Intracellular and Extracellular Angiotensin II Enhance the L-Type Calcium Current in the Failing Heart

Walmor C. De Mello, Jose Monterrubio

Abstract—The influence of intracellular and extracellular angiotensin II (Ang II) on the L-type calcium current of cardiomyocytes isolated from cardiomyopathic hamsters was investigated. The results indicated that Ang II (10⁻⁶ mmol/L), added to the bath, increased the peak inward calcium current (I_{Ca}) density by 37±3.4% (P<0.05), an effect that depends on the activation of protein kinase C. Intracellular administration of the same dose of Ang II (10⁻⁶ mmol/L) also elicited an increase of peak I_{Ca} density but enhanced the rate of I_{Ca} inactivation, an effect not seen with extracellular Ang II. Moreover, in control animals, no change in the rate of I_{Ca} inactivation was seen with intracellular Ang II. Thapsigargin (1 μmol/L), a potent inhibitor of sarcoplasmic reticulum (SR) ATPase, which depletes the SR, decreased the rate of I_{Ca} inactivation elicited by intracellular Ang II, although the cytoplasmic calcium concentration was highly buffered with 10 mmol/L EGTA. These findings might indicate that intracellular Ang II releases calcium from the SR and inactivates I_{Ca}. The effect of intracellular Ang II on peak I_{Ca} was not altered by extracellular losartan (10⁻⁷ mmol/L), supporting the notion that the peptide acted intracellularly. Other studies showed that intracellular Ang I administration (10⁻⁸ mmol/L) enhanced the peak I_{Ca} density and the rate of I_{Ca} inactivation, an effect that was reduced by intracellular enalaprilat (10⁻⁸ mmol/L). Moreover, intracellular enalaprilat by itself reduced the peak I_{Ca} density. These observations might indicate that endogenous Ang II is contributing to I_{Ca} modulation in the failing heart. (Hypertension. 2004;44:1-5.)

Key Words: angiotensin ■ heart failure ■ calcium current

The concept of a cardiac renin-angiotensin system gained support with the demonstration that: (1) angiotensin I (Ang I) is converted to Ang II in the isolated and perfused heart; (2) the angiotensin-converting enzyme (ACE) has been found around the nucleus of heart cells in culture; and (3) ACE inhibitors prevent cardiac remodeling, an effect independent of the change in blood pressure.

However, in normal heart, renin mRNA levels and renin content are negligible in nephrectomized rats. Moreover, no renin is released from the isolated and perfused rat heart, which suggests that cardiac renin is attributable to its uptake from plasma. However, under some conditions, such as stretch, renin gene expression is enhanced, and overexpression of angiotensinogen gene in normal mice leads to hyper trophy of the right and left ventricles and to an increase of Ang II levels in both ventricles without any change in arterial blood pressure. Furthermore, a second renin gene transcript that may code for intracellular renin because it lacks the coding zone of the secretory signal peptide is upregulated in the left ventricle after myocardial infarction. Moreover, cardiac angiotensinogen is upregulated after myocardial infarction. These observations support the notion that renin and Ang II can be formed inside the heart cells under pathological conditions.

Evidence has been presented that Ang II intracellular administration enhances the inward calcium current (I_{Ca}) in cardiomyocytes of normal animals, an effect that varies with species. Intracellular Ang II can be the result of intracellular formation or internalization of extracellular Ang II. No information is available on the effect of Ang II on I_{Ca} in the failing heart. Therefore, it is important to investigate: (1) whether the extracellular or intracellular administration of Ang II changes the I_{Ca} in the failing heart; (2) whether the intracellular and extracellular administration of angiotensins interact on the control of I_{Ca}; and (3) whether endogenous Ang II plays a role in regulation of the I_{Ca} and consequently on heart contractility. In the present work, these problems were investigated in isolated myocytes from the heart of cardiomypathomypathic hamsters, which represent a good model of cardiomyopathy and heart failure in humans.

Methods

Male Syrian cardiomyopathic hamsters (4 months old) with a hemodynamic profile characterized by low cardiac output, eccentric hypertrophy, increased preload, and reduced renal blood flow and age-matched healthy control hamsters were used. Animals were kept at the animal house on a normal laboratory diet and tap water ad libitum. Cells were obtained by enzymatic dispersion of hamster ventricle after the method of Powell and Twist and Tanigushi et
The heart was removed and perfused immediately with normal Krebs solution containing the following (in mmol/L): 136.5 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.53 MgCl₂, 0.3 NaH₂PO₄, 11.9 NaHCO₃, 5.5 glucose, and 5 HEPES, with pH adjusted to 7.3. After 20 minutes, a calcium-free solution containing 0.4% collagenase (Worthington) was recirculated through the heart for 1 hour. The collagenase solution was washed out with 100 mL of recovery solution containing the following (in mmol/L): 10 taurine, 10 oxalic acid, 70 glutamic acid, 25 KCl, 10 KH₂PO₄, 11 glucose, and 0.5 EGTA, with pH adjusted to 7.4. All solutions were oxygenated with 100% O₂.

Ventricles and auricles were minced (1- to 2-mm-thick slices), and the resulting solution was agitated gently with a Pasteur pipette. Suspension was filtered through a nylon gauze and the filtrate centrifuged 4 minutes at 22 g. The cell pellets were then resuspended in normal Krebs solution. All experiments were conducted at room temperature.

Suction pipettes were pulled from microhematocrit tubing (Clark Electromedical Instruments) by means of a controlled puller (Narashige). The pipettes, which were prepared immediately before the experiment, were filled with the following solution (in mmol/L): 120 cesium aspartate, 10 NaCl, 3 MgCl₂, 10 EGTA, 20 tetraethylammonium chloride, 5 Na₂ATP, and 5 HEPES, with pH adjusted to 7.3. The resistance of the pipettes varied from 2.5 to 3.5 MΩ.

Experimental Procedures
All experiments were performed in a small chamber mounted on the stage of an inverted phase-contrast microscope (Diaphot; Nikon). Ventricular cells were placed in a modified cultured dish (volume 0.75 mL) in an open-perfusion microincubator (model PDMI-2; Medical Systems). Cells were allowed to adhere to the bottom of the chamber for 15 minutes and were superfused with normal Krebs solution (3 mL/min), which permits a complete change of the bath in <500 ms. A video system (Diaphot) made it possible to inspect the cells and pipettes throughout the experiments.

Electrical measurements were performed using the patch-clamp technique in a whole-cell configuration with a patch-clamp amplifier (model 200B; Axon Instruments). Leak currents were digitally subtracted by the P/N method (n=5 to 6). Experiments performed without leak subtraction indicated low and stable leak currents. Series resistance originated from the tips of the micropipettes was compensated for electronically at the beginning of the experiment. Current-voltage (I-V) curves were obtained by applying voltage step in 10-mV increments (−40 to 60 mV) starting from a holding potential of −40 mV. All current recordings were obtained after Iᵥ had been stabilized, which was usually achieved ∼8 minutes after cell membrane rupture.

Drugs
Ang I, Ang II, Val-Ala₅-Ang II, thapsigargin, and staurosporine were from Sigma. Losartan was from Merck Sharp & Dohme.

Data Analysis
The output of the preamplifier was filtered at 1 kHz, and data acquisition and command potentials were controlled with pCLAMP 8 software (Axon Instruments).

Statistical Analysis
Data are expressed as mean±SE. Statistical changes induced by Ang II or losartan were analyzed by Student t test, and significance was defined as P<0.05.

Results
The influence of Ang II on Iᵥ was studied by measuring the Iᵥ on isolated ventricular cells of 4-month-old cardiomyopathic hamsters before and after administration of the peptide (10⁻⁸ mmol/L) to the bath. Previous studies showed that in 4-month-old cardiomyopathic hamsters, the cardiac renin angiotensin system is activated and ACE activity enhanced.

The Iᵥ was generated by a test pulse of 400-ms duration from −40 to 0 mV. Figure 1 (top) shows typical examples of voltage- and time-dependent Iᵥ density from 20 control and 20 cardiomyopathic hamsters. Data are expressed as mean±SEM (P<0.05). D, Influence of staurosporine (5 mmol/L) on the effect of extracellular administration of Ang II (10⁻⁸ mmol/L) on peak calcium density of cardiomyopathic hamsters. Each point is the average of 20 cells (3 animals). Mean±SEM.

Figure 1. L-type Ca²⁺ current recorded from ventricular cells of cardiomyopathic hamster (4 months old) under control conditions (A) and after administration of Ang II (10⁻⁸ mmol/L) to the bath (B). The currents were elicited by a test pulse from −40 to 0 mV. C, Voltage dependence of peak Iᵥ density from 20 control and 20 cardiomyopathic hamsters. Data are expressed as mean±SEM (P<0.05).

D, Influence of staurosporine (5 mmol/L) on the effect of extracellular administration of Ang II (10⁻⁸ mmol/L) on peak calcium density of cardiomyopathic hamsters. Each point is the average of 20 cells (3 animals). Mean±SEM.
Inhibitor (staurosporine 5 nmol/L) for 7 minutes, Ang II protein kinase C (PKC) because in cells dialyzed with a PKC
was added to the pipette solution and then dialyzed into the cell
before and after administration of Ang II to the bath. Control
and Ang II current-density relationships show a bell shape and voltage dependence.

The effect of Ang II on I\textsubscript{\text{Ca}} is dependent on the activation of protein kinase C (PKC) because in cells dialyzed with a PKC
inhibitor (staurosporine 5 nmol/L) for 7 minutes, Ang II (10^{-8} mmol/L) added to the bath had no effect on I\textsubscript{\text{Ca}} (n=20; 
P<0.05; Figure 1). Similar results were found previously in control animals. \textsuperscript{13} Val\textsuperscript{5}-Ala\textsuperscript{8} -Ang II (10^{-8} mmol/L) reduced
the effect of Ang II by 33\% (n=9; P<0.05).

The time course of I\textsubscript{\text{Ca}} inactivation was determined by the
decay phase of the current traces elicited by voltage steps. Ang II reduces the rate of decay of the current trace in
ventricular myocytes of 4-month-old cardiomyopathic hamsters. Indeed, at 0 mV, the time constant for the fast
component was 52\pm4.2 ms (n=11) for the control and 69\pm3.6 ms (n=12; P<0.05) for Ang II (10^{-8} mmol/L). This
was true for the whole voltage range. This is an indication that the rate of I\textsubscript{\text{Ca}} inactivation was reduced by Ang II applied
extracellularly. The I\textsubscript{\text{Ca}} time to peak at 10 mV was 6.2\pm0.4 
ms in the control and 7.2\pm0.3 ms (n=6; P<0.05) after administration of Ang II (10^{-8} mmol/L) to the bath.

**Intracellular Ang II Effect on I\textsubscript{\text{Ca}}**

To study the effect of intracellular Ang II on the I\textsubscript{\text{Ca}}, the peptide
was added to the pipette solution and then dialyzed into the cell
using an electrode similar to that described by Irisawa and
Kokubun.\textsuperscript{18} Figure 2 shows that Ang II (10^{-8} mmol/L) increased
I\textsubscript{\text{Ca}} generated by a test pulse from -40 to 0 mV in ventricular
myocytes by 35.4\pm2.8\% (n=24; P<0.05). Significance was
estimated by comparing I\textsubscript{\text{Ca}} values before and after Ang II
administration. The increment of I\textsubscript{\text{Ca}} started within seconds but
required 7 to 8 minutes to reach a steady state. The effect of
intracellular Ang II (10^{-8} mmol/L) on peak I\textsubscript{\text{Ca}} of normal controls (27\pm2.6\%; n=18) was smaller than that found in
cardiomyopathic hamsters (P<0.05). Interestingly, the rate of
decay of the current traces was not reduced by intracellular Ang
II (Figure 2). On the contrary, the rate of decay of the fast and slow components was increased by 23\pm2.4\% and 14\pm3.1\%,
respectively (P<0.05), which indicates that the rate of I\textsubscript{\text{Ca}}
inactivation was incremented by intracellular Ang II. Because
evidence is available that calcium released by the sarcoplasmic
reticulum (SR) can inactivate I\textsubscript{\text{Ca}},\textsuperscript{19} we decided to investigate
the influence of thapsigargin on the inactivation process, a drug
that causes SR depletion.\textsuperscript{19} For this, I\textsubscript{\text{Ca}} measurements were taken at different times before and after administration of
thapsigargin (1 \mu mol/L) to the cytosol. Results showed that the
increased rate of inactivation elicited by intracellular Ang II was
abolished in cells dialyzed with thapsigargin (1 \mu mol/L) for
\geq800 ms before I\textsubscript{\text{Ca}} activation (Figure 3). Experiments
performed on age-matched control animals showed no change in
the inactivation rate after intracellular Ang dialysis II
(10^{-8} mmol/L; Figure 3).

**Is the Effect of Intracellular Ang II Attributable
to Its Diffusion to the Extracellular Space?**

The question of whether the effect of intracellular Ang II is
related to its diffusion to the extracellular fluid and consequent
activation of Ang II type-1 (AT\textsubscript{1}) receptors located at the
surface cell membrane was investigated in isolated cells
exposed to Krebs solution containing losartan (10^{-7} mmol/L),
an inhibitor of AT\textsubscript{1} receptors. After 30 minutes of equilibration
in this medium, Ang II (10^{-5} mmol/L) was dialyzed into
the cell, and its influence on I\textsubscript{Ca} was monitored. As shown in Figure 2, losartan (10\textsuperscript{-7} mmol/L) applied to the extracellular fluid did not influence the effect of the peptide on I\textsubscript{Ca}.

Possible Role of Endogenous Ang II

Because the cardiac renin angiotensin system is activated during heart failure, the question of whether endogenous Ang II modulates I\textsubscript{Ca} in cardiac myocytes of the failing ventricle merits serious consideration. To investigate the contribution of endogenous Ang II on I\textsubscript{Ca} modulation, Ang I (10\textsuperscript{-8} mmol/L) was added to the pipette solution, and the peptide was dialyzed into the cell. As shown in Figure 4, Ang I increased the peak I\textsubscript{Ca} density and enhanced the rate of I\textsubscript{Ca} inactivation, as shown with Ang II. To test whether the effect of Ang I was related to its conversion to Ang II, enalaprilat (10\textsuperscript{-8} mmol/L) was administered into the cell with Ang I. Figure 4 shows that the effects of Ang I on peak I\textsubscript{Ca} density and on the rate of I\textsubscript{Ca} inactivation were reduced greatly by the ACE inhibitor. Moreover, intracellular enalaprilat by itself decreased the peak I\textsubscript{Ca} density by 23\% (n=8; data not shown).

Discussion

The present results indicate that intracellular as well as extracellular Ang II modulate the I\textsubscript{Ca} in the failing heart of cardiomyopathic hamsters at 4 months of age. The increment of I\textsubscript{Ca} elicited by the peptide was dependent on PKC activation, as has been shown in normal controls.\textsuperscript{13} The effect of intracellular administration of Ang II on the peak I\textsubscript{Ca} is not related to its diffusion to the extracellular space and consequent activation of AT1 because losartan added to the bath did not change the effect of the peptide.
Ang II increased the rate of $I_{\text{Ca}}$ inactivation. These findings contrast with those obtained in normal controls in which intracellular dialysis of the same dose of Ang II was unable to change the rate of $I_{\text{Ca}}$ inactivation. Previous studies demonstrated that in normal rat cardiomyocytes, calcium channels are inactivated by calcium release from the SR. Because these results were found in rat cardiomyocytes despite the fact that the cytoplasmic calcium concentration was highly buffered with 10 mmol/L EGTA, it was postulated that there is a cross-signaling between the ryanodine receptor and the dihydropyridine receptor. The present results with thapsigargin, also achieved using 10 mmol/L EGTA in the internal solution, seem to indicate that in cardiomyopathic hamsters, the calcium released from the SR is the major contributor for the increased rate of $I_{\text{Ca}}$ inactivation found with intracellular Ang II.

The question of whether Ang II is formed inside the cardiac cells is of seminal importance. It is conceivable that overexpression of renin and angiotensinogen genes during the process of heart failure lead to formation of Ang I which is then converted to Ang II by ACE. The increase of peak $I_{\text{Ca}}$ density and of the rate of $I_{\text{Ca}}$ inactivation elicited by intracellular Ang II seems to be related to its conversion to Ang II because enalaprilat reduced its effect. Moreover, the decrease of $I_{\text{Ca}}$ caused by intracellular enalaprilat might indicate that endogenous Ang II is contributing to $I_{\text{Ca}}$ modulation. These findings support the notion that there is an intracellular ACE. It is known that there are tissue-bound and soluble forms of ACE, but it is not known whether there is a soluble form of ACE. Intracellular ACE activity is not enhanced by enalaprilat or the other ACE inhibitors used in the present study. Therefore, it is possible to conclude that the pathological condition is involved in the effect of the peptide on the inactivation process. This finding is particularly important because it is known that reduced SR Ca$^{2+}$ release is a characteristic of heart failure.

Considering that intracellular Ang II is probably localized in endosomes or other structures, further studies will be needed to characterize the compartmentalization of the peptide and its relevance to the present findings.

Because the duration of the action potential depends on the rate of calcium current inactivation and the activation of potassium current, the increase in duration of the action potential of hamster heart elicited by extracellular Ang II (W.C.D.M., unpublished data, 2001) might be, at least in part, related to the effect of the extracellular administration of the peptide on $I_{\text{Ca}}$ inactivation. The question of whether Ang II is formed inside the cardiac cells is of seminal importance. It is conceivable that overexpression of renin and angiotensinogen genes during the process of heart failure lead to formation of Ang I which is then converted to Ang II by ACE. The increase of peak $I_{\text{Ca}}$ density and of the rate of $I_{\text{Ca}}$ inactivation elicited by intracellular Ang I seems to be related to its conversion to Ang II because enalaprilat reduced its effect. Moreover, the decrease of $I_{\text{Ca}}$ caused by intracellular enalaprilat might indicate that endogenous Ang II is contributing to $I_{\text{Ca}}$ modulation. These findings support the notion that there is an intracellular ACE. It is known that there are tissue-bound and soluble forms of ACE, but it is not known whether there is a soluble form of ACE. Intracellular ACE activity is not enhanced by enalaprilat or the other ACE inhibitors used in the present study. Therefore, it is possible to conclude that the pathological condition is involved in the effect of the peptide on the inactivation process. This finding is particularly important because it is known that reduced SR Ca$^{2+}$ release is a characteristic of heart failure.

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References

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