Involvement of a GTP-Binding Protein in Stimulating Action of Angiotensin II on Calcium Channels in Vascular Smooth Muscle Cells

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The possible involvement of a GTP-binding protein in the regulation of Ca\(^{2+}\) channels by angiotensin II (Ang II) in vascular muscle cells was investigated by the whole-cell voltage-clamp method. Single cells were freshly isolated from guinea pig portal vein. The pipette solution contained high Cs\(^+\) to inhibit K\(^+\) currents and thereby isolate the Ca\(^{2+}\) channel current. Ba\(^{2+}\) (2 mM) was in the bath solution as a charge carrier for the Ca\(^{2+}\) channel. Application of Ang II (0.1–100 nM) produced an increase in peak amplitude of the Ba\(^{2+}\) current, with a shift of the current–voltage curve in the negative direction. These effects were inhibited by pretreatment with an antagonist of the Ang II receptor, [Sar\(^1\),Ile\(^8\)]-Ang II. Presence of 0.1 mM GTP in the pipette solution stabilized the Ang II action, but 0.3–1.0 mM GDP-\(\beta\)-S and 1.0 mM GTP-\(\gamma\)-S inhibited it. GTP-\(\gamma\)-S alone produced a slowly progressing increase in the basal (unstimulated) current amplitude. Preincubation of muscle tissues with pertussis toxin (1 \(\mu\)g/ml, for up to 6 hours at 36°C) or intracellular application of preactivated pertussis toxin (1 \(\mu\)g/ml plus NAD (1 mM) did not inhibit the Ang II action. Cholera toxin (10 \(\mu\)g/ml) also had no effect on the Ang II action. These results suggest that the Ang II stimulation of Ca\(^{2+}\) channels in smooth muscle of guinea pig portal vein may be mediated by a G protein that is insensitive to both pertussis toxin and cholera toxin. (Circulation Research 1991;68:763–771)

Relatively little is known about mechanisms of action of various hormones on Ca\(^{2+}\) slow channels in vascular smooth muscle cells. Several recent studies showed that some hormones, such as norepinephrine and angiotensin II (Ang II), enhanced the activation of voltage-dependent Ca\(^{2+}\) channels.\(^1\)–\(^3\) This action is considered to be one of the mechanisms for the contraction induced by these agonists. However, other reports suggested that these agonists actually inhibited the calcium channels.\(^4\) Therefore, there is controversy concerning the mechanism of the hormonal modulation of the Ca\(^{2+}\) channels, and the agonists may act by more than one mechanism (multifactor).

Ang II is a potent vasoconstrictor, and application of this hormone depolarized and triggered action potentials and prolonged action potential duration in rat portal venous cells\(^5\) and cultured rat aortic cells.\(^6\) Ang II also was reported to enhance Ca\(^{2+}\) channel currents of rabbit aortic cells.\(^3\) In the present study, we investigated the mechanisms underlying Ang II action on the Ca\(^{2+}\) channels in vascular smooth muscle cells from guinea pig portal vein. The involvement of a GTP-binding protein in Ang II action on the Ca\(^{2+}\) channels was found; this protein is insensitive to pertussis toxin (PT) and cholera toxin (CT).

Materials and Methods

Cell Dispersion

Single smooth muscle cells were freshly isolated from guinea pig (200–300 g) portal vein by collagenase treatment. Details of the methods used were described previously.\(^\text{7,8}\) After isolation, the cells were placed in Ca\(^{2+}\)-free solution and stored in a refrigerator (5–7°C). The composition of this solution was (mM) NaCl 145, KCl 6, glucose 10, and HEPES 10, pH 7.3 (titrated with NaOH), containing 2 mM Mg\(^{2+}\), 0.2% bovine serum albumin (essentially fatty acid free), and 0.1% trypsin inhibitor (type II-S). The cells were used for experiments within 2 hours after dispersion. When the cells were stored in the Ca\(^{2+}\)-
free solution, the enhancement of the calcium channel current by Ang II was observed more consistently than when they were stored in a Ca\(^{2+}\)-containing solution.

Noncontracted cells were spindle-shaped (length, 100–200 \(\mu\)m; diameter, 3–8 \(\mu\)m). In a physiological salt solution containing 2 mM Ca\(^{2+}\), the cells contracted in response to bath application of various agonists, such as norepinephrine (more than 10 \(\mu\)M), Ang II (more than 1 nM), and ATP (more than 30 \(\mu\)M).

**Electrical Recording**

Whole-cell voltage clamp was performed with a suction pipette through a single-electrode voltage-clamp amplifier (EPC-7, List Electric, Darmstadt-Eberstadt, FRG).\(^9\) The recording electrodes were made from borosilicate glass capillary tubing (resistance, 4–6 M\(\Omega\)). Lower resistance electrodes (less than 2 M\(\Omega\)) usually caused loss of the Ang II sensitivity of the cells. Membrane currents were filtered through an eight-pole Bessel-type filter (1–3 KHz) and were digitized and stored in a microcomputer for subsequent analysis. All experiments were performed at room temperature (20–22°C).

To record the Ca\(^{2+}\) channel current, test command potentials were applied every 10 seconds from a holding potential of \(-90\) mV. The maximal peak amplitudes of the Ca\(^{2+}\) channel currents were less than 500 pA (usually 100–300 pA), and the input resistance of the cell was more than 5 G\(\Omega\). Series resistance was partly compensated electrically, usually 60–80%. Leak and residual capacitive currents were subtracted using the currents elicited by small hyperpolarizing or depolarizing pulses. The background noise was reduced by signal averaging. The liquid junction potential between the pipette and bath solution was corrected (approximately 10 mV).

Ba\(^{2+}\) currents did not run down, under the conditions used, for more than 15 minutes (at 15 minutes, the amplitude was 97±6% \([n=12]\) of control).

**Solutions and Chemicals**

To isolate the inward currents, the pipette was filled with a high Cs\(^{+}\) solution of the following composition (mM): Cs\(^{+}\) 130, Na\(^{+}\) 10, ATP 5.1, GTP 0.1, Mg\(^{2+}\) 5.2, EGTA 10, Cl\(^-\) 30, glutamate\(^-\) 112, and HEPES 10, pH 7.2 (titrated with CsOH). The bath solution (Na\(^{+}\)-free, K\(^{+}\)-containing (mM) Tris\(^+\) 100, tetraethylammonium (TEA\(^+\)) 50, Ba\(^{2+}\) 2, HEPES 10, Cl\(^-\) 154, glucose 10, and 4-aminopyridine 3, pH 7.3 (titrated with CsOH).

Ang II and [Sar\(^1\),Ile\(^8\)]-Ang II were from Sigma Chemical Co., St. Louis. GTP (disodium salt), guanosine 5’-O-(3-thiotriphosphate) (GTP-\(\gamma\)-S) (tetra-lithium salt), and guanosine 5’-O-(2-thiodiphosphate) (GDP-\(\beta\)-S) were from Boehringer Mannheim Corp., Indianapolis, Ind. PT, CT, dithiothreitol (DTT), and nicotinamide adenine dinucleotide (NAD) also were from Sigma.

Ang II, [Sar\(^1\),Ile\(^8\)]-Ang II, and CT were applied to the bath solution. The bath (content volume, 0.2 ml) was superfused continuously at a constant flow rate (1–2 ml/min), and the excess fluid was siphoned off. The test solution reached the cell 5–8 seconds after the exchange of bath solutions was started, and it took 15–30 seconds for complete exchange of the whole bath.

Effects of GTP analogues were determined to assess the possible involvement of GTP-binding proteins in the Ang II action on the calcium channels. GTP-\(\gamma\)-S and GDP-\(\beta\)-S were included in the pipette solution instead of GTP. These agents diffused into the cell interior in the whole-cell configuration (cell dialysis). We used two different incubation times for diffusion of these agents—a shorter time (1–3 minutes) and a longer time (5–8 minutes). After such incubation periods, 10 nM Ang II was applied to the bath solution. Then, effects of the GTP analogues on the Ang II action were assessed, comparing the Ang II–induced enhancement of current amplitudes under various conditions.

**Toxin Treatments**

Two methods were used to determine the effect of PT on the Ang II action on the calcium channels. One method was to incubate the smooth muscle tissues with PT before collagenase treatment.\(^10\),\(^11\) We preincubated the smooth muscle tissue with 10 \(\mu\)g/ml PT for 4–6 hours at 36°C in modified Krebs’ solution. The other method was to include preactivated PT in the pipette solution.\(^11\),\(^12\),\(^13\) To preactivate PT, 10 \(\mu\)g/ml PT was incubated with 1 mM NAD and 5 mM DTT for 1 hour in the pipette solution (containing 5.1 mM ATP). Then the solution was diluted 10-fold with fresh pipette solution so that the final concentration of preactivated PT was 1 \(\mu\)g/ml (1 mM NAD, 0.5 mM DTT). Both methods were demonstrated to be effective to inactivate G\(_i\) (or G\(_s\)) protein in whole-cell voltage-clamp studies in single intestinal smooth muscle cells.\(^11\) Inoue and Isenberg\(^11\) used cells of similar diameter (but longer length), similar pipette diameter, and similar time for incubation, but lower concentrations of chemicals, as compared with the present study.

CT was dialyzed for 24 hours at room temperature to remove sodium azide. CT (10 \(\mu\)g/ml) was applied to the cell suspension 30–60 minutes before the voltage-clamp experiment was started at 36°C. This concentration and incubation time was reported to be sufficient to induce responses in tissue preparation of guinea pig mesenteric artery.\(^14\)

Control experiments for toxin treatments were done using the same incubation procedure with the same solutions but without toxins.

**Data Analysis**

Data are given as mean±SEM. Statistical significance was determined by an analysis of variance and then a post hoc test. Values of \(p<0.05\) were considered statistically significant.
Results

With 2 mM Ba\(^{2+}\) in the bath solution, calcium channel currents were recorded by command potentials of more than -50 mV from a holding potential of -90 mV (Figures 1Aa and 1Ba). Maximum amplitude was observed around -10 mV. Decay of the calcium channel current was slow at command potentials of less than 10 mV. Application of 10 nM Ang II in the bath solution gradually enhanced the inward current (Figure 1Ab). Threshold was shifted to -60 mV, and maximum amplitude was observed around -20 mV (Figure 1Ba). Peak amplitudes and amplitudes measured at 200 msec before and after application of Ang II are plotted in Figures 1Ba and 1Bb, respectively. In both cases, the current amplitudes were similarly enhanced by Ang II at all command potential levels (Figure 1Ba versus Figure 1Bb).

Time courses of the increase in the peak current amplitude induced by three different concentrations of Ang II are plotted in Figure 2. The time delay was shorter with higher concentrations of Ang II. At a low concentration (0.1 nM), the increase in the amplitude usually appeared slowly (Figure 2A). Higher concentrations (10 nM and 1 μM) of Ang II produced a much more rapid enhancement of the current amplitude (Figures 2B and 2C). The enhancement of the current by Ang II was transient.

The dose–response relation of the Ang II action on peak amplitudes of the calcium channel currents is plotted in Figure 3 (open circles). Ang II was applied after 1–3 minutes of dialysis of a cell with the pipette solution. The effect was measured at the maximal response and expressed as a relative current amplitude. Ang II (0.1–10 nM) dose-dependently enhanced the calcium channel current. The enhancement was maximal at approximately 100 nM Ang II. This stimulation was more consistent with the presence of 0.1 mM GTP in the pipette solution. Without GTP, 10 nM Ang II enhanced the current by 1.55 times (1.55±0.21, n=10), whereas with GTP, the enhancement was 1.88 times (1.88±0.14, n=18). Pretreatment with 1 μM [Sar\(^{1},\)Ile\(^{8}\)]-Ang II, an antagonist of Ang II, inhibited the action of Ang II (Figure 3, filled circles).

The effects of intracellular GTP-γ-S were examined; GTP-γ-S was dialyzed into the cell for either 1–3 or 5–8 minutes. As a control, the effect of Ang II was examined using GTP (0.1 mM) dialysis for 1–3 or 5–8 minutes. Although the Ca\(^{2+}\) channel current did not run down in this condition, the Ang II enhancement ran down slightly: 10 nM Ang II enhanced the current 1.88 times (±0.14, n=18) at 1–3 minutes and 1.51 times (±0.10, n=7) at 5–8 minutes. When the pipette solution contained GTP-γ-S, the short (1–3
minutes) incubation did not alter the Ang II effect (Figure 4A a). The longer (5–8 minutes) incubation with GTP-γ-S gradually enhanced the basal (unstimulated) current before application of Ang II (Figure 4A b). As illustrated in Figure 4A b, a 7-minute incubation with 1 mM GTP-γ-S inhibited the Ang II action. A summary of the changes in the current amplitudes during GTP-γ-S incubation and Ang II application is plotted in Figure 4B, just after the cell dialysis was started, (0 minutes), before application of Ang II (1–3 or 5–8 minutes), and after application of Ang II (A-II). This figure shows that longer incubation with GTP-γ-S enhanced the basal current with consequent inhibition of the Ang II effect, but that the shorter incubation did not. A summary of the effects of Ang II with different conditions (with 0.1 mM GTP or with 0.3 and 1 mM GTP-γ-S) is presented in Figure 4C. Current amplitudes obtained after Ang II application were normalized to those obtained just before Ang II addition. This figure shows that higher concentration and longer incubation with GTP-γ-S inhibited the Ang II action.

Similar experiments were performed using GDP-β-S. Incubation with GDP-β-S did not change the basal current; 5–8 minutes after incubation with 1 mM GDP-β-S, the amplitude of the current remained at 0.98±0.07 (n=5) of control. After incubation with 1 mM GDP-β-S for 7 minutes, Ang II failed to stimulate the Ca²⁺ channel current (Figure 5A).

FIGURE 2. Time courses of the changes in amplitude of the Ca²⁺ channel current (Ba²⁺ current) evoked by three different concentrations of angiotensin II (A-II): 0.1 nM (panel A), 10 nM (panel B), and 1 μM (panel C). A-II application in the bath solution is indicated by a horizontal bar. Current was evoked by a command potential of −20 mV from a holding potential of −90 mV. Actual current traces are presented in the right column before (trace a), 2 minutes after (trace b), and 5 minutes after (trace c) application of A-II. Current traces a, b, and c correspond to the amplitude indicated by a, b, and c in the left-hand plots. Solutions were the same as in Figure 1.

FIGURE 3. Dose–response relation for the action of angiotensin II (A-II) on the Ca²⁺ channel current. Relative amplitudes (open circles) recorded after application of various A-II concentrations were plotted against log A-II concentration. Currents recorded after A-II application following pretreatment of the cells with the A-II antagonist [Sar²Ile⁷]–A-II (1 μM) also are plotted (filled circles). The horizontal dotted line indicates the basal (unstimulated) Ca²⁺ channel current. The numbers in parentheses indicate the n values.
Effects of Ang II on the calcium channel current under different conditions (with 0.1 mM GTP or with 0.3 and 1 mM GDP-β-S) are plotted in Figure 5B. This figure shows that higher concentration and longer incubation with GDP-β-S inhibited the effect of Ang II.

In an attempt to further clarify the involvement of GTP-binding protein, the bacterial toxins PT and CT were used, as described in “Materials and Methods.” Toxin treatments did not produce significant change in the averaged amplitude of the basal current (unstimulated): 1) PT preincubation: control, 138.3±19.2 pA (n=11) versus toxin, 130.2±22.1 pA (n=10); 2) preactivated PT: control, 135.23±22.0 pA (n=9) versus toxin, 120.9±20.9 pA (n=8); and 3) CT incubation: control, 152.3±21.1 pA (n=5) versus toxin, 148.3±19.3 pA (n=5).

Both methods of introducing PT into the cell—that is, pretreatment of tissues by 1 µg/ml PT (Figure 6Aa) or intracellular application of preactivated PT (1 µg/ml) (Figure 6Ab)—did not change the Ang II effect. Pretreatment of the single cells with 10 µg/ml CT also did not alter the Ang II action. Effects of Ang II with different toxin treatments are summarized in Figure 6B. No significant effects on the Ang II action were observed by these toxin treatments.

Discussion

Ang II contracts vascular smooth muscle cells both by increasing Ca²⁺ influx and by triggering Ca²⁺ release from intracellular store sites. Enhancement of the spontaneous action potential spike activity was reported in rat portal vein without depolarization by low concentrations of Ang II and with
depolarization by high concentrations of Ang II. In rat cultured aortic muscle cells, Ang II enhanced the calcium-dependent action potentials. The present study showed that Ang II enhanced calcium channel currents. This stimulatory effect of Ang II on the calcium channels is in good accord with previous reports.

Besides enhancement of the amplitude, Ang II shifted the current-voltage relation in the negative direction to 5–15 mV. With 2 mM Ba²⁺, threshold was between −60 and −50 mV, and maximum amplitude was observed around −10 mV. With 2 mM calcium, threshold of the current was approximately −40 mV, and maximum amplitude was observed at approximately 0 mV. If simple extrapolation is applied, in physiological solution (2 mM Ca²⁺), the threshold potential should be shifted to −55 to −45 mV. This potential is close to the resting membrane potential reported in this tissue. This also could explain the fact that Ang II can enhance spike activity without depolarization.

In the present study, we did not distinguish between the slow (L-type, high-threshold) and fast (T-type, low-threshold) calcium channel currents, because changing holding potential failed to separate the basal current into two components in unstimulated conditions (data not shown). Furthermore, the shape of current traces at the lower command potential had almost no decay, indicating there was only little fast current in unstimulated conditions (Figure 1). After application of Ang II, the current–voltage curve was shifted in the negative direction, and decay of the current was slightly accelerated. These changes suggest that the fast Ca²⁺ current appeared after stimulation by Ang II. However, the current amplitudes measured at the peak and at the end of the pulse (200 msec) were enhanced equally by Ang II. This argues against the predominant enhance-
ment of the fast current. Another possibility is that the shift of the current–voltage curve and the change in current decays may be due to a kinetic change of the slow Ca\(^{2+}\) channels.

The action of Ang II was time dependent. After application of Ang II, the current at first increased and then decayed slowly. Higher concentrations of Ang II provoked faster increase and faster decay. Two possible mechanisms may be considered to explain the transient action: 1) only an excitatory mechanism exists, and it works transiently, or 2) at least two mechanisms (excitatory and inhibitory) are provoked by Ang II. A similar time-dependent action of Ang II was seen in force recording and in the calcium transient (using fura 2).

It was thought that this transient action was closely related to a tachyphylaxis usually observed in the Ang II response in vascular tissues.

The analogues of GTP, GDP-\(\beta\)-S and GTP-\(\gamma\)-S, are considered to be good tools to examine the involvement of G protein in the receptor coupling with various effectors, including gating of ion channels by G proteins. GTP-\(\gamma\)-S is thought to 1) substitute for endogenous GTP at the binding site of the G protein, 2) be a poor substrate for GTPase, causing a persistent effect, and 3) cause slowly increasing activation of G protein in the absence of agonist stimulation. In electrophysiological studies, a persistent and strong effect of GTP-\(\gamma\)-S was observed in the activation of the G\(_x\) channel in atrial cells and in the inhibition of the calcium currents of neuronal cells. In the present study, GTP-\(\gamma\)-S induced a slowly progressive increase in the basal current amplitude and consequently inhibited the subsequent Ang II action. The progressive increase in the current amplitude might be due to the slow increase in degree of activation of G protein.

Thus, the usual stimulatory effect of Ang II on the Ca\(^{2+}\) channels would be inhibited.

If GTP-binding protein is involved, the effect induced in the presence of GTP-\(\gamma\)-S would be irreversible. However, in the present experiments, the effect induced by Ang II in the presence of GTP-\(\gamma\)-S was transient. This also indicates the presence of a secondary inhibitory effect produced by Ang II, as suggested above.

GDP-\(\beta\)-S is a competitive inhibitor of GTP binding to the G proteins. There are several reports using electrophysiological studies that intracellular GDP-\(\beta\)-S application blocked the agonist-dependent responses. In the present study, GDP-\(\beta\)-S dose-dependently suppressed the effect of Ang II. Therefore, this provides positive evidence of the involvement of G protein.

GDP-\(\beta\)-S had little inhibitory effect on the unstimulated Ca\(^{2+}\) channel current. This might be due to a different regulation of the basal (unstimulated) current and the agonist-stimulated current. In cardiac cells, different regulations of the Ca\(^{2+}\) channels between the unstimulated (basal) condition and the stimulated condition (\(\beta\)-agonist) were observed. It was reported in guinea pig ventricular cells that application of an inhibitor for cyclic AMP-dependent phosphorylation did not suppress the basal current but only inhibited the action of \(\beta\)-agonists. This suggested that basal activity of the Ca\(^{2+}\) channels in ventricular cells may be independent of cyclic AMP–dependent phosphorylation of the channel protein.

High concentration and long incubation of the GTP analogues were needed to inhibit the Ang II action on the Ca\(^{2+}\) channels, and the inhibition was not complete. Also, the GTP-\(\gamma\)-S–induced enhancement was not as large as the Ang II–induced enhancement, although GTP-\(\gamma\)-S should produce a strong enhancement of the calcium channel current. Perhaps substitution by a GTP analogue takes time in the whole-cell patch configuration, or contamination of other chemicals in the GTP-\(\gamma\)-S (and GDP-\(\beta\)-S) may interfere, as suggested. Furthermore, the low temperature (22°C) used in the present experiments might be another possible reason for the weak action of the GTP analogue.

PT is a bacterial toxin that ribosylates the subunit of G, or G-like protein, and consequently inhibits the action of G protein (see Reference 27). Pretreatment of smooth muscle tissue by PT for 4–6 hours at 36°C and application of preactivated PT failed to suppress the Ang II action on the Ca\(^{2+}\) channels. In biochemical and electrophysiological studies, these procedures were demonstrated to be effective in ribosylating the GTP-binding protein in smooth muscle tissues. To confirm our results, we treated the muscle tissues by PT (1 \(\mu\)M) for 24 hours at 36°C and measured contractile force induced by high K\(^+\) and by Ang II. There were no significant differences between control tissue (incubated without PT) and PT–pretreated tissue in the contraction induced by 10 nM Ang II (Y. Ohya, unpublished observations, 1990).

The effects of PT treatment on the contractions and Ca\(^{2+}\) transients (using Ca\(^{2+}\)-sensitive dyes) of vascular smooth muscle in response to agonists such as norepinephrine, serotonin, or Ang II are controversial. In dog and rabbit mesenteric artery, PT treatment abolished the increase in calcium measured by fura 2 and contraction induced by norepinephrine and Ang II. Inhibition of Ca\(^{2+}\) transients also was reported in cultured rat aortic muscle cells in response to norepinephrine, Ang II, and serotonin; however, there was only partial inhibition. Furthermore, even in the same tissue (cultured rat aortic cells), others reported that PT treatment did not alter the [Ca\(^{2+}\)] increase produced by Ang II. Others also reported, in dog coronary artery, that PT pretreatment had little effect on the contraction induced by acetylcholine. In an electrophysiological study, Nozaki and Sperelakis reported that in guinea pig mesenteric artery PT treatment did not change the resting potential, input resistance, or depolarization induced by norepinephrine or ATP. These reports suggest that there may be differences
in PT effects among species and regional vascular beds. They also suggest that the [Ca], increase and the contraction produced by agonists may be mediated by mechanisms that are PT-sensitive and PT-insensitive.

Another important toxin for investigation of the involvement of GTP-binding protein in the agonist response is CT. CT ribosylates G protein. It was reported that G protein had a direct stimulatory action on the calcium channels of cardiac muscles. In vascular smooth muscle tissues, CT was reported to inhibit an agonist-induced contraction, probably because of increasing cytosolic cyclic AMP level. Application of CT in the present study did not alter the Ang II response, suggesting that G protein does not play an important role in this preparation.

In the present study, we demonstrated that activation of the Ang II receptor stimulated, dose-dependently and time-dependently, the voltage-dependent Ca\(^{2+}\) channel of vascular smooth muscle cells from guinea pig portal vein. This stimulatory effect was mediated by a G protein that is insensitive to PT and CT. It remains to be determined what type of second messenger system is involved in this G protein-mediated response. G proteins regulate various enzyme systems, such as adenylate cyclase, which regulates the production of cAMP, and phospholipase C, which regulates the turnover of phosphatidylinositol metabolites. Another possible mechanism is the direct regulation or gating of Ca\(^{2+}\) channels by G protein, as suggested in cardiac cells and neurons. Ang II also may induce a second inhibitory response after the stimulatory effect on the calcium channels.

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