Intracellular Angiotensin II Regulates the Inward Calcium Current in Cardiac Myocytes

Walmor C. De Mello

Abstract—The influence of intracellular administration of angiotensin II (Ang II) on the inward calcium current (I\textsubscript{Ca}) was investigated in single myocytes isolated from adult rat ventricle. Comparative studies were also made in ventricular cells of Golden hamsters. The I\textsubscript{Ca} was measured in single cells using the whole-cell voltage clamp configuration. The results indicated that Ang II (10\textsuperscript{-6} mmol/L) dialyzed into the rat myocytes reduced the peak I\textsubscript{Ca} by 35±5.5% (n=20; P<0.05). Losartan (10\textsuperscript{-7} mmol/L) added to the bath did not suppress the effects of Ang II, indicating that the peptide is acting intracellularly. Moreover, the intracellular dialysis of losartan (10\textsuperscript{-6} mmol/L) or [Sar\textsuperscript{1} Val\textsuperscript{5} Ala\textsuperscript{8}] Ang II (10\textsuperscript{-6} mmol/L) did not change the effect of Ang II. Stimulation of I\textsubscript{Ca} by exogenous cAMP or inhibition of protein kinase C did not alter the effect of Ang II on I\textsubscript{Ca}. Zaprinast (100 μmol/L), an inhibitor of cGMP phosphodiesterase, when added to the bath solution increased appreciably the effect of Ang II on I\textsubscript{Ca} (P<0.05). In ventricular myocytes of Golden hamsters, in which Ang II has a positive inotropic action, the intracellular administration of Ang II (10\textsuperscript{-8} mmol/L) increased I\textsubscript{Ca} by 36±2.4% (n=20; P>0.05). The effect of the peptide was not altered by the intracellular administration of losartan (10\textsuperscript{-6} mmol/L), by [Sar\textsuperscript{1} Val\textsuperscript{5} Ala\textsuperscript{8}] Ang II (10\textsuperscript{-6} mmol/L), or by the inhibitor of protein kinase A. The inhibition of protein kinase C, however, prevented the effect of Ang II I\textsubscript{Ca} in the hamster myocytes. The results particularly suggest that the activation of the cardiac renin-angiotensin system regulates I\textsubscript{Ca} and myocardial contractility, an effect that varies with the species. (Hypertension. 1998;32:976-982.)

Key Words: angiotensin II, intracellular calcium current, myocytes

There is increasing evidence that there is a local renin-angiotensin system in the heart.\textsuperscript{1} Indeed, genes of angiotensinogen and renin are coexpressed in isolated cardiac myocytes.\textsuperscript{2,5} Angiotensin I (Ang I), angiotensin II (Ang II), and the angiotensin-converting enzyme have been found around the nucleus of heart cells in culture,\textsuperscript{6} and Ang I is converted to Ang II in the isolated and perfused rat heart.\textsuperscript{7}

In the last 5 years evidence has been accumulated that the plasma and the cardiac renin-angiotensin system contribute to the regulation of intercellular communication in heart muscle. For example, Ang II reduces whereas enalapril, an angiotensin-converting enzyme inhibitor, increases the gap junction conductance not only in cells of the adult rat ventricle\textsuperscript{8} but also in cardiomyopathic hamsters.\textsuperscript{9} Further studies showed that the intracellular administration of Ang I decreases cell communication, an effect suppressed by intracellular dialysis of enalaprilat,\textsuperscript{10} supporting the view that the decline in junctional conductance is related to the conversion of Ang I to Ang II inside the cardiac myocyte.

No information is available on whether the synthesis of Ang II inside the heart cells regulates other important membrane functions such as the inward calcium current (I\textsubscript{Ca}). This is particularly important because Ang II has a positive inotropic action in different preparations,\textsuperscript{11} whereas in the rat heart the peptide reduces the action potential duration\textsuperscript{12} and has a negative inotropic action.\textsuperscript{13} Previous observations by Allen et al\textsuperscript{14} indicated that Ang II when added to the bath increases the L-type calcium current in cultured neonatal heart cells. Recently, Habuchi et al\textsuperscript{15} demonstrated that in the sinoatrial node of the rabbit, Ang II reduces I\textsubscript{Ca} when added to the extracellular fluid.

In the present study, the possible effect of intracellular administration of Ang II on I\textsubscript{Ca} was investigated in myocytes isolated from adult rat ventricle and also from the ventricle of Golden hamsters.

Methods

Adult rats (Sprague-Dawley, Indianapolis, Ind) weighing 125 to 150 g were used. In some experiments adult Golden hamsters (Biobreeders, Watertown, Mass) of similar weight were used. The animals were kept in air-conditioned facilities on a normal laboratory animal diet and given tap water ad libitum. The animals were anesthetized with sodium pentobarbital (50 mg/kg IP), and the heart was removed with the animals under deep anesthesia. The animals were kept in air-conditioned facilities on a normal laboratory animal diet and given tap water ad libitum. The animals were anesthetized with sodium pentobarbital (50 mg/kg IP), and the heart was removed with the animals under deep anesthesia.

Cells were obtained by enzymatic dispersion of the ventricle according to the methods of Powell and Twist\textsuperscript{16} and Tanigushi et al.\textsuperscript{17}

The heart was removed and immediately perfused with normal Krebs’ solution containing (mmol/L) NaCl 136.5, KCl 5.4, CaCl\textsubscript{2}...
The pipettes, which were prepared immediately before the experiment, were filled with the following solution (mM): potassium aspartate 120, NaCl 10, MgCl₂ 3, EGTA 10, tetraethylammonium chloride 20, Na₂ATP 5, and HEPES 5, with pH adjusted to 7.3. In some experiments, cesium aspartate replaced potassium aspartate. Ang II was dialyzed into the cells.

To investigate the possible influence of intracellular Ang II on ICₐ, losartan, a specific Ang II AT₁ receptor antagonist (10⁻⁶ mmol/L), was added to the perfusion fluid, and after 5 minutes of equilibration with this compound, Ang II (10⁻⁶ mmol/L) was dialyzed into the cell while the ICₐ was monitored. The results indicated no influence of losartan on the effect of Ang II on ICₐ (n=8, P>0.05; not shown). In 7 experiments, losartan (10⁻⁶ mmol/L) was added to the pipette solution, the compound was dialyzed into the cell for 4 minutes, and then Ang II (10⁻⁶ mmol/L) was administered intracellularly. The results indicated no influence of losartan on the effect of Ang II on ICₐ (P>0.05; not shown).

These observations led to the idea that the effect of intracellular administration of Ang II on ICₐ is related to some intracellular action of the peptide.

Biochemical studies have shown previously that Ang II when added extracellularly inhibits cAMP production in rat myocardium (see Reference 19). To investigate whether the decline in ICₐ described above is related to the inhibition of cAMP production, isolated cells were exposed to dibutyryl-cAMP (10⁻⁶ mmol/L) for several minutes, and as soon as the increase in ICₐ elicited by dibutyryl-cAMP reached a maximal and steady value, the peptide (10⁻⁶ mmol/L) was dialyzed into the cell. Figure 3 demonstrates that despite the stimulation of ICₐ by exogenous cAMP, the effect of Ang II on ICₐ was not changed (P>0.05). In other experiments in which the cells had been previously exposed to Krebs’ solution containing forskolin (10⁻⁶ mmol/L), an activator of adenyl cyclase, the effect of internal administration of Ang II (10⁻⁶ mmol/L) on ICₐ was similar to that seen in the control cells (P>0.05; not shown).

The possibility that Ang II reduces ICₐ by activating protein kinase C (PKC) was also investigated. The pseudosubstrate of the kinase, an inhibitor of PKC (20 µg/mL), was dialyzed into the cell for 4 minutes before the addition of Ang II (10⁻⁶ mmol/L) to the internal solution. The results from 8 experiments indicated that the pseudosubstrate of PKC did not alter the effect of Ang II on ICₐ (Figure 3). Staurosporine (50 µmol/L), a nonpeptide inhibitor of PKC, dialyzed into the cell for 4 minutes was also unable to suppress the effect of intracellular Ang II on ICₐ. Indeed, in 6 experiments with

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**Drugs**

Dibutyryl-cAMP, forskolin, the pseudosubstrate of protein kinase C, staurosporine, Ang II, [Sar¹ Val⁵ Ala⁸] Ang II, the inhibitor of protein kinase A, phorbol 12-myristate 13-acetate, and zaprinast were from Sigma Chemical Co. PD 123,319 was from Fluka Laboratories, and losartan was a gift from DuPont Merck (West Point, Pa).

**Experimental Procedures**

All experiments were performed in a small chamber mounted on the stage of an inverted phase-contrast microscope (Diaphot; Nikon). A video system (Diaphot; Nikon) made it possible to inspect the cells and the pipettes throughout the experiments. The electrical measurements were carried out in single ventricular myocytes using the whole-cell configuration. Series resistance originated from the tips of the micropipettes was compensated for electronically at the beginning of the experiment. Current/voltage curves were obtained by applying voltage steps in 8-mV increments (−40 to +36 mV) starting from a holding potential of −40 mV. All current recordings were obtained after ICₐ had been stabilized, which was usually achieved approximately 5 minutes after the rupture of the cell membrane. Data from experiments in which the stabilization was not achieved within this time were discarded.

**Data Analysis**

The output of the preamplifier was filtered at 2 kHz, and data acquisition and command potentials were controlled with PCLAMP software (Axon Instruments). Voltage and current were displayed simultaneously on an oscilloscope (Tektronix 5113; Tektronix). The output of the preamplifier was filtered at 2 kHz, and data acquisition and command potentials were controlled with PCLAMP software (Axon Instruments). Voltage and current were displayed simultaneously on an oscilloscope (Tektronix 5113; Tektronix).

**Statistical Analysis**

Numerical data were expressed as mean±SEM. Student’s t test was used to estimate statistical significance, defined as P<0.05.

**Results**

To investigate the possible influence of intracellular Ang II on ICₐ in rat myocytes, the peptide was added to the pipette solution and then dialyzed into the cytosol using an electrode similar to that described by Irisawa and Kokubun. Figure 1 shows that Ang II at a concentration of 10⁻⁶ mmol/L reduced the peak ICₐ generated by a test pulse from −40 to 0 mV by 35±5.5% (n=20; P<0.05). The significance was estimated by comparing values of ICₐ before and after the administration of Ang II. The reaction to the peptide, which was dose dependent (Figure 2), began within seconds but reached a maximal and steady value 4 minutes later (Figure 1). Figure 1 (bottom) shows the effect of intracellular administration of Ang II on the current-voltage relationship for Ca²⁺ current (ICₐ). The peptide reduced ICₐ for different values of transmembrane voltage. [Sar¹ Val⁵ Ala⁸] Ang II (10⁻⁶ mmol/L), an Ang II antagonist, when dialyzed into the cell (n=7) had no effect on ICₐ and did not influence the effect of Ang II on ICₐ (P>0.05; not shown). Moreover, the dialysis into the cell of PD 123,319 (10⁻⁶ mmol/L), an angiotensin type 2 (AT₂) receptor blocking agent, performed in 6 myocytes did not alter the effect of the peptide on ICₐ (n=6; P>0.05; not shown). Because there is evidence that Ang II when added to the bath reduces ICₐ in cardiac pacemaker cells, it is reasonable to think that the peptide dialyzed into the cytosol might leave the cell and interact with AT₁ receptors located at the surface cell membrane.

To investigate this possibility, losartan, a specific Ang II AT₁ receptor antagonist (10⁻⁶ mmol/L), was added to the perfusion fluid, and after 5 minutes of equilibration with this compound, Ang II (10⁻⁶ mmol/L) was dialyzed into the cell while the ICₐ was monitored. The results indicated no influence of losartan on the effect of Ang II on ICₐ (n=8; P>0.05; not shown). In 7 experiments, losartan (10⁻⁶ mmol/L) was added to the pipette solution, the compound was dialyzed into the cell for 4 minutes, and then Ang II (10⁻⁶ mmol/L) was administered intracellularly. The results indicated no influence of losartan on the effect of Ang II on ICₐ (P>0.05; Figure 2). In other experiments, the peptide (10⁻⁶ mmol/L) was administered to the bath solution and its influence on ICₐ was investigated. As seen in Figure 2, Ang II caused a reduction in the ICₐ by 20±3.3% (n=14), an effect that was abolished by losartan (10⁻⁶ mmol/L; not shown).

These observations led to the idea that the effect of intracellular administration of Ang II on ICₐ is related to some intracellular action of the peptide.

Biochemical studies have shown previously that Ang II when added extracellularly inhibits cAMP production in rat myocardium (see Reference 19). To investigate whether the decline in ICₐ described above is related to the inhibition of cAMP production, isolated cells were exposed to dibutyryl-cAMP (10⁻⁶ mmol/L) for several minutes, and as soon as the increase in ICₐ elicited by dibutyryl-cAMP reached a maximal and steady value, the peptide (10⁻⁶ mmol/L) was dialyzed into the cell. Figure 3 demonstrates that despite the stimulation of ICₐ by exogenous cAMP, the effect of Ang II on ICₐ was not changed (P>0.05). In other experiments in which the cells had been previously exposed to Krebs’ solution containing forskolin (10⁻⁶ mmol/L), an activator of adenyl cyclase, the effect of internal administration of Ang II (10⁻⁶ mmol/L) on ICₐ was similar to that seen in the control cells (P>0.05; not shown).

The possibility that Ang II reduces ICₐ by activating protein kinase C (PKC) was also investigated. The pseudosubstrate of the kinase, an inhibitor of PKC (20 µg/mL), was dialyzed into the cell for 4 minutes before the addition of Ang II (10⁻⁶ mmol/L) to the internal solution. The results from 8 experiments indicated that the pseudosubstrate of PKC did not alter the effect of Ang II on ICₐ (Figure 3). Staurosporine (50 µmol/L), a nonpeptide inhibitor of PKC, dialyzed into the cell for 4 minutes was also unable to suppress the effect of intracellular Ang II on ICₐ. Indeed, in 6 experiments with
staurosporine, Ang II elicited a decline of $I_{Ca}$ of 34.2 ± 4.9%, an effect not significantly different from control ($P > 0.05$). The possibility that the activation of PKC per se influences $I_{Ca}$ was investigated by adding phorbol 12-myristate 13-acetate (300 nmol/L) to the bath and monitoring $I_{Ca}$. Results from 4 experiments indicate a small increase of $I_{Ca}$ (13.8 ± 3.9%; $P < 0.05$) at the end of 4 or 5 minutes (not shown; see References 14 and 20).

Because evidence is available that cGMP protein kinase is involved in the regulation of $I_{Ca}$,21-23 the possibility that the effect of Ang II on $I_{Ca}$ is related to the activation of this kinase was investigated. For this, myocytes were perfused with Krebs’ solution containing zaprinast (100 µmol/L), a selective inhibitor of cGMP phosphodiesterase, and after 4 minutes of equilibration with this compound, Ang II (10⁻⁸ mmol/L) was dialyzed into the cell. As shown in Figure 4, the effect of Ang II on $I_{Ca}$ was significantly increased by zaprinast ($P < 0.05$), whereas zaprinast by itself at this concentration did not change $I_{Ca}$ ($P > 0.05$).

The question of whether intracellular Ang II increases $I_{Ca}$ in other species in which the peptide has a positive inotropic action was also investigated. For this, myocytes isolated from the ventricle of normal adult hamsters were used. Previous studies in these animals have indicated that Ang II added to the bath increases the strength of the heart beat (W.C. De M., unpublished observations, 1997).

Comparative experiments performed on myocytes isolated from the ventricle of normal Golden hamsters indicated that intracellular administration of Ang II (10⁻⁸ mmol/L) increased $I_{Ca}$ by 36 ± 2.4% (n=20) as shown in Figure 4. The effect of the peptide required 8 to 10 minutes to reach a maximal and steady level, and in 4 experiments the increase in $I_{Ca}$ was transitory. Losartan (10⁻⁶ mmol/L) added to the internal solution did not influence the effect of the peptide on $I_{Ca}$ (Figure 5). In 5 experiments in which PD 123,319 (10⁻⁶ mmol/L) was dialyzed into the cell for 5 minutes, no change in the effect of Ang II (10⁻⁸ mmol/L) was found ($P > 0.05$; not shown). Moreover, Ang II (10⁻⁸ mmol/L) added to the bath increased $I_{Ca}$ by 18 ± 1.9% (n=6; $P < 0.05$), an effect suppressed by losartan (10⁻⁷ mmol/L) added to the bath (not shown).

To investigate the idea that the increase in $I_{Ca}$ is related to the activation of cAMP cascade, experiments were performed on hamster myocytes previously dialyzed with an inhibitor of protein kinase A (20 µg/mL) for 4 minutes. The addition of

![Figure 1. Top left, Effect of intracellular administration of Ang II (10⁻⁸ mmol/L) on $I_{Ca}$ in a single rat myocyte. Control $I_{Ca}$ with horizontal arrow indicating zero current (A). $I_{Ca}$ after 4 minutes of Ang II administration (B). Top right, Average results from 15 experiments. Vertical line at each point shows SEM. Bottom, Voltage dependence of $I_{Ca}$ in rat myocytes in the absence (A) and presence (B) of Ang II (10⁻⁸ mmol/L). Amplitudes of maximal values of inward current were plotted against test potentials. Holding potential = −40 mV.]
Ang II (10^{-8} \text{ mmol/L}) to the internal solution under these conditions elicited an effect on $I_{\text{Ca}}$ similar to that of the controls (n=8; $P > 0.05$; not shown).

Because evidence exists that activation of PKC increases $I_{\text{Ca}}$ in cardiac myocytes,^{14} it was important to investigate whether the increase in $I_{\text{Ca}}$ seen with intracellular administration of the peptide was related to the activation of this kinase. For this experiment, myocytes isolated from hamster ventricles were dialyzed with the pseudosubstrate of PKC (20 \mu g/mL), an inhibitor of the kinase, and $I_{\text{Ca}}$ was monitored. As shown in Figure 5, the inhibitor by itself caused a decline of $I_{\text{Ca}}$ of 15\% within 3 minutes. As soon as the effect of the inhibitor reached a steady level, Ang II (10^{-8} \text{ mmol/L}) was added to the internal solution. Figure 5 shows that under these conditions Ang II was unable to increase $I_{\text{Ca}}$. In other experiments staurosporine (50 \mu mol/L) was dialyzed into the cell before the addition of Ang II (10^{-8} \text{ mmol/L}) to the internal solution. The results from 5 experiments indicated that Ang II under these conditions increased $I_{\text{Ca}}$ by only 1.89±0.97\% ($P > 0.05$; not shown). Moreover, the activation of PKC per se elicited by the addition to the bath of phorbol 12-myristate 13-acetate (300 nmol/L) increased $I_{\text{Ca}}$ by 9.8±3.8\% (n=6; $P < 0.05$; not shown) within 6 minutes. Control measurements made with the same concentration of DMSO used to dilute the phorbol ester showed no effect on $I_{\text{Ca}}$. Experiments made with [Sar^{1} Val^{5} Ala^{8}] Ang II (10^{-8} \text{ mmol/L}) in the internal solution showed no change in the effect of Ang II on $I_{\text{Ca}}$ (n=6; $P > 0.05$; not shown).

**Discussion**

The present results indicate that the administration of Ang II to the cytosol of ventricular myocytes of normal adult rats

![Graph 1](image1.png)

![Graph 2](image2.png)
reduces $I_{Ca}$ in an appreciable fashion. Conceivably, the synthesis of Ang II inside cardiac myocytes induced by activation of the cardiac renin-angiotensin system leads to similar decreases $I_{Ca}$ and a decrease in rat heart contractility. Moreover, the addition of Ang II to the perfusion fluid also caused a decline in $I_{Ca}$, but the effect was smaller than that elicited by the intracellular administration of the peptide.

The possibility that Ang II diffuses out of the cell and activates AT1 receptors located at the surface cell membrane with a consequent decrease in $I_{Ca}$ seems unlikely because losartan when added to the bath did not reduce the effect seen with the intracellular administration of Ang II. The plausible conclusion to be drawn from these experiments is that the peptide is acting intracellularly. Because losartan ($10^{-6}$ mmol/L) when added to the internal solution did not change the effect of Ang II on $I_{Ca}$, it is possible to conclude that an intracellular Ang II receptor similar to AT1 is not involved in the effect of the peptide. This finding seems to explain why [Sar1,Val5,Ala8] Ang II ($10^{-6}$ mmol/L) did not alter the effect of Ang II on $I_{Ca}$.

Previous work19 has indicated that Ang II administered to the extracellular fluid inhibits cAMP production in rat heart, an effect mediated by the inhibition of adenylate cyclase. Recently, it has been reported that Ang II inhibits the intracellular increase in cAMP produced by isoproterenol in heart cells.24 Our present studies in dialyzing Ang II into the cytosol show that the decrease in $I_{Ca}$ produced by the peptide was not altered by the exogenous administration of cAMP (dBcAMP) or even forskolin. Furthermore, the inhibition of PKC did not change the effect of Ang II on $I_{Ca}$ in rat myocytes.
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Figure 5. Top, Lack of action of losartan (10⁻⁶ mmol/L) added intracellularly on the effect of Ang II (10⁻⁶ mmol/L) on I_Ca recorded from hamster myocytes. Each point is the average of 13 experiments. Vertical line at each point is 3×SEM. Bottom, Effect of intracellular dialysis of the pseudosubstrate of PKC (20 μg/mL) on I_Ca of normal hamsters. At right is shown the lack of action of Ang II (10⁻⁶ mmol/L) on I_Ca in cells previously dialyzed with the PKC inhibitor. Each point is the average of 17 experiments. Vertical line at each point is 3×SEM.

It is known that the increase in intracellular Ca²⁺ decreases the amplitude of the inward calcium current.¹⁸ Although the concentration of EGTA used in the internal solution might preclude a change in free intracellular calcium, this possibility cannot be completely ruled out, and further studies are needed to clarify this point.

An alternative hypothesis to explain the decline in I_Ca is that Ang II activates the cGMP-dependent protein kinase. Evidence is available that Ca²⁺ current in mammalian heart cells is regulated by cGMP-dependent protein kinase.²⁰-²³ The finding that zaprinast, which is an inhibitor of cGMP phosphodiesterase, enhanced the effect of intracellular Ang II on I_Ca in normal hamsters supports the view that in Golden hamster myocytes, as in rat myocytes, the effect of Ang II on I_Ca is not related to the activation of an intracellular Ang II receptor similar to AT₁. Further studies will be needed to identify all the factors involved in the effect of the peptide in other species.

In summary, the effect of intracellular administration of Ang II on I_Ca described above seems to be related to an intracellular mechanism and suggests that the activation of the cardiac renin-angiotensin system plays an important role on the regulation of heart contractility.

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References


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