Effects of the Renin-Angiotensin System on the Current $I_{to}$ in Epicardial and Endocardial Ventricular Myocytes From the Canine Heart

Hangang Yu, Junyuan Gao, Hongsheng Wang, Randy Wymore, Susan Steinberg, David McKinnon, Michael R. Rosen, Ira S. Cohen

Abstract—The Ca$^{2+}$-independent portion of transient outward K$^+$ current ($I_{to}$) exhibits a transmural gradient in ventricle. To investigate control mechanisms for this gradient, we studied canine epicardial and endocardial ventricular myocytes with use of the whole-cell patch-clamp technique. $I_{to}$ was larger in amplitude, had a more negative voltage threshold for activation, and had a more negative midpoint of inactivation in epicardium. Recovery from inactivation was $>10$-fold slower in endocardium. Incubation of epicardial myocytes with angiotensin II for 2 to 52 hours altered $I_{to}$ to resemble unincubated endocardium and reduced the amplitude of the phase 1 notch of the action potential. In contrast, incubation of endocardial myocytes with losartan for 2 to 52 hours altered $I_{to}$ to resemble unincubated epicardium and induced a phase 1 notch in the action potential. With RNAse protection assays, we determined that incubations with angiotensin II or losartan did not alter mRNA levels for either Kv4.3 or Kv1.4; thus, a change in the $\alpha$ subunit for $I_{to}$ is unlikely to be responsible. To test whether posttranslational modification produced the effects of angiotensin II, we coexpressed Kv4.3 and the angiotensin II type 1a receptor in Xenopus oocytes. Incubation with angiotensin II increased the time constant for recovery from inactivation of the expressed current by 2-fold with an incubation time constant of 3.7 hours. No effect on activation or inactivation voltage dependence was observed. These results demonstrate that the properties of $I_{to}$ in endocardium and epicardium are plastic and likely under the tonic-differing influence of the renin-angiotensin system. (Circ Res. 2000;86:1062-1068.)

Key Words: angiotensin, epicardium, endocardium, current

Differences in the action potential duration of myocytes from the endocardium (ENDO) and the epicardium (EPI) of the canine heart contribute to the morphology and polarity of the T wave of the ECG. 1,2 The ionic currents responsible for these differences are determinants of the T wave. One major difference between EPI and ENDO myocyte ion currents is in the Ca$^{2+}$-independent transient outward current (referred to here as $I_{to}$). Previous investigations have demonstrated a lower density and slower recovery from inactivation in ENDO compared with in EPI. 3,4 We recently identified the molecular basis of $I_{to}$ to in canine ventricle as a member of the Shaker family of K channels, Kv4.3. 5

Among the potential modulators of the T wave is the renin-angiotensin system and its active hormone, angiotensin II (Ang II). 6 Ang II has direct actions on cardiac membrane currents, increasing chloride current 7 and decreasing Na$^+$/K$^+$ exchange current 8 in rabbit ventricular myocytes. These actions occur through the Ang II type 1 (AT$_1$) receptor, which is prevalent in cardiac tissues of many species. 9,10 Not only is the AT$_1$ receptor present in heart, but also an autocrine renin-angiotensin system is present in rat ventricular myocytes. 11–13 This suggests that mammalian cardiac myocytes or nonmyocyte elements in mammalian hearts can produce this hormone, which modulates repolarizing currents.

In the present study, we first describe the properties of $I_{to}$ in canine EPI and ENDO, outlining differences in both current density and gating properties. We then demonstrate that the differences between these 2 tissues are entirely plastic; that is, long-term exposure of EPI to Ang II or of ENDO to the AT$_1$ receptor blocker losartan can convert properties of $I_{to}$ of either region into those of the other, thereby providing a basis for an altered transmural gradient of repolarization in the ventricle and resultant T wave changes.

Materials and Methods

Cell Preparation

Adult dogs of either sex were euthanized with an injection of sodium pentobarbital. EPI and ENDO ventricular myocytes were dissociated...
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### \( I_{\text{to}} \) Plasticity in ENDO and EPI

<table>
<thead>
<tr>
<th>Conductance-voltage slope, pS/(pF/mV)</th>
<th>EPI</th>
<th>EPI + Ang II</th>
<th>ENDO</th>
<th>ENDO + Losartan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Threshold for activation, mV(^\dagger)</td>
<td>(-25 \pm 2) (n = 24)</td>
<td>(-10 \pm 1) (n = 17)</td>
<td>(4 \pm 3) (n = 15)</td>
<td>(-29 \pm 2) (n = 24)</td>
</tr>
<tr>
<td>(\tau_{\text{inact}}), ms(...)</td>
<td>(27 \pm 6) (n = 9)</td>
<td>(492 \pm 56) (n = 12)</td>
<td>(764 \pm 103) (n = 6)</td>
<td>(27 \pm 5) (n = 15)</td>
</tr>
<tr>
<td>Steady-state inactivation ((V_{1/2})/slope), mV(...)</td>
<td>(-43 \pm 3/5 \pm 1) (n = 12)</td>
<td>(-27 \pm 2/6 \pm 1) (n = 11)</td>
<td>(-29 \pm 3/9 \pm 1) (n = 13)</td>
<td>(-42 \pm 2/9 \pm 1) (n = 13)</td>
</tr>
</tbody>
</table>

All indicates Ang II; LOS, losartan.

\*Linear fit takes the first and last 2 points of conductance-voltage relationship.

\(\dagger\)Compared with EPI, \(P_{\text{EPI}+\text{Ang II}} < 0.05, P_{\text{ENDO}} < 0.05\); compared with ENDO, \(P_{\text{ENDO}+\text{Losartan}} < 0.05\).

\(\ddagger\)Compared with EPI, \(P_{\text{EPI}+\text{Ang II}} < 0.05, P_{\text{ENDO}} < 0.05\); compared with ENDO, \(P_{\text{ENDO}+\text{Losartan}} < 0.05\).

\(\S\)For midpoint voltage compared with EPI, \(P_{\text{EPI}+\text{Ang II}} < 0.05, P_{\text{ENDO}} < 0.05\); compared with ENDO, \(P_{\text{ENDO}+\text{Losartan}} < 0.05\).

For slope, compared with EPI, \(P_{\text{EPI}+\text{Ang II}} = 0.74, P_{\text{ENDO}} = 0.84\); compared with ENDO, \(P_{\text{ENDO}+\text{Losartan}} = 0.02\).

### Results

#### Gating Properties of \( I_{\text{to}} \) in ENDO Differ From Those in EPI

We began our investigation by characterizing \( I_{\text{to}} \) in both ENDO and EPI ventricular myocytes. We first examined the voltage dependence of activation. We held the cells at \(-65\) mV and first briefly depolarized (5 ms) to \(-45\) mV to inactivate \( I_{\text{to}} \) and then depolarized to voltages from \(-40\) to \(+50\) mV in 10-mV increments for 300 ms. The average threshold for first observation of transient outward current (see Materials and Methods for definition) was \(-25 \pm 2\) mV (n = 24) in EPI myocytes and \(+4 \pm 3\) mV (n = 15) in ENDO myocytes. As previously reported,\(^3\) the amplitude of transient outward current is also larger in the EPI myocyte. The larger \( I_{\text{to}} \) was determined in the Table by measuring the dependence of the transient outward conductance on voltage, which increases almost 4-fold more rapidly in EPI than in ENDO. Our results are provided in Figures 1C and 1E for EPI and ENDO myocytes and 104 \pm 8 pF (n = 26) in EPI myocytes.

We also examined the voltage dependence of inactivation of \( I_{\text{to}} \). We held the membrane at \(-65\) mV and used a 3-pulse protocol. We first depolarized for 5 ms to \(-45\) mV to inactivate \( I_{\text{to}} \) and then used a first (conditioning) pulse, which either depolarized or hyperpolarized for 2 seconds to bring the membrane to a new starting value for inactivation; with the second (test) pulse, we depolarized to \(+10\) mV for 400 ms (see Figure 1A). \( I_{\text{to}} \) amplitude normalized to \( I_{\text{to}} \) for the most negative conditioning pulse is plotted against each conditioning voltage in Figure 1B, along with the fits of a Boltzmann 2-state model. \( I_{\text{to}} \) exhibited a more positive midpoint of inactivation in ENDO myocytes and a reduction in steepness of the inactivation curve.

The time constant of inactivation of \( I_{\text{to}} \) did not differ between ENDO and EPI. We then used a 2-pulse protocol to investigate the kinetics of recovery from inactivation. The cycle length was 8 seconds. The first depolarizing pulse of 300 ms in duration was applied from the holding potential of \(-65\) mV to the test potential of \(+5\) mV for 400 ms (see Figure 1A). \( I_{\text{to}} \) amplitude normalized to \( I_{\text{to}} \) for the most negative conditioning pulse is plotted against each conditioning voltage in Figure 1B, along with the fits of a Boltzmann 2-state model. \( I_{\text{to}} \) exhibited a more positive midpoint of inactivation in ENDO myocytes and a reduction in steepness of the inactivation curve.

#### Data Analysis

Isolated cells were maintained at 32\( \pm 1\)°C (±0.5°C in each experiment).\(^6\) \( I_{\text{to}} \) and action potentials were recorded with use of the whole-cell patch-clamp technique. \( I_{\text{to}} \) was defined as the difference to activation. The threshold was the most negative test voltage that elicited an \( I_{\text{to}} \) of \( >10 \) pA. The pipettes were filled with solution containing (in mmol/L) NaCl 6, K-aspartate 130, NaCl 5, EGTA 0.5, MgCl 2 4, CaCl 2 2, and creatine 5, titrated to pH 7.2 with KOH.\(^5\) at room temperature.

### Measurement of \( I_{\text{to}} \), Action Potentials, and Data Analysis

Cells were stored in KB solution (which contained [in mmol/L] KCl 83, K-HPO 4 30, MgSO 4 5, Na-pyruvic acid 5, β-OH butyric acid [Na-salt] 5, taurine 20, glucose 10, EGTA 0.5, HEPES 5, Na 2 ATP 5, and creatine 5, titrated to pH 7.2 with KOH).\(^5\) at room temperature for at least 1 hour before electrophysiological experiments. Cells were incubated in KB solution at room temperature the first day of experiments (2 to 5 hours) and then at 4°C for experimentation on days 2 and 3 (6 to 52 hours). Ang II and saralasin were purchased from Sigma Chemical Co, and losartan was kindly provided by Merck.

### RNase Protection Assays

ENDO and EPI myocytes were incubated for 24 hours either with or without 2 μmol/L Ang II (EPI) or 2 μmol/L losartan (ENDO). mRNA was isolated with the use of paramagnetic poly(dT) beads (Dynal). RNA probes for Kv1.4 and Kv4.3 were prepared as described previously.\(^5\) RNase protection assays were performed and quantified as described previously.\(^5\)

### Heterologous Expression in Oocytes

Oocytes were prepared from \( X. laevis \) as previously described.\(^4\) Oocytes were injected with 50 nL Kv4.3 mRNA or an equimolar ratio of Kv4.3 and AT 1a mRNA. Injected oocytes were incubated at 18°C for 24 to 48 hours. In the study of Ang II treatment, oocytes were then incubated in OR3 solution containing 4 μmol/L Ang II for 25 to 32 hours at 18°C before testing.

Oocytes were voltage-clamped with the use of a 2-microelectrode voltage clamp. The extracellular recording solution was OR2.\(^5\)

Data are presented as mean±SEM. Statistical significance was tested with Student’s t tests. P < 0.05 was considered statistically significant.

An expanded Materials and Methods section is available online at http://www.circresaha.org.
much slower rate in ENDO than in EPI myocytes. This difference in kinetics is quantified in Figures 1D and 1F. For this example, recovery from inactivation of EPI $I_o$ to is fit with a time constant of 30 ms, whereas the recovery from inactivation for ENDO $I_o$ to is fit by a constant of 950 ms. The average results summarized in the Table suggest that $I_o$ in EPI is more dense, activates more negatively, inactivates more negatively, and recovers faster from inactivation than does the same current in ENDO.

Incubation of EPI Myocytes With Ang II Alters $I_o$ so That Its Gating Properties Resemble Those Found in ENDO

Superfusion of EPI myocytes with 1 μmol/L Ang II for 5 to 20 minutes induced no change in the activation or density of $I_o$ (n=8). Recovery from inactivation was also studied in 4 myocytes, where no change was observed. We then studied the effects of chronic exposure of EPI myocytes to Ang II by storing myocytes for a period of 2 to 52 hours in KB medium containing 0.5 to 2 μmol/L Ang II, after which electrophysiological studies of $I_o$ were performed in the absence of Ang II at 32° to 35°C. We also incubated EPI myocytes from the same animals in the same storage solution and for the same time period but without Ang II. These myocytes showed no significant difference from the control values for EPI myocytes studied acutely. Figure 2A provides sample data from EPI myocytes incubated in control solution or with Ang II for 24 hours (Figure 2B). Figure 2C clearly shows the current density is reduced by Ang II and that the activation has been shifted to more positive potentials (see Table).

We also examined the steady-state voltage dependence of inactivation and the recovery from inactivation in the Ang II–incubated myocytes; the results are presented in Figure 3. Steady-state inactivation is shifted to more positive potentials, and the recovery from inactivation is dramatically slowed by incubation with Ang II.

Figure 1. Steady-state voltage dependence of $I_o$ inactivation. Schematic of the protocol is provided in the inset. A, Sample current traces in an EPI myocyte. The conditioning pulses are from −95 to +15 mV in 10-mV increments. The test pulse is +10 mV. B, Steady-state inactivation curves fit by a Boltzmann 2-state model. Kinetics of recovery from inactivation. Schematic of the typical 2-pulse protocol is provided as an inset. C, EPI myocyte. E, ENDO myocyte. Recovery from inactivation can be fit to the function $1-e^{-t/t_r}$ (D, EPI; F, ENDO). Legends for all figures appear in more detail online (see http://www.circresaha.org).

Figure 2. Effect of chronic exposure to Ang II (AII) on $I_o$ activation and current density in EPI. A, Control myocyte, incubated for 24 hours. $I_o$ is activated at −30 mV, and the current amplitude is increased with further membrane depolarizations up to +50 mV. B, After incubation of another myocyte with Ang II for 24 hours, identical depolarizing pulses to those used in A were applied. $I_o$ begins to activate at −10 mV, and its amplitude is increased with further depolarization up to +50 mV. C, Averaged results before and after incubation; current-voltage relationship shows the averaged current density of $I_o$ in EPI myocytes pretreated with Ang II is reduced.

Figure 3. Effects of chronic exposure to 1 μmol/L Ang II (AII) on voltage dependence of inactivation and recovery from inactivation of $I_o$. A, Steady-state inactivation in 11 myocytes incubated for 24 hours with Ang II is shifted to more positive potentials than in control (12 myocytes). B and C, $I_o$ recovers much more slowly from inactivation.

Figure 2A provides sample data from EPI myocytes incubated in control solution or with Ang II for 24 hours (Figure 2B). Figure 2C clearly shows the current density is reduced by Ang II and that the activation has been shifted to more positive potentials (see Table).
**Incubation of ENDO Myocytes With the AT₁ Receptor Blocker Losartan Alters I_{to} Such That Its Properties Resemble Those of EPI**

Given the effects of incubation with Ang II on EPI myocytes and the existence of an autocrine renin-angiotensin system, we explored the possibility that blockade of the normal Ang II pathway via the AT₁ receptor might modulate steady-state inactivation and recovery from inactivation of 

\[ I_{to} \] 

in ENDO myocytes. Losartan incubation converts most properties of \( I_{to} \) in ENDO myocytes to resemble those of unincubated EPI myocytes.

Two additional control experiments were performed. In 1 experiment (\( n=10 \)), EPI was incubated for 2 to 52 hours with losartan, and in the other (\( n=5 \)), ENDO was incubated with Ang II for the same time period. No effect was observed of either incubation on \( I_{to} \) (data not shown).

**Action Potential Notch Is Influenced by Ang II**

\( I_{to} \) is responsible for phase I rapid repolarization. Given the effects of Ang II and of losartan on EPI and ENDO \( I_{to} \), respectively, changes in the action potential contour might be expected. We recorded action potentials from 11 control EPI and 10 control ENDO myocytes as well as from 5 EPI myocytes incubated for 24 hours with 1 \( \mu \)mol/L Ang II and from 6 ENDO myocytes incubated for 24 hours with 1 \( \mu \)mol/L losartan. Representative results are illustrated in Figure 6. Control EPI action potentials always demonstrated a notch, whereas control ENDO action potentials did not (a difference noted previously by others¹). EPI myocyte incubation with Ang II resulted in loss of the notch, whereas ENDO myocyte incubation with losartan produced a notch. These changes are consistent with the changes in \( I_{to} \) that we described earlier.

**mRNA levels for Kv4.3 and Kv1.4**

The patch-clamp results revealed changes in \( I_{to} \) induced by incubation with either Ang II (EPI) or losartan (ENDO). However, they do not provide a mechanism for the observed effects. One possibility is a change in the molecular correlate of the current. We previously demonstrated that Kv4.3 underlies \( I_{to} \) in the canine ventricle. However, Kv1.4 is present in canine myocytes and recovers from inactivation much more slowly. We therefore examined the effects of...
incubation of EPI myocytes with 2 μmol/L Ang II for 24 hours and the effects of incubation of ENDO myocytes with 2 μmol/L losartan for the same period on the mRNA level for these K⁺ channel subunits. The mRNA levels were quantified from 3 samples with the use of RNase protection assays. For EPI, (EPI + Ang II)/EPI = 1.00 ± 0.07 for Kv4.3 and 0.98 ± 0.09 for Kv1.4. For ENDO, (ENDO + losartan)/ENDO = 1.01 ± 0.04 for Kv4.3 and 1.02 ± 0.10 for Kv1.4. Neither incubation resulted in any change in mRNA levels for either transcript.

Heterologous Expression of Kv4.3 With the AT₁ Receptor in X laevis Oocytes

If a change in the molecular correlate of Ito does not occur, possibly the changes could be induced by posttranslational modification of the existing protein. To test this hypothesis, we heterologously expressed the dominant molecular correlate of canine Ito, Kv4.3, in X laevis oocytes along with the AT₁ receptor.

Figure 7 shows the results of our study of the effects of incubation with 4 μmol/L Ang II for up to 32 hours on recovery from inactivation with the following protocol. Membrane potential was depolarized to −20 mV for 600 to 900 ms from a holding potential of −90 mV to completely inactivate Kv4.3 current. The recovery potential was −100 mV followed by test steps to −20 mV at various intervals. The recovery kinetics were slower for the treated oocyte (τ = 361 ms) compared with the control oocyte (τ = 141 ms) (Figures 7A and 7B). The average ≈2-fold slowing of the time constant for recovery from inactivation is provided in Figure 7C for all oocytes studied. Figure 7C also illustrates that this effect is not observed in the absence of either Ang II or the AT₁ receptor. Figure 7D illustrates the time course of AT₁ receptor modulation of Kv4.3 channel recovery kinetics. Increase in recovery time constant of the Kv4.3 channel was normalized to the maximum increase for each batch of oocytes and was plotted against Ang II incubation time. τ = 3.7 hours.

Discussion

In agreement with previous studies,³,⁴ we found a smaller density and a slower recovery from inactivation of Ito in ENDO than in EPI. We also observed a more positive threshold for Ito activation in ENDO. The smaller current density, more positive activation, and slower recovery from inactivation all contribute to the absence of a notch in the ENDO action potential during steady-state ventricular beating. One additional difference between these 2 tissue types is
the voltage dependence of $I_{\text{to}}$ inactivation, which is more positive in ENDO.

The critical findings in our study were that, first, Ang II converts many of the properties of EPI $I_{\text{to}}$ to those normally seen in ENDO, and second, losartan converts many of the properties of ENDO $I_{\text{to}}$ to those normally seen in EPI. Important to these observations is that neither exposure of ENDO to agonist nor exposure of EPI to antagonist had any effect and that exposure of EPI to agonist plus antagonist also had no effect. Moreover, none of the effects on EPI or ENDO were acute: incubation was required. Such incubation of EPI in Ang II (1) decreases the $I_{\text{to}}$ current density, (2) shifts the threshold for $I_{\text{to}}$ activation to more positive voltages, (3) shifts the voltage dependence of $I_{\text{to}}$ inactivation to more positive voltages, and (4) slows $I_{\text{to}}$ recovery from inactivation by $>1$ order of magnitude. Although the acute actions of Ang II on cardiac membrane currents in other species are mediated by activation of protein kinase C, and may include mitogen-activated protein kinase in the signaling cascade, the signaling pathway that underlies the chronic action of Ang II on $I_{\text{to}}$ remains to be determined.

Chronic incubation of ENDO with losartan induces (1) an increase in $I_{\text{to}}$ density, (2) a more negative $I_{\text{to}}$ activation, (3) a more negative voltage dependence of $I_{\text{to}}$ inactivation, and (4) a dramatic speeding of $I_{\text{to}}$ recovery from inactivation. The resultant ENDO $I_{\text{to}}$ resembles that observed in normal EPI. This observation is all the more remarkable because it occurs in the absence of applied agonist. One plausible explanation is that tonic activation of the AT$_1$ receptor of normal ENDO myocytes occurs both in vivo and in the incubate and that the source of this tonic activation is an autocrine renin-angiotensin system present in the canine ventricular myocytes or in nonmyocyte cells that remain in the incubate.

An autocrine renin-angiotensin system contributes to modulation of the T wave, then ENDO is likely exposed to a larger tonic influence of Ang II than EPI. This supposition is based on the observation that the conversion of ENDO $I_{\text{to}}$ to EPI $I_{\text{to}}$ involves AT$_1$ receptor blockade, whereas the converse action requires AT$_1$ receptor activation. This raises the possibility that these 2 cell types are normally under a chronically differing influence of the renin-angiotensin system. An autocrine renin-angiotensin system has already been reported in rat ventricular myocytes. Either Ang II production, AT$_1$ receptor density, or coupling of the AT$_1$ receptor to its second messenger pathway might be more efficient in the ENDO, leading to a larger tonic effect. A higher activity of ACE in ENDO than in EPI has been reported in rat ventricle. Also, angiotensin mRNA is higher in ENDO than in EPI in human ventricle. Preliminary experiments with ACE inhibition in dogs have demonstrated alterations in the T wave vector.

The changes we observed in EPI and ENDO $I_{\text{to}}$ could have been caused by at least 3 alternatives: (1) a change in the molecular correlate of $I_{\text{to}}$, (2) posttranslational modification of the channel protein, and (3) an auxiliary ($\beta$) subunit. We consider each in turn. (1) Regarding a change in the molecular correlate of $I_{\text{to}}$, in canine myocytes, $I_{\text{to}}$ has been identified as Kv4.3, although in control conditions, there also is some expression of Kv1.4. Because Kv1.4 recovers from inactiva-

References
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