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Intracrine action of angiotensin II in the intact ventricle of the failing heart: angiotensin II changes cardiac excitability from within

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Abstract

The influence of intracellular injection of angiotensin II (Ang II) on electrical properties of single right ventricular fibers from the failing heart of cardiomyopathic hamsters (TO2) was investigated in the intact ventricle of 8-month-old animals. Intracellular injection was performed using pressure pulses (40–70 psi) for short periods of time (20 ms) while recoding the action potential simultaneously from the same fiber. The results indicated that intracellular Ang II caused a hyperpolarization of $7.7 \text{ mV} \pm 4.3 \text{ mV}$ ($n = 39$) (4 animals) ($P < 0.05$) followed by a small fall in membrane potential. The action potential duration was significantly increased at 50% and at 90% repolarization, and the refractoriness was significantly enhanced. The effect of intracellular Ang II on action potential duration was related to the inhibition of potassium conductance through PKC activation because Bis-1 (360 nM), a selective PKC inhibitor, abolished the effect of the peptide. Injections performed in different fibers of the same ventricle showed a variable effect of Ang II on action potential duration and generated spontaneous rhythmicity. The effect of intracellular Ang II on action potential duration and cardiac refractoriness remains for more than 1 h after interruption of the intracellular injection of the peptide.

Keywords

Failing heart; Intracellular injection; Angiotensin II; Action potential; Refractoriness

Introduction

Classically, the systemic RAS basically depends upon its rate-limiting enzyme renin that is produced in the juxta-glomerular (JG) apparatus of the kidney and is released under conditions of intravascular volume decrease, reduced arterial blood pressure, and hypokalemia. The release of renin from the JG apparatus acts on its protein substrate angiotensinogen to generate the decapeptide angiotensin I that in turn is converted to angiotensin II by the angiotensin converting enzyme (ACE) in the pulmonary circulation. The result is freely circulating angiotensin II that promotes vasoconstriction and stimulation of the adrenal cortex and consequent release of aldosterone to restore intravascular volume and arterial pressure.

Accumulating evidence supports the notion that there is a local RAS in the heart [1–3] and that different components of the renin angiotensin system are taken up by different tissues

[4] thereby influencing the synthesis of Ang II locally [3–5]. Moreover, the synthesis of several of these components has been detected in the heart [4] and previous studies performed in pigs, indicated that as much as 75% of cardiac Ang II is synthesized at tissue sites [5].

Several observations indicate that the local renin angiotensin system in the heart has a functional intracrine component [3, 6–9, 14]. Indeed, when angiotensin II is dialyzed into a cardiomyocyte from the failing heart, there is cellular uncoupling elicited by a drastic decline of gap junctional conductance [6, 7, 11]. The increase in gap junctional resistance explains the decline in conduction velocity and the generation of reentrant rhythms elicited by the peptide [17]. Intracellular Ang II also changes the inward calcium current in cardiac myocytes [6] substantiating the view that the peptide has an intracrine action. Furthermore, the concept that endogenous levels of Ang II plays an important role on cell functions is supported by studies in which intracellular administration of enalaprilat was performed [11].

Although, there is evidence that an intracrine renin angiotensin aldosterone system is present in cardiac myocytes [3, 4, 6–8, 14], particularly of the failing heart [11], no information is available if intracellular Ang II alters the electrical properties of cardiac cells in the intact heart in which the myocardial structure is preserved. This is of fundamental importance to heart cell biology because the integrity of extracellular matrix and cell contacts is vital for cellular and tissue function.

In the present work, this problem was investigated in the intact ventricle of the failing heart of cardiomyopathic hamsters at an advanced stage of the disease.

Methods

Male Syrian TO-2 cardiomyopathic (CM) hamsters (8 month old) and healthy male F1B controls (Biobreeders, MA) were used. During this advanced stage of heart failure, ventricular dilatation is found and sudden death is usually seen due to malignant ventricular arrhythmias. The animals were kept at the animal house on a normal laboratory animal diet and tap water ad libitum. Constant veterinary care was provided, and the recommendations of NIH were followed. The animals were anaesthetized with 45 mg/kg of ketamine + 5 mg/kg of xylazine (i.p.), and the heart was removed under deep anesthesia. The heart was transferred to a bath containing oxygenated Krebs solution (37°C), and the right ventricle was dissected out and transferred to a transparent chamber through which oxygenated Krebs solution (37°C) flowed continuously.

Measurements of transmembrane potentials

Intracellular potassium chloride (KCl) microelectrodes connected to a high impedance DC amplifier (8100 single electrode amplifier) (Dagan, Co) were used to measure the transmembrane potentials. The right ventricular muscle was dissected and transferred to a bath through which oxygenated Krebs solution (36°C) flowed continuously. The composition of Krebs solution was as follows (mM): NaCl—150; KCl—5.4; CaCl₂—1.8; MgCl₂—1; HEPES—5; glucose—5; pH adjusted to 7.3. The solution was saturated with 100% O₂. The ventricle was stimulated with rectangular pulses (3 ms duration, 0.6 Hz) generated by an electronic stimulator and applied gently to the tissue through a bipolar platinum electrode. The determination of the stimulus strength was achieved by amplifying the voltage drop across a 10 M Ω resistor placed between the muscle and ground.

Measurements of refractoriness

To investigate the influence of intracellular angiotensin II on cardiac refractoriness, strength–interval curves were obtained from the same ventricular cell before and after the

injection of the peptide. The refractoriness was measured by applying a second current pulse of constant duration (3 ms) and variable intensity at different moments of the action potential and determining the minimal current strength required to elicit a clear propagated response. The intervals were previously selected and kept constant. The current strength was plotted against the interval, and strength–interval curves were obtained for control and experimental conditions.

Injection of angiotensin II

The intracellular injection of Ang II was performed following a procedure similar to that described previously [13]. A Picospritzer (General Valve Corp) connected to a compressed nitrogen tank was employed for pressure regulation (up to 100 psi) and Ang II injection. Microelectrode filled with Ang II solution was connected to an 8100 single electrode amplifier (Dagan, Co) that permitted to inject current pulses and record the action potential from the same cell simultaneously. The injection micropipette connected to the compressed nitrogen tank was beveling to a resistance of 10 M Ω or less and filled with a solution containing KCl (3 M) plus angiotensin II (10^{-8} M). The beveling of the micropipettes facilitates the ejection of the solution into the cell. Pressure pulses (40–60 psi) were applied to the injection electrode for 20 s to inject the peptide. The volume ejected and the intensity of pulse pressure were linearly related, and positive results were obtained in about 85% of the injected cells. In about 15% of the experiments, clogging of the microelectrode was produced with consequent increase in electrode resistance and the experiment was rejected. The ejection volumes were quantified by measuring the diameter of the aqueous droplets delivered into mineral oil previously to cell impalement. A precise quantification of the injected volume was difficult due to expected differences between injection into the air and intracellularly (see also [13]). The use of micropipettes with a resistance of 8 M Ω reduced this problem.

The protocol used in these experiments consisted in impaling the cell and recording the action potential of the same endocardial ventricular fiber before and after the intracellular injection of the peptide. During the injection of the peptide, the microelectrode was kept inside the cell. The experiments were considered acceptable if: (a) the value of the resting potential was maintained immediately after the injection indicating no cell injury; (b) the ejection of a droplet of fluid from the tip of the micropipette into air (about 200 nl) was observed under the microscope during pressure pulse (45 psi for 20 s) before impalement of the micropipette; (c) control experiments injecting the KCl solution into cardiac cells (using pressure of 45 psi, for 20 s) in absence of the peptide showed no change in the electrical parameters.

Measurements of potassium current in isolated cardiomyocytes

Cell isolation procedure—To investigate the possible effect of intracellular Ang II on potassium, current experiments were performed on cardiomyocytes isolated from 8-month-old cardiomyopathic hamsters. Cells were obtained from the ventricle of cardiomyopathic hamsters (TO-2). 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; HEPES 5, pH adjusted to 7.3. After 20 min, a Ca-free solution containing 0.4% collagenase (Worthington Biochemical Corp) was recirculated through the heart for 1 h. The collagenase solution was washed out with 100 ml of recovery solution containing (mM): taurine 10; oxalic acid 10; glutamic acid 70; KCl 25; KH₂PO₄ 10; glucose 10; EGTA 0.5; pH 7.4. All solutions were oxygenated with 100% O₂. Ventricles were minced (1–2 mm thick slices), the resulting solution was agitated gently, the suspension was filtered through a nylon gauze, and the filtrate centrifuged for 4 min at 22×g. The cell pellets were then resuspended in normal Krebs solution. Suction pipettes were

pulled from microhaematocrit tubing by means of a controlled puller (Narashige). The pipettes that were prepared immediately before the experiments were filled with the following solution (mM): cesium aspartate 120; MgCl_2 3; EGTA 10; HEPES 5; pH 7.3.

The pipette was filled with KCL (3 M) containing Ang II (10^{-8} M), and the peptide was dialyzed into the cell. The resistance of the micropipette varied from 2.5 to 3.5 M Ω .

Experimental procedures used in isolated cells—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 in an open perfusion microincubator (Model PDMI-2, Medical System). Cells were allowed to adhere to the bottom of the chamber for 15 min and were superfused with normal Krebs solution. The electrical measurements were carried out using a patch-clamp technique (pCLAMP #10) (Axon Instruments, CA) in a whole cell configuration with an Axon (model 200B) amplifier and Digidata 1400A (Axon Instruments, CA). Series resistance originating from the tips of the micropipettes was compensated for electronically. The delayed outward K current was recorded in Na, Ca-free solution containing (mM): methyl-D-glucamine—150; MgCl_2 —5; Hepes—5, pH 7.4. Current–voltage (I–V) curves were obtained by applying voltage steps in 10 mV increments (–40 to +60 mV) starting from a holding potential of –40 mV.

Drugs—Angiotensin II and bisindolylmaleimide (Bis-1) were from Sigma Chemical Co. Losartan was from Merck.

Statistical analysis

Data are mean $\pm$ SEM. Comparison between groups was done by the analysis of variance (ANOVA), and differences were considered significant when $P < 0.05$.

Results

As shown in Fig. 1, the intracellular injection of Ang II in a ventricular cell of the failing heart caused an increase of the resting potential of $7.7 \text{ mV} \pm 4.3 \text{ mV}$ ($n = 39$) (4 animals) ($P < 0.05$) and an increment of the action potential duration at 50% and at 90% repolarization, respectively (see Fig. 1). These effects of the peptide were seen within 3 min of intracellular injection. The hyperpolarization lasts for about 15 min followed by a fall in membrane potential (see Fig. 1). The effect of the peptide on action potential duration persisted for a long time even when the micropipette was removed far away from the cell. As shown in Fig. 2, re-impalement of the same spot 60 min after the interruption of Ang injection still showed increased values for action potential duration.

No changes in resting potential or action potential duration were found during intracellular injection in absence of the peptide inside the pipette solution. Of particular interest is the finding that despite the criteria used in the injection procedure and no clogging of the micropipettes was seen, in some ventricular cells no effect of intracellular injection of Ang II was found (see Fig. 3). In this particular experiment, the pulse pressure was incremented from 45 to 60 psi in order to confirm the lack of action of the peptide.

To investigate the possible role of intracellular Ang II AT1 receptor on the change of electrophysiological parameters caused by Ang II, losartan (10^{-8} M) was added to the micropipettes solution containing Ang II (10^{-8} M) and both compounds were injected into the cell. As shown in Fig. 4 the effect of Ang II on action potential duration was inhibited by losartan.

To investigate whether the increase in action potential duration was related to a decline in potassium conductance, preliminary studies were performed on myocytes ($n = 15$) isolated from the ventricle of 3 cardiomyopathic hamsters (TO2) at 8 month of age. As shown in Fig. 5, a decrease in the density of the delayed rectifier K current, which is the major repolarizing outward current, was found after 5 min of intracellular dialysis of Ang II (10^{-8} M).

With repeated injections of Ang II in the intact ventricle, an increase in threshold and refractoriness was found. As shown in Fig. 6, the peptide caused a displacement of the strength-duration curve to the right what means that the cardiac refractoriness was significantly increased followed by a blockade of impulse propagation in some cells. In all these experiments, if the impalement was lost during the injection procedure, the experiment was rejected. No change in refractoriness was seen with repeated intracellular injections without Ang II inside the pipette. The increase in refractoriness elicited by Ang II persists for more than 60 min after interruption of the peptide injection and removal of the electrode from the cell (Fig. 2).

To investigate the role of PKC activation on the effect of Ang II on action potential duration, the ventricle was superfused with oxygenated Krebs solution containing bis-indolylmaleimide (Bis-1) (360 nM), a selective PKC inhibitor, for 15 min and then the peptide was injected into the cell. As shown in Fig. 1 (right), the effect of the peptide on the action potential duration was suppressed by Bis-1.

In some experiments in which intracellular injection of Ang II was repeated in several cells to investigate the variability of the effect of Ang II throughout the ventricle, spontaneous rhythmicity was frequently recorded (see Fig. 1).

Experiments performed in the intact ventricle of normal Golden hamsters (F1B), 8 month old, showed a smaller increment of action potential duration with intracellular injection of Ang II (10^{-8} M) as shown in Fig. 7.

Discussion

The present results show, by the first time, that intracellular injection of Ang II performed in the intact ventricle of the failing heart caused a significant change in resting potential, in action potential duration and refractoriness. The effect of the peptide was larger in the failing heart. The importance of these results relies on the demonstration that Ang II has an intracrine effect in the intact heart in which the structure of the extracellular space and the integrity of cell membrane were preserved.

It is classically known that prolongation of the plateau of the cardiac action potential is related to a decrease in the inward Ca current (I_{Ca}) inactivation or to a decrease in potassium conductance. Although both possibilities might explain the increment in duration of the action potential, the first alternative is unlikely because intracellular Ang II increases, not decreases I_{Ca} inactivation [6]. Therefore, a blockade of K channels leading to a small hyperpolarization and increase of the refractory period is a plausible explanation for our results. Our preliminary studies performed in isolated myocytes from the ventricle of a cardiomyopathic hamster (TO2) support this hypothesis.

The activation of the intracellular AT₁ receptors elicited by Ang II seems involved in the effect of the peptide on action potential duration because intracellular losartan abolish it. On the other hand, evidence is available that chronic stimulation of AT₁ receptor located at the cell membrane directly leads to an increased incidence of cardiac arrhythmia associated with delayed repolarization [15, 16]. Moreover, Ang II produces an inhibitory effect on I_{Kr} /hERG currents via AT₁ receptors linked to the PKC pathway in ventricular myocytes [16]. The

influence of PKC activation seems particularly important in the failing heart because previous studies of Wang et al. [18] have shown an increase in PKC activity during this pathological condition. Recent studies support the notion that the inhibitory effect of Ang II on potassium conductance in cardiac myocytes is mediated by the PKC pathway [16] and our results with bisindolylmaleimide -a selective PKC inhibitor, substantiated this view.

Although practically all the effects of Ang II on heart function are related to the activation of AT1 receptors located at the surface cell membrane, there is evidence that there is an intracellular Ang II receptor [10, 19, 20]. Moreover, AT1 receptor is able to internalize into nonclathrin-coated vesicles and stimulate directly nuclear AT1 receptors to increase in vitro transcription of mRNAs of TGF1 and MCP-1 in renal tissue [21–23].

The present results support the notion that endogenous Ang II [12] or internalization of the Ang II-AT1 receptor complex is able to alter ionic channels with consequent changes in heart excitability in the intact ventricle. Further studies are, however, needed to clarify the sources of intracellular Ang II generation in cardiac tissues particularly during different pathological conditions.

Although the increment in action potential duration produced by intracellular Ang II might be beneficial because it decreases the incidence of cardiac arrhythmias, the prolongation of the plateau beyond certain limits can generate early after depolarizations and torsade de pointes. Spontaneous rhythmicity, which was seen after intracellular injections of Ang II in different spots of the ventricular wall, is probably explained by the dispersion of action potential duration elicited by the different durations of the plateau.

The mechanism involved in the variability of the effect of intracellular Ang II injection on action potential duration is not known but the variability in potassium conductance found in different cardiac cells throughout the ventricle is a plausible hypothesis. Despite the possibility that variations in the effect of intracellular injection of Ang II might be related to changes in the amount of Ang II injected, it is surprising that in some ventricular fibers, no effect of intracellular Ang II on action potential was found despite the lack of clogging of the micropipette. This observation opens the possibility that different levels of activation of the intracrine renin angiotensin system, different expression of intracellular Ang II receptor or different degrees of internalization of the Ang II-AT1 receptor complex throughout the ventricle, be an important cause of changes in the effect of Ang II.

A significant finding is that the effect of intracellular Ang II on action potential duration persists even after 60 min or more of the interruption of the injection. Although this result might be related to a long lasting activation of PKC and intracellular signaling pathways, the implication of this observation for the failing heart is not known.

The relevance of these findings to clinical cardiology is several: (a) intracellular Ang is able to decrease the potassium conductance in the intact ventricle in which the structure of the extracellular space, cell contacts, and surface cell membrane is preserved; (b) the activation of the intracrine renin angiotensin system might generate reentrant rhythms through an increased dispersion of the action potential duration. Furthermore, it might potentiate the effects of drugs like quinidine that increases the action potential duration, facilitating the generation of arrhythmias like torsade de pointes; (c) the results might indicate that intracellular Ang II contributes to the well-known prolongation of the action potential found in the failing heart.

Acknowledgments

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Fig. 1.

Top, Left—influence of intracellular Ang II injection on transmembrane action potential duration recorded from a single fiber of the right ventricle. A—control B—after 4 min of intracellular Ang II administration (for 20 s); C—spontaneous rhythmicity recorded after the performance of intracellular injections in different areas of the *right* ventricle. *Vertical* calibration in A 34 mV. *Horizontal* calibration in A, B, and C 50 ms. In C, the sweep velocity was smaller. *Right*—each *bar* shows the effect of intracellular injection of Ang II on action potential duration recorded from 40 *right* ventricular fibers (4 animals). The effect of Bis-1 (360 nM), a selective PKC inhibitor, on the effect of Ang II is also shown. Each *bar* for this experiment is the average from 15 fibers (3 animals). *Vertical line* at each *bar* SEM. $P < 0.05$. *Bottom*—influence of intracellular Ang II injection on the resting potential. Each point is the average from 39 fibers (4 animals). *Vertical line* at each point SEM

Fig. 2.
Long-lasting effect of intracellular Ang II injection on action potential duration. Each *bar* is the average from 32 fibers (4 animals). *Vertical line* at each *bar* SEM. $P < 0.05$

Fig. 3. Lack of action of intracellular administration of Ang II in single ventricular fiber of the right ventricle from a cardiomyopathic hamster. A—control; B—recorded after 5 min of intracellular Ang II injection (45 psi, for 20 s). *Vertical* calibration in A 40 mV. *Horizontal* calibration in A and B 50 ms

Fig. 4. Influence of intracellular administration of losartan on the effect of intracellular Ang II on action potential duration in the intact ventricle. Each *bar* is the average from 35 fibers (4 animals). *Vertical line* at each *bar* SEM. $P > 0.05$

Fig. 5. Current/voltage relationship recorded from myocytes isolated from the ventricle of 8-month-old cardiomyopathic hamsters (TO2) showing the effect of intracellular injection of Ang II on potassium current. Each point is the average from 20 cardiomyocytes (3 animals). Vertical line at each point SEM. $P < 0.05$

Fig. 6.
Effect of intracellular Ang II injection on cardiac refractoriness. Each *bar* is the average from 35 fibers (4 animals). *Vertical line* at each *bar* SEM

Fig. 7.
Influence of intracellular injection of Ang II on action potential duration recorded from the right ventricle of F1B control hamsters. Each *bar* is the average from 20 fibers (3 animals).
Vertical line at each *bar* SEM