Autocrine Stimulation of Cardiac Na\(^{+}\)-Ca\(^{2+}\) Exchanger Currents by Endogenous Endothelin Released by Angiotensin II

Ernesto A. Aiello, María C. Villa-Abrille, Horacio E. Cingolani

The goal of the present study was to evaluate the effects of Ang II on the current produced by the Na\(^{+}\)-Ca\(^{2+}\) exchanger (I\(_{\text{NCX}}\)) in the reverse mode and the possible autocrine role played by the release of endothelin (ET) in these actions. I\(_{\text{NCX}}\) was studied in isolation in cat cardiac myocytes. Angiotensin II (Ang II) (100 nmol/L) increased I\(_{\text{NCX}}\) at potentials higher than 0 mV (at +60 mV: 2.07±0.22 pA/pF in control versus 2.73±0.22 pA/pF in Ang II, n=9; P<0.05). The increase in I\(_{\text{NCX}}\) induced by Ang II was prevented by the treatment of the cells with the unspecific blocker of the ET receptors, TAK 044 (1 μmol/L) (at +60 mV: 2.15±0.27 pA/pF in control versus 2.01±0.26 pA/pF in Ang II, n=5, NS). These results show, for the first time, that the effect of Ang II on I\(_{\text{NCX}}\) is the result of the autocrine actions of ET released by the octapeptide.

Previous experimental evidence indicates that some cardiovascular effects initially thought to be mediated by angiotensin II (Ang II) were, in fact, due to the release of endogenous endothelin (ET). In cultured neonatal rat cardiomyocytes, Ito et al. reported inhibition of Ang II-induced hypertrophy with a blocker of ET receptors (BQ123) or antisense oligonucleotides directed against mRNA of preproET. In rats, the elevation of blood pressure induced by the infusion of Ang II was reversed by the ETA blocker PD proET. In rats, the elevation of blood pressure induced by ET \(^1\) was partially inhibited by both forward and reverse modes of NCX, as it was previously reported for this concentration of the agent. Lower concentrations of KBR were proven to block selectively the reverse mode of NCX.\(^8\) Consistently, no effects of this drug on the forward mode were observed when 5 μmol/L KBR was used (shown in the online data supplement). Ni\(^{2+}\) (10 mmol/L), another NCX antagonist, blocked the current recorded at +60 mV by 85±5% (n=11). On average, the apparent reversal potentials (E\(_{\text{rev}}\)) of I\(_{\text{NCX}}\) were −44.4±2.5 mV (n=6) and −39.5±4.1 mV (n=11) estimated as the zero current potential of the KBR- and Ni\(^{2+}\)-sensitive currents, respectively. These values are similar to the I\(_{\text{NCX}}\) E\(_{\text{rev}}\) values reported by other authors working in similar conditions to those of the present study.\(^4\) The above experiments allowed us to conclude that the whole-cell currents registered were mainly underlain by the NCX (I\(_{\text{NCX}}\)).

Ballard and Schaffer\(^9\) reported that Ang II or ET increased the activity of the NCX in canine cardiac sarcomemal vesicles. Thus, we next examined whether Ang II was able to affect I\(_{\text{NCX}}\) in isolated cat cardiac myocytes. Figure 2A depicts the time course of the effect of Ang II (100 nmol/L) on the current recorded at +60 mV in a single myocyte. Ang II induced a gradual increase in the current that reaches steady-state values after 5 minutes of administration of the peptide. Average data of the current recorded at +60 mV in the absence and presence of Ang II are shown in Figure 2C. This current increased by 24.7±4.9% (n=26) in the presence of Ang II. The Ang II type I receptor (AT \(^1\)) antagonist losartan (1 μmol/L) reversed the effects of Ang II (data not shown). The outward current recorded at +60 mV is mostly but not totally I\(_{\text{NCX}}\) because a small remnant current is present after the effects of the NCX blockers Ni\(^{2+}\) or KB\(^{10}\). Thus, the possibility that the Ni\(^{2+}\)-insensitive current could be affected by Ang II was evaluated. No increase in outward current was induced by Ang II (100 nmol/L) in the presence of Ni\(^{2+}\).
(10 mmol/L) (0.75±0.31 pA/pF in Ni²⁺ and 0.78±0.36 pA/pF in Ni²⁺ plus Ang II, n=5; NS), indicating that the current affected by Ang II is most likely \( I_{\text{NCX}} \). Moreover, to confirm that Ang II affects \( I_{\text{NCX}} \), we studied the effects of this peptide on the KBR-sensitive currents. Figure 2D shows average current density-voltage relations obtained in 9 myocytes exposed to 10 μmol/L KBR after having obtained the increase in the whole-cell current by Ang II. Ang II induced a significant increase in outward currents at potentials positive to 0 mV. KBR blocked the currents recorded after Ang II to the same extent of those recorded in the absence of the peptide (Figure 1). Figure 1E shows the effects of Ang II on the average current-voltage relation for the KBR-sensitive currents, corresponding to \( I_{\text{NCX}} \), obtained by subtracting

**Figure 1.** Characterization of \( I_{\text{NCX}} \). A, Families of representative whole-cell currents evoked by depolarizing pulses between −80 to +100 mV from a holding potential of −40 mV, recorded in a myocyte in the absence and presence of KBR (10 μmol/L). Pulses (260 ms in duration) were delivered at 0.1 Hz. B, Voltage dependence of the average current density measured in 6 myocytes exposed to KBR (10 μmol/L). C, Average current-voltage relations of the KBR-sensitive currents, representing \( I_{\text{NCX}} \) (n=6).

**Figure 2.** Ang II effects on \( I_{\text{NCX}} \). A, Time course of effects of Ang II on the current registered at +60 mV from a holding potential of −40 mV, recorded in a single myocyte. Pulses were delivered at 0.1 Hz. B, Representative traces of whole-cell currents corresponding to the points indicated in panel A. C, Average data of current density recorded at +60 mV before and after the addition of Ang II (100 nmol/L) (n=26). D, Ang II (100 nmol/L) and KBR (10 μmol/L) effects on average current-voltage relations evoked by the voltage protocol of Figure 1 (n=9). E, Ang II effects on average current-voltage relations for the KBR-sensitive current, corresponding to \( I_{\text{NCX}} \) (n=9). *Ang II statistically different from control.
currents in KBR to those recorded in both control and in Ang II. Ang II significantly increased \( I_{NCX} \) at potentials positive to 0 mV. At 160 mV, \( I_{NCX} \) increased by 38.7±14.6% in the presence of Ang II (\( n=9 \)).

We next examined whether the Ang II–induced increase in \( I_{NCX} \) observed in the present study was produced by release of endogenous ET. For this purpose, we pretreated the cells with the nonselective blocker of ET receptors, TAK 044 (1 \( \mu \)mol/L). This blocker did not affect basal current (at 160 mV: 2.59±0.15 pA/pF in control versus 2.45±0.12 pA/pF in TAK 044-treated myocytes, \( n=13 \); NS). Figure 3A shows the time course of effects of Ang II in the presence of TAK 044 on the current registered at 160 mV. In the presence of the ET receptor blocker, Ang II failed to induce enhancement of this current. On average, no effects of Ang II were observed in the presence of TAK 044 on the current recorded at 160 mV (Figure 3C) or at any of the tested voltages (Figure 3D).

In addition, no effects of Ang II in the presence of TAK 044 were observed on the Ni\(^{2+}\)-sensitive currents, representing \( I_{NCX} \) (at 160 mV: 2.15±0.27 pA/pF in TAK 044 versus 2.01±0.27 pA/pF in Ang II plus TAK 044, \( n=5 \); NS).

To rule out the possibility that the ET receptor blocker TAK 044 is acting on the Ang II receptors, we performed experiments in which the L-type calcium current (\( I_{Ca} \)) was measured in the absence and presence of Ang II (100 nmol/L) with or without TAK 044 (1 \( \mu \)mol/L) in the extracellular solution. We have recently reported that Ang II enhances \( I_{Ca} \) via activation of \( AT_1 \) receptors in cat cardiac myocytes.\(^3\)

Under the recording conditions of the present study, the peak \( I_{Ca} \) recorded at 0 mV increased by 23±8% (\( n=5 \)) after 10 minutes in the presence of Ang II. A similar increment in \( I_{Ca} \) after 10 minutes of Ang II was observed in the presence of TAK 044 (21±7%, \( n=5 \)). These results suggest that this ET receptor blocker did not unspecifically bind to \( AT_1 \) receptors and that the endogenous ET released by Ang II did not affect \( I_{Ca} \). The previously reported failure of ET-1 to affect \( I_{Ca} \) when standard whole-cell configuration is used can account for the last result.\(^10\)

Taken together, the above results allow us to suggest that in cardiac myocytes the stimulation of \( I_{NCX} \) induced by Ang II is, in fact, mediated by endogenous ET released by Ang II. The major finding of this study is the demonstration at the cardiac single-cell level of the existence of an autocrine mechanism in the heart involving the myocyte as the source and target of ET. Additional observations and extended discussion of the results are presented in the online data supplement.

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References


**Key Words**: cardiac myocytes, angiotensin II, endothelin, sodium-calcium exchanger.
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AUTOCRINE STIMULATION OF CARDIAC Na⁺/Ca²⁺
EXCHANGER CURRENTS BY ENDOGENOUS ENDOTHELIN
RELEASED BY ANGIOTENSIN II

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ONLINE DATA SUPPLEMENT

METHODS

Isolated cat ventricular myocytes were diluted and placed in a perfusion chamber and superfused with bath solution at a flow rate of 1.5 ml/min. The standard whole-cell configuration of the patch clamp technique was used for voltage-clamp recordings with a patch-clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, Calif.). Whole-cell currents (filtered at 1 kHz) were digitally recorded directly to hard disk via an analog-to-digital convertor (Digidata 1200, Axon Instruments) interfaced with an IBM clone computer running pClamp and Axotape software (Axon Instruments). Data analysis was performed with pClamp (Clampfit).

Isolation of \( I_{\text{NCX}} \): The external solution contained (in mmol/L): NaCl 140, MgCl₂ 1, CaCl₂ 2, glucose 10, HEPES 5, pH 7.4 with Tris base. This solution also
contains (in μmol/L): nifedipine 10, 4-acetamido-4'-isothiocyanoato-stilbene-2,2'-disulfonic acid (SITS) 100 and ouabain 0.5 in order to block L-type Ca$^{2+}$, Cl$^-$/ and Na$^+$/K$^+$-ATPase currents, respectively. The NCX blockers KB-R7943 and Ni$^{2+}$ (NiCl$_2$) were added to the extracellular solution in some experiments. The internal solution contained (in mmol/L): NaCl 20, CsCl 110, MgCl$_2$ 0.4, CaCl$_2$ 1.75, glucose 5, HEPES 10, Mg ATP 5, tetraethylammonium (TEA) 20 and BAPTA 5, pH 7.2 with Tris. The mixture of 5 mmol/L BAPTA and 1.75 mmol/L Ca$^{2+}$ gave a free [Ca$^{2+}$] of 200 nmol/L (calculated using the Maxchelator program, D. Bers, Loyola University, Chicago, Ill). The liquid junction potential (-2.3 mV) was not corrected. The currents were measured at the end of the depolarizing pulses. In some experiments L-type Ca$^{2+}$ current (I$_{Ca}$) was recorded with the same internal and external solutions, except for the exclusion of nifedipine in the latter. I$_{Ca}$ was registered and measured as previously described (1). For each cell, capacitative current was recorded to determine the membrane capacitance, and the currents were normalized for cell capacitance. The average cell capacitance was 129±6 pF (n=23). All data are presented as means±SE. Comparison between groups was assessed by a paired Student’s t-test. A value of P<0.05 was taken to indicate statistical significance.

**RESULTS**

Figure 1 shows the inhibitory effects of the NCX blocker KB-R7943 (KBR, 5 μmol/L) on whole-cell currents recorded under conditions of I$_{NCX}$ isolation. This concentration of KBR was proven to block selectively the reverse mode of NCX (2). Consistently, no effects of this drug on the forward mode were observed when 5 μmol/L KBR was used, as evidenced in the current-voltage relations for
the total whole-cell current (Panel B) and for the KBR-sensitive current (Panel C).

In another set of experiments, the addition to the extracellular solution of a different non-selective ET receptor antagonist PD 142,893 (1 \textmu mol/L) partially reversed the Ang II (100 nmol/L) induced increase in $I_{\text{NCX}}$. On average (at +60 mV), the percentage increase of $I_{\text{NCX}}$ after 5-7 minutes of Ang II (respect to control) was 29±7 % and decreased to 10±6 % after 5-7 minutes in PD 142,893 in the continuous presence of Ang II (n=6; p<0.05). PD 142,893 did not affect basal current values (at +60 mV, 2.4±0.5 pA/pF and 2.2±0.4 pA/pF before and after the addition of PD 142,893 to the bath solution, respectively; n=6, NS).

**DISCUSSION**

The subcellular mechanisms by which endogenous ET released by Ang II increases $I_{\text{NCX}}$ were not evaluated in the present study. We have recently reported that the increase in $I_{\text{Ca}}$ induced by Ang II is a PKC dependent mechanism via the activation of a Ca$^{2+}$-dependent isoform (1). Whether or not the activation of the NCX is also a PKC-dependent mechanism was not explored by us. However, the possibility of the activation of a different PKC isoform could be speculated.

The functional relevance of the Ang II/ET-induced stimulation of the reverse mode of the NCX was not investigated in the present study. Other results from our group failed to demonstrate any participation of the NCX or the Na$^{+}$/H$^{+}$ exchanger (NHE) in the Ang II-induced positive inotropic effect (3). A possible explanation for this discrepancy is that the effect of Ang II was examined at maximal dose of the peptide in the experiments by Vila Petroff et
al. (3). When pH changes are minimized, the positive inotropic effect of Ang II is probably the result of an increase in the Ca$^{2+}$ transient (CaT). This increase is the result of two mechanisms: a) the increase in $I_{Ca}$ induced by Ang II per se and b) the stimulation of the NCX in reverse (increase in [Na$^+$]) and direct activation) mediated by the autocrine release of ET. At the plateau of the CaT-Force relationship, the suppression of the contribution of the NCX by KBR is probably not inducing significant changes in force. However, when the effects of KBR are examined at submaximal doses of Ang II, this agent induces a decrease in the response to Ang II. Unpublished experiments by us are supporting this position.

We previously reported that myocardial stretch elicited an autocrine-paracrine mechanism beginning with Ang II release, and followed by ET release/formation and NHE stimulation. The NHE activation increases Na$^+$, driving the NCX in reverse. In these experiments, the prevention of the [Na$^+$] increase by blocking the NHE abolished the slow force response (4,5). This indicates that an increase in [Na$^+$], is a mandatory step to drive the NCX in reverse. On the other hand, the control of the intracellular milieu in the whole-cell experiments of the present study likely prevented changes in [Na$^+$], as evidenced by the lack of effects of Ang II on the $I_{NCX} E_{rev}$, supporting in this case the contention of a direct modulation of the NCX by ET. Thus, at the light of the present results, a contribution of the activation of the NCX in reverse independent of the increase in [Na$^+$], can be postulated. Furthermore, Ang II increases $I_{Ca}$. Actually, we published before (1) and we are presenting evidence here that Ang II increases $I_{Ca}$. It can be argued that if Ang II increases $I_{Ca}$, the SFR would not be suppressed by interfering with NCX. The possible
explanation is that low doses of Ang II mainly stimulate ET release/formation without significant effects on $I_{Ca}$. 

REFERENCES


FIGURE LEGENDS

**Figure 1**: Characterization of $I_{NCX}$. A: Families of representative whole-cell currents evoked by depolarizing pulses between -80 to +100 mV from a holding potential of -40 mV, recorded in a cat myocyte in the absence and presence of
KBR (5 μmol/L). Pulses (260 ms duration) were delivered at 0.1 Hz. **B**: Voltage dependence of the average current density measured in 5 myocytes exposed to KBR (5 μmol/L). **C**: Average current-voltage relations of the KBR-sensitive currents, representing $I_{NCX}$ (n=5).
Figure 1 (online data supplement)