Beneficial versus harmful effects of Angiotensin (1-7) on impulse propagation and cardiac arrhythmias in the failing heart

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Abstract

Introduction. The presence of Angiotensin (1-7) (Ang 1-7) and ACE 2 in the ventricle of cardiomyopathic hamsters as well as the influence of Ang (1-7) on membrane potential, impulse propagation and cardiac excitability were investigated.

Methods. Histology and immunochemistry were used to demonstrate the presence of Ang (1-7) and ACE 2 in the ventricle of cardiomyopathic hamsters. Measurements of transmembrane potentials, conduction velocity and refractoriness were made using conventional intracellular microelectrodes. The influence of Ang (1-7) on sodium pump current was investigated in voltage-clamped myocytes isolated from the ventricle.

Results. The results indicated the presence of Ang (1-7) and ACE 2 in myocytes of cardiomyopathic hamsters. Moreover, Ang (1-7) (10−8 M) hyperpolarised the heart cell, increased the peak pump current density was investigated in voltage-clamped myocytes isolated from the ventricle.

Conclusions. Ang (1-7) has beneficial effects on the failing heart by activating the sodium pump, an effect related to the activation of an electrogenic sodium pump and increasing the conduction velocity. These effects as well as the increment of refractoriness indicate that Ang (1-7) has antiarrhythmic properties. At higher concentrations (10−7 M), however, the heptapeptide induced early-after depolarisations, which leads to the conclusion that an optimal generation of Ang (1-7) must be achieved to permit a protective role of Ang (1-7) on cardiac arrhythmias.

Introduction

Previous studies showed that the activation of the renin-angiotensin system (RAS) impairs cell communication and impulse propagation with consequence generation of malignant re-entrant rhythms. Indeed, angiotensin II (Ang II) decreases conduction velocity and enhances the dispersion of action potential duration in the failing heart, an important mechanism of cardiac arrhythmias. The mechanism of the enhanced dispersion is probably related, at least in part, to different expression of AT1-receptors throughout the ventricular wall. In addition, the morphologic abnormalities caused by Ang II such as fibrosis and rupture of cell contacts among myocytes lead to changes in geometry of the ventricular wall with consequent impairment of impulse conduction and the generation of anisotropic propagation.

Angiotensin-converting enzyme 2 (ACE 2) is a new enzyme with a high homology to angiotensin-converting enzyme 1 (ACE) and able to hydrolyze Ang II to Ang 1-7, which is present in the failing human heart, restores cardiac dysfunction after myocardial infarction in rats. Moreover, up-regulating cardiac ACE 2 messenger ribonucleic acid (mRNA) by an AT1-receptor mechanism has been described in post-myocardial infarction.

Previous studies indicated that Ang (1-7) has antiarrhythmic properties. Moreover, the heptapeptide re-establishes impulse conduction in rat heart during ischaemia-reperfusion, an effect related to the activation of an electrogenic sodium pump.

Since evidence is available that Ang (1-7) counteracts the harmful effects of Ang II on cardiac muscle, we decided to investigate if...
Ang (1-7) ameliorates the propagation of the electrical impulse, which is impaired in the failing heart. In the present work this problem was studied in the heart of cardiomyopathic hamsters (TO-2) which represents a good model of cardiomyopathy and heart failure in humans.11

Methods
Male cardiomyopathic hamsters (TO-2) (Biobreeders, MA) weighing 125–150 g were used. The animals were kept in the Animal House at constant temperature (24°C) and humidity following the recommendations of the National Institute of Health. Animals were kept on a normal laboratory animal diet and given tap water ad libitum. The animals were anaesthetised with sodium pentobarbital (50 mg/kg, i.p.), and the heart was removed with the animals under deep anaesthesia. A small piece of the right ventricle was dissected out and transferred to a transparent chamber through which oxygenated Krebs solution flowed continuously (37°C).

Measurements of transmembrane potentials, refractoriness and conduction velocity
Intracellular potassium chloride (KCl) microelectrodes connected to a high impedance DC amplifier (WP Instruments, Model 750 Dual Probe with capacitance neutralisation) were used to measure the transmembrane potentials. The right ventricular muscle was dissected and transferred to a bath through which oxygenated Krebs solution flowed continuously. The composition of Krebs solution was as follows (mM): NaCl 150; KCl 5.4; CaCl2 1.8; MgCl2 0.53; NaH2PO4 0.3; glutamic acid 70; oxalic acid 10; KCl 25; KH2PO4 10; NaHCO3 11.9; glucose 5.5; and HEPES 5 with pH adjusted to 7.4. The solution was agitated gently with a Pasteur pipette. The composition of Krebs solution was replaced by Krebs solution containing (mM): NaCl 136.5; KCl 5.4; CaCl2 1.8; MgCl2 0.53; NaH2PO4 0.3; NaHCO3 11.9; glucose 5.5; and HEPES 5 with pH adjusted to 7.4. After 30 minutes of equilibration in Krebs solution, the membrane potential was recorded from superficial endocardial fibers. The conduction velocity was determined with two micro-electrodes impaled at a fixed distance.

The strength of the current was plotted against the cycle interval and strength-interval curves were obtained for control and experimental conditions in the same muscles and in many cases in the same cells. Voltage calibration was determined by injecting known voltages between the Krebs solution and the ground. Changes in membrane potential were displayed and stored for further analysis.

Influence of Ang (1-7) on the sodium pump

Changes in membrane potential
To investigate the possible influence of Ang (1-7) on the sodium pump, small pieces of the right ventricle (6–8 mm in length, 2–4 mm in diameter) of four month-old cardiomyopathic hamsters were used. The small size of the preparation combined with a fast flow system made it possible to induce changes in sodium pump activity and study its influence on the resting membrane potential of quiescent fibers. To confirm the role of the sodium pump on the increment in resting potential, ouabain was added to Krebs solution.

Influence of Ang (1-7) on sodium pump current

To study the direct influence of Ang (1-7) on the sodium pump, myocytes were isolated from the ventricle of cardiomyopathic hamsters and the membrane potential was clamped at -40 mV. The whole cell voltage clamp configuration was used. The sodium pump was suppressed by superfusing the cells with potassium free (K-free) solution for ten minutes and then the K-free solution was replaced by Krebs solution containing 5.4 mM of K ions which caused a reactivation of the sodium pump. The peak pump current was measured by the maximal current generated by pump reactivation. In some experiments Ang (1-7) was added to the Krebs solution.

Cell isolation procedure

Cells were obtained by enzymatic dispersion of the ventricle following the method of Powell and Twist12 and Tanigushi et al.13 The heart was removed and immediately perfused with normal Krebs solution containing (mM): NaCl 136.5; KCl 5.4; CaCl2 1.8; MgCl2 0.53; NaH2PO4 0.3; NaHCO3 11.9; glucose 5.5; and HEPES 5 with pH adjusted to 7.4. After 20 minutes, a calcium-free solution containing 0.4% collagenase (Worthington Biochemical Corp.) was re-circulated through the heart for one hour. The collagenase solution was washed out with 100 ml of recovery solution containing (mM): tauroine 10; oxalic acid 10; glutamic acid 70; KCl 25; KH2PO4 10; glucose 11; EGTA 0.5 with pH adjusted to 7.4. All solutions were oxygenated with 100% O2. Ventricles were minced (1–2 mm thick slices) and the resulting solution was agitated gently with a Pasteur pipette.
The suspension was filtered through a nylon gauze and the filtrate centrifuged for four minutes at 22 g. The cell pellets were then resuspended in normal Krebs solution. All the 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 experiments were filled with the following solution (mM): 120 caesium aspartate; 10 NaCl; 3 MgCl₂; 10 EGTA, 20 tetraethylammonium chloride, 5 Na₂ATP and 5 HEPES, pH adjusted to 7.3. The resistance of the pipettes varied from 1.5–2 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 superwashed with normal Krebs solution (3 ml/min) which permits a complete change of the bath in less than 500 ms. A video system (Diaphot) made it possible to inspect the cells and the pipettes throughout the experiments.

The electrical measurements were carried out using the patch-clamp technique in a whole-cell configuration with an Axon (model 200B) patch-clamp amplifier. Series resistance originated from the tips of the micropipettes was compensated for electronically at the beginning of the experiment.

**Histology and immunochemistry**

The cardiac tissue was left in 4% formalin for 48 hours before being transferred to 70% ethanol. Blocks of cardiac tissue were imbedded in paraffin; 5 μm sections were transferred to subbed slides and deparaffinised by sequential washes with xylene, 100% ethanol, 95% ethanol, 75% ethanol and double-distilled water. Tissue sections were incubated with 3% hydrogen peroxide for five minutes, washed with Phosphate-buffered saline (PBS) (pH 7.2), dried, and then incubated with 5% normal goat serum for one hour at room temperature. Sections were incubated with 3% hydrogen peroxide for five minutes, washed with Phosphate-buffered saline (PBS) (pH 7.2), dried, and then incubated with 5% normal goat serum for one hour at room temperature. Sections were washed with PBS and incubated overnight at 4°C with a high affinity-purified rabbit polyclonal antibody to Ang (1-7) at 1:100 dilution of the antibody in bovine serum albumin (BSA). The Ang (1-7) antibody was purified and characterised as previously described. Additional sections were incubated with a 1:100 dilution of an affinity-purified rabbit polyclonal antibody to ACE 2. The next day, tissues were washed with PBS and incubated for three hours at 4°C with a biotinylated anti-rabbit antibody at a dilution of 1:100 in 1% BSA. Slides were rinsed with PBS, dried, and reacted immunocytochemically by the avidin biotin method (Vector Laboratories, Burlingame, CA, USA) and stained brown with 3,3’-diaminobenzidine (Sigma Chemical Co.) in PBS buffered saline (0.05 mol/L, pH 7.7). The reaction was stopped in PBS and sections were rinsed in double-distilled water before being counter-stained with haematoxylin (Sigma-Aldrich, St. Louis, MO, USA). Tissue sections were dehydrated in ethanol (70–100%) and Histoclear (National Diagnostics, Atlanta, GA, USA). Finally they were mounted under coverslips with Histamount (National Diagnostics). Tissue sections were examined under a Leica DM 4000 light microscope and photographed with AxioCam (Carl Zeiss MicroImaging Inc. Thornwood, NY, USA) digital camera and Simple PCI software (Complix Inc. Cranberry Township, PA, USA).

**Drugs**

Ang (1-7) and ouabain were obtained from Sigma Chemical Co. Losartan was from a gift from Merck Sharp and Dohme and (D-Ala7)-Ang (1-7) was obtained from Bachem Laboratory.

**Statistical analysis**

Data are expressed as mean±SEM. Statistical changes induced by Ang 1-7 were analysed by Student’s t-test and significance was defined as p<0.05. Comparison between groups was done by analysis of variance (ANOVA). Differences were considered significant when p<0.05.

**Results**

**Presence of Ang (1-7) and ACE 2 on the ventricle of CM hamsters**

As illustrated in figure 1, immunoreactivity for Ang (1-7) (figure 2a) and ACE 2 is found in cardiac myocytes. Solid bar 100 μm.
detectable in adjacent sections of the left ventricular free wall. The immunostaining for both Ang (1-7) and ACE 2 was predominantly localised in the myocytes of the cardiomyopathic hamsters. Additionally, similar staining was visualised in the interventricular septal region and right ventricular free wall.

Effect of Ang (1-7) on resting potential, impulse propagation and refractoriness

Measurements of resting potential performed on the wall of the right ventricle, showed that Ang (1-7) (10^{-8} M) hyperpolarises the muscle fibers from -67±2.1 mV (n=20)–76±2.4 mV (p<0.05) within 20 minutes of drug administration. Moreover, the amplitude of the action potential was increased by 10±1.1 mV (n=22) (p<0.05). Concurrently, the action potential duration was transiently reduced at 90% of repolarisation from 175±2 ms (n=22) in the control to 156±2 ms (n=20) after Ang (1-7) (10^{-8} M) (p<0.05). With higher concentration of Ang (1-7) (10^{-7} M), however, the action potential duration was enhanced by 36±3% (n=13) (p<0.05) (see figure 2) and early-afterdepolarisations were found (see figure 2) after 40 minutes of equilibration with the heptapeptide (see figure 2). The conduction velocity, measured longitudinally, was significantly increased by Ang (1-7) (10^{-8} M) as shown in figure 2, while measurements of cardiac refractoriness performed before and after the administration of Ang (1-7) (10^{-8} M) to the bath, showed a displacement of the strength-interval curves to the right, indicative of an increase in cardiac refractoriness (see figure 3). Preliminary experiments performed with the mas receptor inhibitor D-Ala7 Ang (10^{-7} M) (p<0.05) (n=4) indicated a significant decline, but not a suppression of the increment in cardiac refractoriness (figure 3). Further studies are needed to clarify the role of mas receptor on the increment in cardiac refractoriness as well as in other electrophysiologic parameters.

To investigate the possibility that the effect of high concentration of Ang (1-7) on cardiac excitability was related to the activation of Ang II
Measurements of the membrane current were performed on isolated cardiomyocytes. To study further the influence of Ang (1-7) on Na-pump, direct measurements of pump current were performed on isolated cardiomyocytes. Measurements of the membrane current generated by the reactivation of the sodium pump previously abolished by K-free solution, made it possible to investigate the influence of Ang (1-7) on the sodium pump activity. For this, quiescent myocytes isolated from the ventricle were voltage clamped at -40 mV and then superfused with K-free solution for ten minutes to block the sodium pump. Since the time of exposure to K-free solution influences the peak amplitude of the transient outward pump current, the exposure to isolated cells to K-free solution was kept constant (ten minutes). After this, the K-free solution was replaced by normal Krebs solution (K_e=5.4 mM) which elicited an outward current generated by the reactivation of the electrogenic sodium pump. Considering that variations in cell size might influence the pump current, its value was normalised to the membrane capacitance (Cm). Values of Cm in the cardiomyopathic hamsters at four months of age, ranged from 117–134 pF. As it can be seen in figure 4, the relative pump current density was significantly larger in presence of Ang (1-7) (10–8 M) than that found in normal Krebs solution. To confirm the view that the increment of the outward current was related to the activation of an electrogenic sodium pump, the myocytes were exposed to ouabain (10^-7M) for ten minutes and then the preparation was superfused with Krebs solution containing Ang (1-7) (10^-7 M) plus ouabain (10^-7 M). As shown in figure 4, the effect of Ang (1-7) on pump current was abolished by ouabain. To determine the degree of possible contamination by different ionic currents the K-activated current was examined at different potentials using 100 ms duration step changes in membrane potential between -120 to +60 mV. Figure 5a shows the steady-state currents elicited by changing the extracellular K concentration from 0–10 mM in absence of ouabain indicating that K-dependent currents were voltage-sensitive. In cells incubated with ouabain (10^-8M) for ten minutes the background current was insensitive to changes extracellular potassium concentration from 0–10 mM (figure 5b) as well as to the presence of Ba^{2+} (2 mM) added to the bath solution (not shown). These findings indicate that the small residual K-activated current found in presence of ouabain at a holding potential of -40 mV represents only a fraction of the total K-activated current and does not interfere with the interpretation of the results.

### Discussion

The present results document for the first time, the presence of Ang (1-7) and ACE 2 in myocytes from the failing heart of cardiomyopathic hamsters and demonstrate that Ang (1-7) hyperpolarises the heart cell and increases the conduction velocity in the failing heart. This finding as well as the increment of cardiac refractoriness caused by Ang (1-7), indicates that the heptapeptide has important antiarrhythmic properties which clearly contrasts with the decline of conduction velocity found with Ang II in the same preparation. The beneficial effects of Ang (1-7) are dose-dependent because at a concentration of 10^-7 M the heptapeptide caused...
of high concentration of Ang (1-7) (10^{-7} M) on the failing heart seems unlikely because: a) losartan did not change the effect of Ang (1-7) on action potential duration as well as on the resting potential and on generation of early-afterdepolarisations. Indeed, the activation of AT1-receptors by the heptapeptide would reduce not increase the resting potential; b) the mas receptor inhibitor reduced significantly the effect of Ang (1-7) on cardiac refractoriness, but further studies will be needed to test the effect of the inhibitor on other parameters; c) Ang II decreases the pump current through AT1 activation while Ang (1-7) activates the pump even at high concentration (10^{-7} M) (not shown).

The increase of the resting potential elicited by Ang (1-7) is related to the activation of the electrogenic sodium pump because: a) direct measurements of the pump current density performed in isolated cells of the failing ventricle showed that Ang (1-7) incremented it beyond the levels achieved under normal control conditions; b) these effects of Ang (1-7) were blocked by ouabain.

It is known that Ang II regulates the selectivity of the sodium pump in cardiac muscle to intracellular sodium and reduces the pump current^{25} through activation of PKC. The present results indicate that, at the level of the sodium pump, Ang (1-7) has an effect opposite to that of Ang II, but similar to that described with ACE-inhibitors which activates the pump^{25}. The intimate mechanism by which Ang (1-7) enhances the sodium pump is not known. However, it is important to mention that previous studies showed that inhibition of ACE increases the plasma concentration of Ang (1-7) since the enzyme is a primary factor in the metabolism of the heptapeptide^{25}.

The increase of cardiac refractoriness elicited by Ang (1-7), which was found after 30 minutes of equilibration with the heptapeptide, might involve changes in threshold and cable properties of the fiber. Moreover, because the external stimulation electrode was located about 1 mm from the recording cell it is conceivable that changes of intracellular or extracellular resistance alter the amount of current that reaches the recording cell. Additional information on these parameters is needed before a conclusion can be reached on the mechanism involved in the alteration of refractoriness.

The large increase in duration of the action potential and the consequent generation of early-afterdepolarisations induced by high doses of Ang (1-7) lead us to think that overexpression of ACE 2 with consequent excessive formation of Ang (1-7) may have a deleterious effect on cardiac rhythm. Therefore, an optimal degree of expression of ACE 2 must be achieved in order to

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**Figure 5**

Voltage clamped myocyte with a sodium concentration of 10 mM inside the pipette; holding potential -40 mV, a steady state voltage-current relationships for K-activated current in absence of ouabain. b: Current-voltage relationships in presence of ouabain (10^{-8} M) and changing the extracellular K concentration from 0–10 mM. An appreciable increase of action potential duration and early-afterdepolarisations. This finding is of clinical relevance because triggered activity, which is due to afterdepolarisations, causes Torsade de Pointes. Furthermore, it probably explains the generation of ventricular tachycardia and sudden death found in animals overexpressing ACE 2.26 The effect of Ang (1-7) on refractoriness was reduced, but not abolished by a mas receptor antagonist D-Ala^{7}-Ang (1-7). This observation might indicate that part of the effect of Ang (1-7) on refractoriness is related to the activation of another Ang (1-7) receptor.19

Studies performed on vascular smooth muscle indicated that pharmacological doses of Ang (1-7) can induce the activation of AT1-receptors.20,21 How valid is the extrapolation of these results to the electrical properties of cardiac myocytes is not known. Moreover, recently, the mas receptor was described as a physiological antagonist of AT1-receptors and that down-regulation of AT1-receptors can be elicited by pharmacological concentrations of Ang (1-7),22 which indicates that the role of AT1-receptor activation on the responses to high doses of Ang (1-7) is a controversial issue. The possible role of Ang II AT1-receptors on the effect of high concentration of Ang (1-7) (10^{-7} M) on the failing heart seems unlikely because: a) losartan did not change the effect of Ang (1-7) on action potential duration as well as on the resting potential and on generation of early-afterdepolarisations. Indeed, the activation of AT1-receptors by the heptapeptide would reduce not increase the resting potential; b) the mas receptor inhibitor reduced significantly the effect of Ang (1-7) on cardiac refractoriness, but further studies will be needed to test the effect of the inhibitor on other parameters; c) Ang II decreases the pump current through AT1 activation while Ang (1-7) activates the pump even at high concentration (10^{-7} M) (not shown).

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The large increase in duration of the action potential and the consequent generation of early-afterdepolarisations induced by high doses of Ang (1-7) lead us to think that overexpression of ACE 2 with consequent excessive formation of Ang (1-7) may have a deleterious effect on cardiac rhythm. Therefore, an optimal degree of expression of ACE 2 must be achieved in order to
protect the failing heart against reentrant arrhythmias. This interpretation agrees with the demonstration that transduction with lentivirus ACE 2 in rats resulted in significant attenuation of Ang II-induced cardiac hypertrophy and fibrosis. However, the question remains if the plasma levels of Ang (1-7) similar to $10^{-7}$ M, as described above, are achievable during pathological conditions.

In conclusion, Ang (1-7) benefits the failing heart by increasing the resting potential with consequent increment of action potential amplitude and by enhancing the conduction velocity and electrical synchronisation. Moreover, the increment of cardiac refractoriness increases the arrhythmogenic action of the compound. With higher doses of the drug, however, earlyafterdepolarisations were elicited showing the arrhythmogenic action of Ang (1-7). This finding might indicate that an optimal expression of ACE 2 must be achieved to permit a protective role of Ang (1-7) on cardiac arrhythmias.

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**References**


