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^{-8} \text{ mmol/L}) added to the bath, increased the peak inward calcium current (I_{Ca}) density by 37\pm3.4\% (P<0.05), an effect that depends on the activation of protein kinase C. Intracellular administration of the same dose of Ang II (10^{-8} \text{ 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 \mu\text{mol/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 \text{ 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} \text{ mmol/L}), supporting the notion that the peptide acted intracellularly. Other studies showed that intracellular Ang I administration (10^{-8} \text{ 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} \text{ 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;44:360-364.)*

Key Words: angiotensin \textbullet} heart failure \textbullet} calcium current

The concept of a cardiac renin-angiotensin system\textsuperscript{1} gained support with the demonstration that: (1) angiotensin I (Ang I) is converted to Ang II in the isolated and perfused heart;\textsuperscript{2} (2) the angiotensin-converting enzyme (ACE) has been found around the nucleus of heart cells in culture\textsuperscript{3}; and (3) ACE inhibitors prevent cardiac remodeling, an effect independent of the change in blood pressure.\textsuperscript{4}

However, in normal heart, renin mRNA levels\textsuperscript{5} and renin content are negligible in nephrectomized rats.\textsuperscript{6} Moreover, no renin is released from the isolated and perfused rat heart,\textsuperscript{7} which suggests that cardiac renin is attributable to its uptake from plasma. However, under some conditions, such as stretch, renin gene expression is enhanced,\textsuperscript{8} and overexpression of angiotensinogen gene in normal mice leads to hypertrophy of the right and left ventricles and to an increase of Ang II levels in both ventricles without any change in arterial blood pressure.\textsuperscript{9,10} 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.\textsuperscript{11} Moreover, cardiac angiotensinogen is upregulated after myocardial infarction.\textsuperscript{12} 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 influences the inward calcium current (I_{Ca}) in cardiac myocytes of normal animals, an effect that varies with species.\textsuperscript{13} 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 cardiomyomopathic hamsters, which represent a good model of cardiomyopathy and heart failure in humans.\textsuperscript{14}

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\textsuperscript{15} 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 tetrabutylammonium 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 ICa had been stabilized, which was usually achieved in 8 minutes after cell membrane rupture.

**Drugs**

Ang I, Ang II, Val5-Ala8-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 ICa was studied by measuring the ICa 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 studies17 showed that in 4-month-old cardiomyopathic hamsters, the cardiac renin angiotensin system is activated and ACE activity enhanced.
Inhibitor (staurosporine 5 nmol/L) for 7 minutes, Ang II was added to the pipette solution and then dialyzed into the cell and voltage dependence.

The I Ca time to peak at 10 mV was 6.2 ± 1.1 ms (n = 110) for the control and 8.6 ± 0.3 ms (n = 69; P < 0.05; Figure 1). Similar results were found previously in control animals. Val5-Ala8-Ang II (10⁻⁸ mmol/L) reduced the time constant for the fast component was 52 ± 7.8% (n = 24) after administration of Ang II (10⁻⁸ mmol/L). Six measurements were taken from each animal (n = 5 hamsters). Mean ± SEM. E, Lack of influence of extracellular administration of losartan (10⁻⁷ mmol/L) on the effect of intracellular administration of Ang II (10⁻⁸ mol/L) on peak I Ca density (n = 25; 5 animals) Mean ± SEM.

Intracellular Ang II Effect on I Ca

To study the effect of intracellular Ang II on the I 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. Figure 2 shows that Ang II (10⁻⁸ mmol/L) increased I Ca generated by a test pulse from −40 to 0 mV in ventricular myocytes by 35.4 ± 2.8% (n = 24; P < 0.05). Significance was estimated by comparing I Ca values before and after Ang II administration. The increment of I 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 Ca of normal controls (27 ± 2.6%; 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 ± 4.1% and 14 ± 3.1%, respectively (P < 0.05), which indicates that the rate of I Ca inactivation was reduced by Ang II. Because evidence is available that calcium released by the sarcoplasmic reticulum (SR) can inactivate I Ca,19,20 we decided to investigate the influence of thapsigargin on the inactivation process, a drug that causes SR depletion.19 For this, I Ca measurements were taken at different times before and after administration of thapsigargin (1 μmol/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 μmol/L) for ≥800 ms before I 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 (AT1) 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 AT1 receptors. After 30 minutes of equilibration in this medium, Ang II (10⁻⁸ mmol/L) was dialyzed into
the cell, and its influence on I_{Ca} was monitored. As shown in Figure 2, losartan (10^{-7} mmol/L) applied to the extracellular fluid did not influence the effect of the peptide on I_{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_{Ca} in cardiac myocytes of the failing ventricle merits serious consideration. To investigate the contribution of endogenous Ang II on I_{Ca} modulation, Ang II (10^{-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_{Ca} density and enhanced the rate of I_{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^{-8} mmol/L) was administered into the cell with Ang I. Figure 4 shows that the effects of Ang I on peak I_{Ca} density and on the rate of I_{Ca} inactivation were reduced greatly by the ACE inhibitor. Moreover, intracellular enalaprilat by itself decreased the peak I_{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_{Ca} in the failing heart of cardiomyopathic hamsters at 4 months of age. The increment of I_{Ca} elicited by the peptide was dependent on PKC activation, as has been shown in normal controls. 13 The effect of intracellular administration of Ang II on the peak I_{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.

It is important to emphasize that although intracellular or extracellular Ang II administration enhance I_{Ca}, there is a significant difference between the site of administration of the peptide in terms of I_{Ca} inactivation. Indeed, only the intracellular...
Ang II increased the rate of I_{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_{Ca} inactivation. Previous studies\(^{19,20}\) 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 crosstalk between the release of calcium from the SR and Ang II. 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_{Ca} inactivation found with intracellular Ang II.

The reason for the difference between the effects of intracellular and extracellular Ang II on the inactivation process is not known. A possible explanation for these results is that intracellular Ang II activates ryanodine receptors with consequent release of calcium from the SR. Because no change in the rate of I_{Ca} inactivation was found with intracellular Ang II in control animals, 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.\(^{21}\)

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 the extracellular 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_{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 II, which is then converted to Ang II by ACE. The increase of peak I_{Ca} density and of the rate of I_{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_{Ca} caused by intracellular enalaprilat might indicate that endogenous Ang II is contributing to I_{Ca} modulation. These findings support the notion that there is an intracellular ACE. It is known that these are tissue-bound and soluble forms of ACE,\(^{22}\) but it is not known whether there is a soluble form of the enzyme inside the cell. Recently,\(^{23,24}\) evidence was provided that intracellular administration of enalaprilat to 2-month-old cardiomyopathic hamsters in which ACE activity is not enhanced did not change the cell coupling, whereas the same amount of enalaprilat increased the junctional conductance by 72±6.2% in 6-month-old cardiomyopathic hamsters in which ACE activity is appreciably increased. These findings and the present observations support the notion that endogenous Ang II contributes to the modulation of I_{Ca} and cell coupling in the failing heart.

Acknowledgments

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References

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