular administration of CV-11974 (1 \(\mu\)mol/L, \(n=11\)); CV-11974 (ex), extracellular administration of CV-11974 (1 \(\mu\)mol/L, \(n=6\)); and PD123319 (in), intracellular administration of PD123319 (1 \(\mu\)mol/L, \(n=5\)). Currents were recorded in the same conditions as in Figure 1A. Data are mean±SEM. *\(P<0.01\). B, Current traces were recorded at 3 minutes and at 10 minutes with intracellular CV-11974 (top), extracellular CV-11974 (middle), and intracellular PD123319 (bottom) in addition to intracellular 10 nmol/L Ang II. C, Summary of effects of CV-11974 and PD123319 is shown. Relative amplitudes obtained at 10 minutes under various conditions are shown as follows (from left to right): without any drugs (control), Ang II only, Ang II and intracellular CV-11974, and with intracellular Ang II and intracellular PD123319. Current was evoked by the same protocol as described in Figure 1A. *\(P<0.05\) vs control.

**Figure 3.** Effects of intracellular Ang I on Ca\(^{2+}\) channel currents. The current was evoked by the same protocol as described in Figure 1A. A, Relative amplitudes of the currents at various times are plotted. Concentrations are as follows: Ang I only, 10 nmol/L (\(n=8\)); Ang I and enalaprilat, 1 \(\mu\)mol/L (\(n=5\)); and Ang I and CV-11974, 1 \(\mu\)mol/L (\(n=5\)). All drugs were administered in the test pipette solution. *\(P<0.01\). B, Current traces recorded at 3 minutes and at 10 minutes under various conditions are shown. C, Summary of the effects of Ang I action is shown. Relative amplitudes obtained at 10 minutes under various conditions are shown as follows (from left to right): without any drugs (control), Ang I only, Ang I and enalaprilat, and Ang I and CV-11974. *\(P<0.05\) vs control.

**Figure 4.** Effects of U73122, calphostin C, PKC inhibitor (PKCI) peptide-[19-36], and KT5720 on the intracellular Ang II action. Current was evoked by the same protocol as described in Figure 1A. Effects of various inhibitors in the presence of 10 nmol/L Ang II in the test pipette solution were assessed. A, Bars indicate relative amplitudes of the currents at 10 minutes under various conditions (mean±SEM). Control indicates without any drugs in the pipette; Ang II, with 10 nmol/L Ang II in the pipette solution. Other bars show results of concomitant dialysis of U73122 (10 \(\mu\)mol/L), calphostin C (100 nmol/L), PKC peptide-[19-36] (1 \(\mu\)mol/L), and KT5720 (100 nmol/L) with intracellular Ang II (10 nmol/L). *\(P<0.05\) vs control. B, Current traces at 3 minutes and at 10 minutes under various conditions.
antagonist and the extracellular AT₁ receptor antagonist did not affect the intracellular Ang II action. Thus, intracellular Ang II enhances Ca²⁺ channel current via intracellular Ang II–binding sites similar to the AT₁ receptor. In cultured rat aortic muscle cells, an injection of Ang II increased the cytosolic Ca²⁺ levels. This Ang II action was inhibited by intracellular administration of the AT₁ receptor antagonist, whereas the AT₁ receptor antagonist was not examined. In the rat aorta, an intracellular administration of Ang II caused contraction. A concomitant application of the AT₁ receptor antagonist inhibited this contraction, although the AT₂ receptor antagonist also inhibited the Ang II action with a weaker potency. The high sensitivity to the AT₁ receptor antagonist in those previous studies is in good agreement with our present observations. Because the AT₁ receptor antagonist did not affect the intracellular Ang II action in the present study, species and/or regional differences might exist.

Ang II has been shown to exist in the submembrane vesicle–like structure (endosome/lysosome) and in the nuclei of vascular smooth muscle cells. In addition, Haller et al have shown that after microinjection of Ang II into the cell, Ang II spreads throughout the cytosol and is then distributed in submembrane lysosomes and nuclei in cultured aortic muscle cells. Thus, it is possible that intracellular Ang II binding sites, which are responsible for the present results, can be located in submembrane lysosomes or nuclei.

It has not been clarified what type of Ang II–binding sites are involved in the intracellular Ang II action in the present study. A soluble Ang II–binding protein was reported first in the liver and then in various tissues, including vascular tissues. This protein has no significant homology with the plasma membrane AT₁ receptor and does not bind to losartan, an AT₁ receptor antagonist. Because the AT₁ receptor antagonist was effective in the present study, it is unlikely that this protein is involved. It has also been reported that Ang II–binding sites exist on nuclear membranes, which are sensitive to the AT₁ receptor. Because intracellular Ang II action appeared within several minutes of administration in the present study, nucleus receptors regulating gene transcription may not be involved. Another possible Ang II–binding site is the internalized AT₁ receptor. Binding of Ang II to the AT₁ receptor causes a rapid internalization of the receptor in various cell types, including vascular smooth muscle cells. Because the internalized receptors exist in endosomes/lysosomes, it could be speculated that intracellularly administered Ang II is distributed to lysosomes and binds to the internalized AT₁ receptors.

In vascular smooth muscle cells, agonists that stimulate phospholipase C, including Ang II and norepinephrine, enhance L-type Ca²⁺ channel current. The stimulatory action is explained, in most studies, by the activation of PKC. Stimulation of cAMP-dependent protein kinase also enhances L-type Ca²⁺ channel currents; however, in some conditions, such stimulation inhibits or does not affect L-type Ca²⁺ channel currents. In the present study, an inhibition of cAMP-dependent protein kinase by KT5720 did not affect the L-type Ca²⁺ channel currents, but that of phospholipase C (U-73122) or PKC (calphostin C and PKC inhibitory peptide) inhibited the intracellular Ang II action. Thus, an activation of phospholipase C and PKC is likely to be involved in the intracellular action of Ang II. It is interesting that internalization of the AT₁ receptor with Ang II appears to be essential to the full expression of Ang II action in vascular smooth muscle, which includes the sustained activation of PKC. Another possible pathway is through the activation of tyrosine kinase; however, information about the role of tyrosine kinase on the regulation of Ca²⁺ channels in smooth muscle cells has not been accumulated.

Although endocytosis or internalization is an important source of intracellular Ang II in vascular muscle cells, Ang II is also considered to be generated inside the cells. Components required for Ang II generation, such as angiotensinogen, renin, and ACE, have been shown to exist in the cell. In the present study, an intracellular administration of Ang I stimulated the Ca²⁺ channel current; this stimulation was inhibited by enalaprilat and CV-11974. Our observation suggested that ACE activity exists in the cytosol and plays an essential role in converting Ang I to Ang II. Ang II but not Ang I acts on the intracellular Ang II–binding sites. Although Ang II–generating enzymes including chymase do play a role in various tissues, their contribution may be limited in guinea pig vascular smooth muscle cells. These results are in contrast to the observation of Brailoiu et al that captopril failed to inhibit the intracellular Ang I action on contraction of the aorta. The reason for this discrepancy is not known at present.

The present study showed that intracellular Ang II concentrations of ≥1 nmol/L were effective in enhancing the Ca²⁺ channel currents. These concentrations are nearly the same as those reported to be necessary for stimulating Ca²⁺ influx in cultured aortic muscle cells and contraction of the aorta. It has been also shown that tissue concentrations of Ang II become higher than plasma concentrations after AT₁ receptor–dependent internalization in the kidney and adrenal tissues. Thus, the concentrations examined in the present study could be sufficient to stimulate Ca²⁺ channels in the physiological condition. In the present study, a dose-response relationship for the intracellular Ang II action was not apparent. Although the reason for this is not known, it is possible that a slow diffusion of Ang II from the pipette to the cytosol and accumulation of Ang II inside the cell may mask the dose-response relationship.

In summary, we have demonstrated that intracellular administration of Ang II and Ang I enhances L-type Ca²⁺ channel currents. Ang I could be converted to Ang II by ACE and affect Ca²⁺ channels. Our observation also suggests that intracellular binding sites similar to the AT₁ receptor, which is associated with phospholipase C and PKC, are involved in the intracellular Ang II action.

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**References**


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