Original Article

Inhibition of Inward Rectifier K^+ Currents by Angiotensin II in Rat Atrial Myocytes: Lack of Effects in Cells from Spontaneously Hypertensive Rats

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We examined the effects of angiotensin II (Ang II) on inward rectifier K^+ currents (I_{K1}) in rat atrial myocytes. [^{125}I]Ang II–binding assays revealed the presence of both Ang II type 1 (AT_1) and type 2 (AT_2) receptors in atrial membrane preparations. Ang II inhibited I_{K1} in isolated atrial myocytes with an IC_{50} of 46 nmol/l. This inhibition was abolished by the AT_1 antagonist RNH6270 but not at all by the AT_2 antagonist PD123319. Treatment of cells with pertussis toxin or a synthetic decapeptide corresponding to the carboxyl-terminus of $G_{i\alpha\text{-}3}$ abolished the inhibition by Ang II, indicating the role of a G_i-dependent signaling pathway. Accordingly, Ang II failed to inhibit I_{K1} in the presence of forskolin, dibutyryl-cAMP or protein kinase A catalytic subunits. In spite of the increased binding capacities for [^{125}I]Ang II, Ang II failed to affect I_{K1} in cells from spontaneously hypertensive rats (SHR). AT_1 immunoprecipitation from atrial extracts revealed decreased amounts of $G_{i\alpha\text{-}2}$ and $G_{i\alpha\text{-}3}$ proteins associated with this receptor in SHR as compared with controls. The reduced coupling of AT_1 with $G_{i\alpha}$ proteins may underlie the unresponsiveness of atrial I_{K1} to Ang II in SHR cells. (*Hypertens Res* 2006; 29: 923–934)

***Key Words*: angiotensin II, inward rectifier K^+ currents, angiotensin II type 1, $G_{i\alpha}$, hypertension**

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Introduction

The inward rectifier K^+ current (I_{K1}) is responsible for both terminal repolarization of the action potential and maintenance of the resting potential in excitable and nonexcitable cells (*1*). Perturbation of this current may be involved in arrhythmogenesis in disease conditions such as coronary heart diseases (*2*) or dilated cardiomyopathy (*3*). This arrhythmogenesis plays the pivotal role for atrial arrythmias, since the mutation of Kir2.1 encoding I_{K1} has been reported to cause the inherited atrial fibrillation (*4*).

The potent vasoconstrictor angiotensin II (Ang II) can modulate various cardiac membrane currents, and its effects on I_{K1} have been examined in previous studies with contradictory results. For example, Ang II did not modulate I_{K1} in rat atrial myocytes (*5*), whereas it inhibited the same current in porcine endothelial cells and mouse renal juxtaglomerular cells (*6*, *7*). Still another report described stimulatory effects of Ang II on I_{K1} in rabbit ventricular myocytes (*8*). Ang II binds to type 1 (AT_1) and type 2 (AT_2) receptors. Each of these receptor subtypes is coupled with a subset of G proteins which activates various signaling pathways, including the conventional phospholipase C/Ca^{2+}/protein kinase C (PLC/ Ca^{2+}/PKC) and adenylate cyclase/cAMP/protein kinase A (AC/cAMP/PKA) signaling pathways. Since I_{K1} is regulated by both PKC and PKA (*9*, *10*), it is reasonable to assume that selective activation of signaling pathways in a given cell type may underlie the differential effects of Ang II.

It appears, however, that the selective activation of signaling pathways is not the sole determinant of cells' responses, because it has been shown that I_{K1} can be modulated in opposite ways in response to the activation of the same signaling pathway. For example, PKA activation increased I_{K1} through Kir2.1 expressed in Xenopus oocytes (*9*), whereas it decreased I_{K1} through endogenous channels in guinea pig ventricular myocytes (*11*). Kir channels are composed of heterogeneous subunits (*10*), and it is likely that the molecular nature of the channel proteins in a given cell type is a basis for the differential effects of Ang II.

Although the atria have a higher binding capacity for Ang II than do ventricles in the guinea pig heart (*12*), there is limited information on the effects of Ang II on atrial I_{K1} (*13*, *14*). The initial purpose of the current study was to determine whether Ang II influences rat atrial I_{K1}. Following confirmation of the inhibitory effects of Ang II, we attempted to determine the receptor subtypes and signaling pathways responsible for the effects. Given the altered characteristics of I_{K1} in spontaneously hypertensive rats (SHR) (*15*), we also examined the effects of Ang II on I_{K1} in SHR atrial myocytes.

Methods

Cell Isolation

Eight week-old Wistar-Kyoto (WKY) rats (*16*) (systolic blood pressure = 140 ± 8 mmHg, mean ± SEM; $n = 8$) or SHR (175 ± 7 mmHg, $n = 8$) weighing 120–180 g were anesthetized with pentobarbital and the hearts were quickly isolated. Atrial myocytes were enzymatically isolated as described previously (*17*). Cells were dispersed in the recording bath and superfused with Tyrode's solution containing (in mmol/l) 140 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES and 5.5 glucose (pH 7.4).

All procedures were performed in accordance with the Tottori University Animal Care Guidelines, which conform to the NIH Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1985).

Membrane Preparations and [^{125}I]-SarIle-Ang II–Binding Assays

Membrane preparations were obtained from pooled atrium samples as described previously (*18*). They were incubated with increasing concentrations (0.05–1 nmol/l) of [^{125}I]-SarIle-Ang II for 2 h at 20°C in buffer containing (in mmol/l) 50 Tris-HCl, 100 NaCl, 10 $MgCl_2$, and 1 EDTA, supplemented with 0.25 mg/ml BSA and 10 mg/ml bacitracin (pH 7.6). Bound and unbound ligands were separated by rapid absorptive filtration (*19*). Specific binding was defined as the difference between binding capacities in the absence and presence of excess Ang II (10 μmol/l). Dissociation constant (K_d) and maximum binding capacity (B_{max}) values were estimated by Scatchard analysis of the saturation data. For inhibition isotherms, membrane preparations were incubated with 10 nmol/l [^{125}I]-SarIle-Ang II and increasing concentrations of the AT_1 antagonist losartan or the AT_2 antagonist CGP42112A (Fig. 1).

Measurement of I_{K1} by Whole-Cell Patch Clamp

Patch pipettes were prepared by pulling borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany). The pipette solution contained (in mmol/l) 140 K aspartate, 5 $MgCl_2$, 5 K_2ATP, 5 EDTA, and 5 HEPES (pH 7.2). Patch pipettes had a resistance of 2–4 MΩ when filled with the pipette solution. After rupture of the cell membrane, whole-cell membrane currents were recorded at room temperature using ramp or square pulses. Ramp clamping was performed with a functional generator (FG-122; NF Corporation, Yokohama, Japan) with a pulse range between −120 and 0 mV and a holding potential of −40 mV. The ramp pulses of 1.5 V/s were applied every 3 s as shown in the inset of Fig. 2A. We also used voltage steps ranging from −120 to 0 mV in 10 mV increments from a holding potential of −40 mV (Fig. 2D).

The test pulse duration was 300 ms and the test voltage steps were delivered every 5 s. The membrane potential was not corrected for the liquid junction potential, which was estimated to be < 10 mV. Data were acquired and analyzed using a personal computer (PC98XL; NEC, Tsukuba, Japan) equipped with an analog/digital converter (PCM-DP16; SHOSHIN EM Corporation, Okazaki, Japan). I_{K1} was defined as a Ba^{2+}-sensitive current obtained by subtracting the current measured in the presence of 3 mmol/l Ba^{2+} (c in Fig. 2B) from the total current measured in the absence of Ba^{2+} (a in Fig. 2B) (*20*). The slope conductance of I_{K1} was calculated from the linear region of the current-voltage (*I-V*) curves. We focused on the inward portion of the Ba^{2+}-sensitive currents, because the outward portion may also include other K^+ currents, such as delayed-rectifier K^+ currents and transient outward currents (*21*).

Measurement of Single I_{K1} Channel Currents

Single I_{K1} channel currents were recorded in both cell-attached and inside-out patch configurations. For the cell-attached patch, the experimental chamber was continuously perfused (1–2 ml/min) with Tyrode's solution. Superfusion with high K^+ solution depolarized the membrane potential so that it was maintained at a holding potential of −80 mV by performing the voltage clamp, creating the inside-out patch mode. For the inside-out patch, the bath solution contained (in mmol/l) 120 K aspartate, 25 KCl, 3 Na_2ATP, 5 EGTA, 5 HEPES and 1 $MgCl_2$ (pH 7.2) and the pipette solution contained 145 KCl and 5 HEPES (pH 7.4). Signals were filtered off-line with a cut-off frequency of 2 kHz through an eight-pole low-pass Bessel filter (48 dB/octave, model 902-LPF; Frequency Devices, Inc., Haverhill, USA), digitized with 14-bit resolution at a sampling rate of 10 kHz, and stored on an LSI 11/73 computer (Digital Equipment Corporation, Maynard, USA).

Intracellular Ca^{2+} Concentration Measurement

The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured using Indo-1 and a dual wavelength spectrophotometric method (*22*). Cells were loaded with 3 μmol/l Indo-1 acetoxymethyl ester (Molecular Probes, Inc., Eugene, USA) for 20 min at 37°C. The Indo-1–loaded cells were placed in the recording chamber and superfused with Tyrode's solution. Fluorescence (at > 500 nm) from sequential illumination (408 and 485 nm) at 0.3 Hz was measured using a spinning wheel spectrophotometer (Cairn Research, Ltd., Faversham, UK).

Data Analysis

The concentration-dependence of the Ang II–induced decrease in the slope conductance of I_{K1} was fitted to the following equation:

$$B(\%) = B_{max} \cdot [\text{Ang II}]^n / (IC_{50}{}^n + [\text{Ang II}]^n),$$

where $B(\%)$ represents the percent reduction in the slope conductance of I_{K1} induced by Ang II at a concentration of [Ang II], and B_{max} represents the maximum attainable reduction. IC_{50} and n are the half-maximal inhibitory concentration and Hill coefficient, respectively.

Measurement of cAMP

Cells were incubated in Kraftbruhe (KB) buffer containing (in mmol/l) 115.9 KOH, 80 glutamic acid, 10 taurine, 14 oxalic acid, 10 KH_2PO_4, 10 HEPES, 25 KCl, 11 glucose and 0.5 EGTA (pH 7.4), with or without Ang II (100 nmol/l) at 37°C for 30 min. The reaction was terminated by the addition of 6% (v/v) trichloroacetic acid. The extracts were centrifuged at 3,000 rpm for 15 min and cAMP contents in the supernatants were determined by using an Eiken cAMP Kit (Eiken Kagaku Co., Tokyo, Japan).

Immunoblot

The atrium was homogenized with a polytron in the buffer (PBS supplemented with 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstain and 1 mmol/l phenylmethylsulfonylfluoride), and insoluble materials were removed by centrifugation. Immunoprecipitation with anti-human AT_1 (*23–26*) (sc-1173AC; Santa Cruz Biotechnology, Santa Cruz, USA) was carried out in PBS/1% Triton X-100, 0.5% SDS, 0.25% sodium deoxycholate, 1 mmol/l EDTA, and protease inhibitors for 2 h at 4°C. The immunocomplexes were collected with protein G-agarose (Pharmacia, Piscataway, USA), separated on 7.5% SDS-polyacrylamide gels under a reducing condition including β-mercaptoethanol, and electrotransferred to a nitrocellulose membrane. Membranes were probed with antibodies against $G_{i\text{-}2\alpha}$ (NeoMarkers, Fremont, USA) or $G_{i\text{-}3\alpha}$ (Santa Cruz Biotechnology). They were developed by using an enzymatic chemiluminescence (ECL) detection system (Amersham International, Buckinghamshire, UK).

Drugs

[^{125}I]-SarIle-Ang II was from Amersham (Piscataway, USA), losartan was from LKT Laboratories (St. Paul, USA) and CGP42112A was from Sigma (St. Louis, USA). RNH6270 was kindly supplied by Sankyo Co. (Tokyo, Japan). PD123319 was from Warner-Lambert Co. (Ann Arbor, USA). Pertussis toxin (PTX) was from Seikagaku Co. (Tokyo, Japan). All other reagents were from Sigma or Wako, Ltd. (Osaka, Japan). A synthetic decapeptide identical to the C-terminal sequence of $G_{i\alpha\text{-}3}$ (EC peptide, "KNNLKECGLY") (*27*) was synthesized and analyzed by Funakoshi Lab. (Tokyo, Japan).

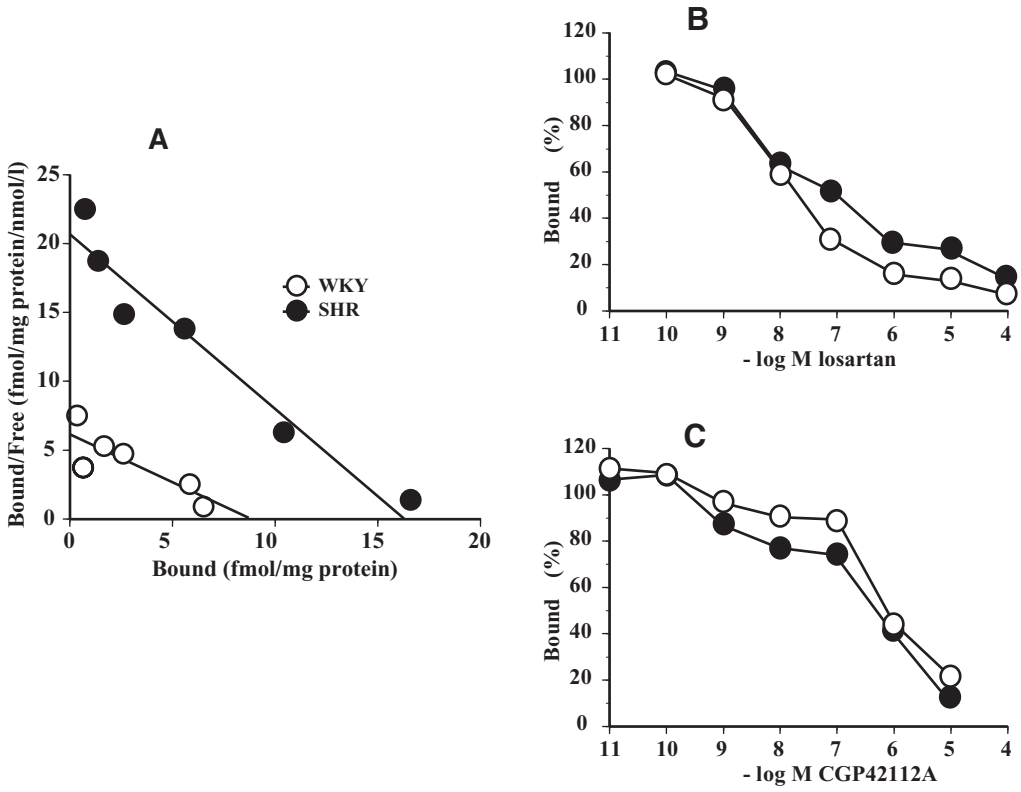

Fig. 1. *[^{125}I]-SarIle-Ang II binding to atrial membranes. A: Scatchard plots of the specific [^{125}I]-SarIle-Ang II binding to atrial membranes of WKY (open circles) and SHR (closed circles). B: Inhibition by losartan. C: Inhibition by CGP42112A. The representative results shown were reproduced in at least 10 tissues. The* K_d *and* B_{max} *values from the saturation isotherm and* K_i *values for the inhibition are given in the text.*

Statistical Analysis

Two-way ANOVA test and Fisher's exact test for post hoc analysis were carried out for multiple comparisons among groups. All data are derived from more than 4 experiments and expressed as the mean±SEM, with values of $p<0.05$ being considered statistically significant.

Cell Transfection

An expression construct pcDNA3.1/rat AT_1 was used for transfection experiments (*28*). COS7 cells were maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, USA)/10% fetal bovine serum at 37°C in a 5% CO_2 incubator. Cells were transfected by using lipofectamine (Gibco BRL) according to the manufacturer's instructions. Forty-eight hours after transfection, the cell extracts were prepared for immunoblotting using the same extraction buffer as used for immunoprecipitation.

Results

Predominant Expression of AT_1 in the Rat Atrium

Ang II receptor densities were assessed by [^{125}I]-SarIle-Ang II–binding assays using membrane preparations from rat atria. When non-specific binding was defined with excess cold Ang II, Scatchard analysis of the saturation data revealed a linear blot with K_d values of 1.30±0.04 nmol/l and maximum-binding capacities of 7.8±0.3 fmol/mg protein (means±SD, $n=13$) (Fig. 1A). Inhibition isotherms with the AT_1-selective antagonist losartan gave a biphasic curve (Fig. 1B). Nonlinear least-square regression analysis suggested the presence of AT_1 and AT_2 with 50% inhibitory concentration (K_i) values of 15 nmol/l and 89 μmol/l for losartan. The relative binding capacities for the two subtypes were estimated to be 9:1. The same analysis with the AT_2-antagonist CGP4112A again showed the presence of both AT_1 and AT_2, with K_i values of 0.5 μmol/l and 0.4 nmol/l for CGP42112A (Fig. 1C). In good agreement with the results using losartan, the relative binding capacities were estimated to be 9:1.

Scatchard analysis of the data from SHR membrane prepa-

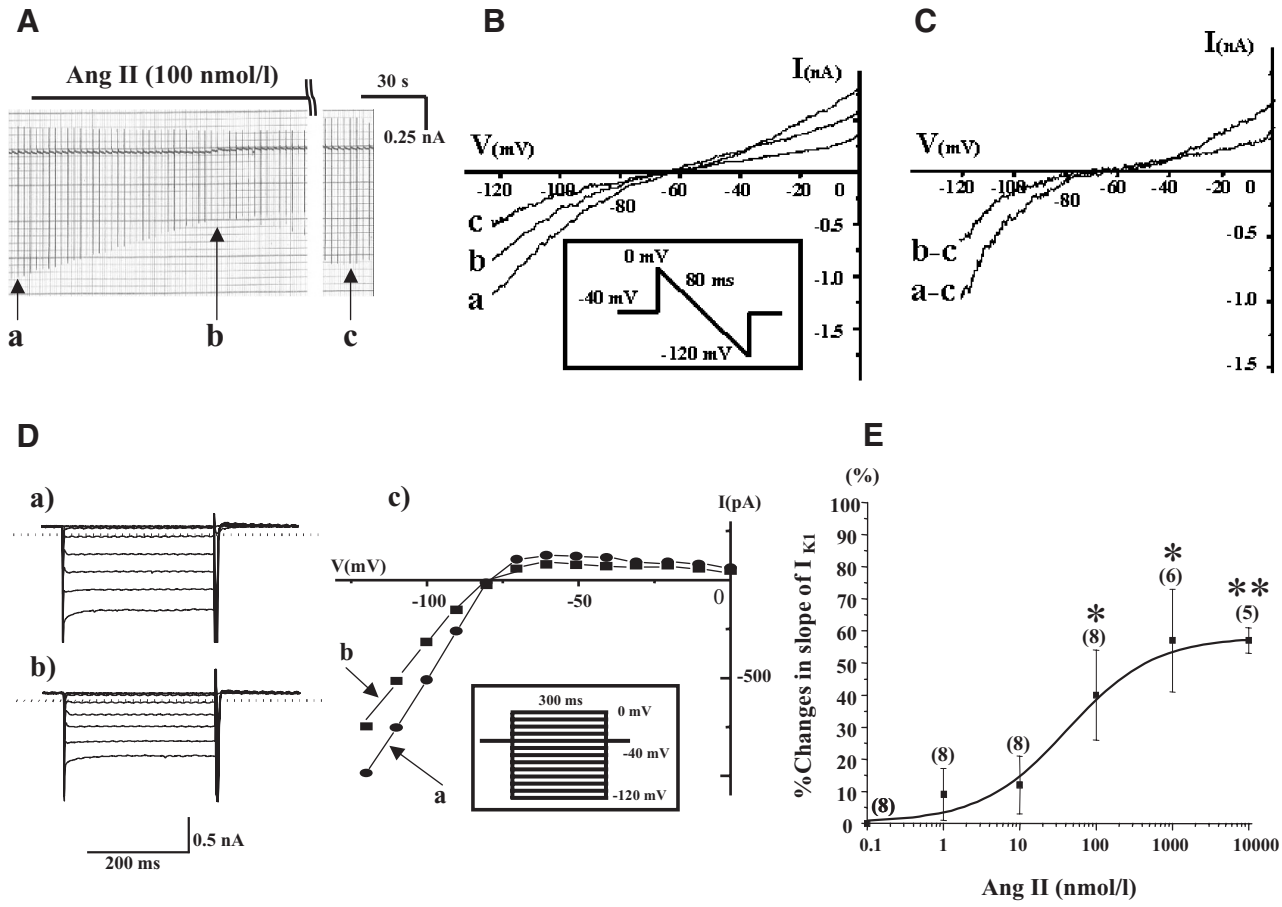

Fig. 2. *Effects of Ang II on atrial* I_{K1}. *The concentration of Ang II was 100 nmol/l in A–D. A: Steady-state membrane currents recorded by ramp pulses (a) before, (b) during and (c) after exposure to Ang II. B:* I-V *relationships determined by ramp pulses in the absence (a) or presence (b) of Ang II, or in the presence (c) of Ang II and* Ba^{2+} *(3 mmol/l). C:* I-V *relations for* Ba^{2+}*-sensitive currents: a–c,* Ba^{2+}*-sensitive currents in control cells corresponding to the difference between current (a) and current (c) in panel B; b–c,* Ba^{2+}*-sensitive currents in cells treated with Ang II corresponding to the difference between current (b) and current (c) in panel B. D: Effects of Ang II on* I_{K1} *elicited by voltage step pulses. The currents were recorded in the absence (a) or presence (b) of Ang II. (c)* I-V *relationships of* Ba^{2+}*-sensitive currents in the absence (a) or presence (b) of Ang II; the amplitude was measured at the steady-state during each test pulse. Similar results were obtained from 4 independent experiments. E: Concentration-dependence. The % reductions in* I_{K1} *conductance were plotted as a function of the concentrations of Ang II. Each point represents the mean ±SEM, with the number of experiments indicated in parentheses.* $^*p < 0.05$, $^{**}p < 0.01$.

rations revealed a linear blot with K_d values of 0.79±0.02 nmol/l and maximum binding capacities of 16.0±0.5 fmol/mg protein (means±SD, n=13) (Fig. 1A). Inhibition isotherms with losartan and nonlinear least-square regression analysis suggested the presence of AT_1 and AT_2, with K_i values of 23 nmol/l and 66 µmol/l for losartan, and the relative binding capacities of 8:2 (Fig. 1B). The same analysis with CGP42112A again showed the presence of both AT_1 and AT_2, with K_i values of 1.1 µmol/l and 0.8 nmol/l and relative binding capacities of 8:2 (Fig. 1C). These data suggested that the relative binding capacities of the two receptor subtypes were not altered, but the densities of both receptors were increased approximately two-fold in SHR atrial membrane preparations as compared with controls.

Inhibition of I_{K1} by Ang II in Rat Atrial Myocytes

Ang II (100 nmol/l) reduced the amplitude of both inward and outward portions of the steady-state currents elicited by ramp pulses (a–b in Fig. 2A, B). The amplitude was restored following withdrawal of Ang II (b–c in Fig. 2A). Neither cadmium (100 µmol/l) nor tetrodotoxin (10 µmol/l) affected the Ang II–induced changes in the slope conductance, excluding an involvement of voltage-gated Ca^{2+} or Na^+ channels. The *I-V* curves showed that Ang II reduced the steady-state current over the whole potential range (a–b in Fig. 2B) and the subse-

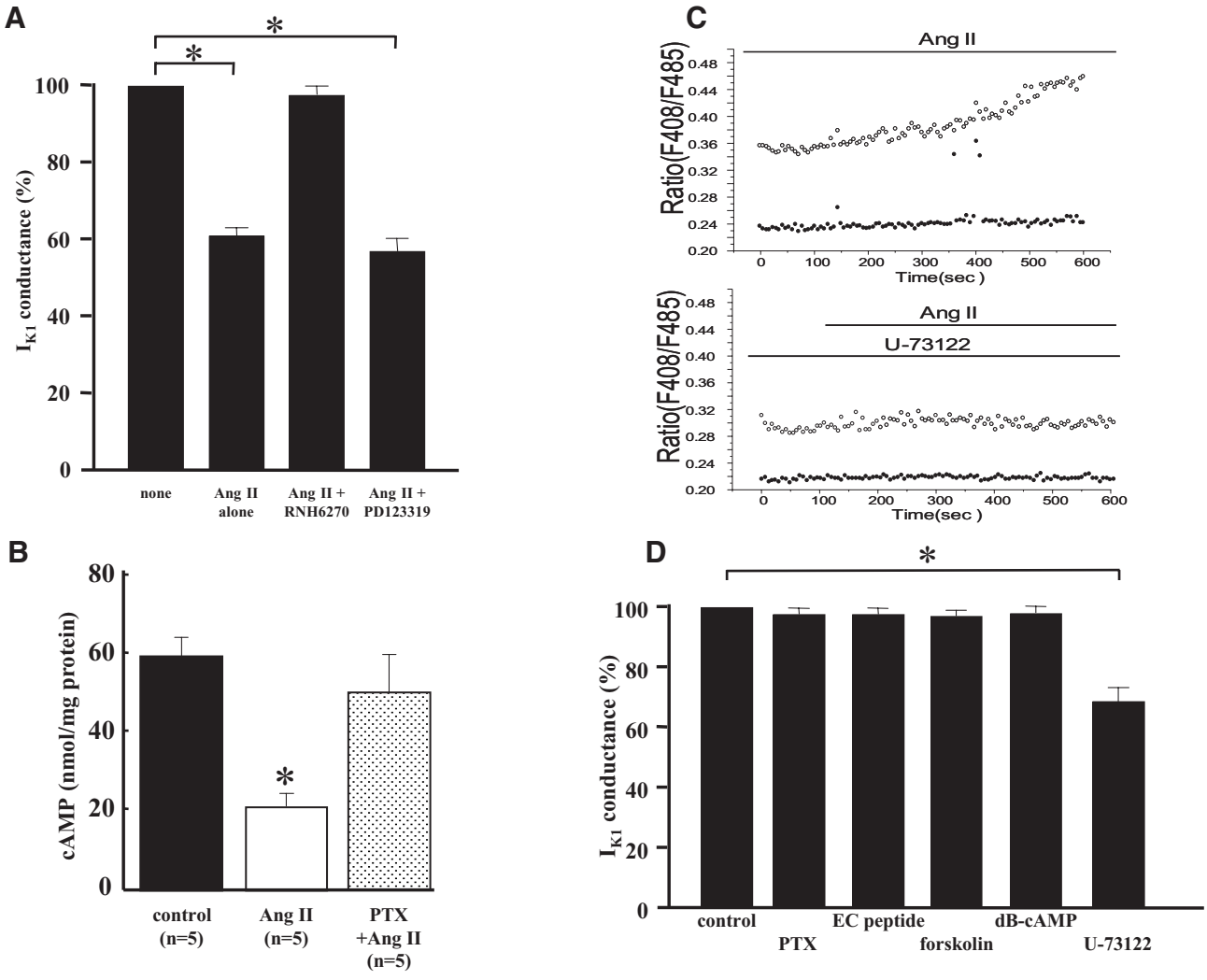

Fig. 3. *Effects of various agents on the Ang II–induced changes in atrial* I_{K1}*, cAMP production and* $[Ca^{2+}]_i$ *in rat atrial myocytes. The concentration of Ang II was 100 nmol/l. A: Effects of* AT_1 *and* AT_2 *blockers. The ordinate indicates the % conductance of* I_{K1} *normalized to the control (100%). Each bar represents the mean±SEM of more than 5 experiments. B: Effect of Ang II on cAMP production. Each bar represents the mean±SEM of more than 5 experiments. C: Effect of Ang II on* $[Ca^{2+}]_i$*.* $[Ca^{2+}]_i$ *was measured at the electrically stimulated state (open circles) or the unstimulated state (closed circles) with or without Ang II. In separate sets of cells, U-73122 (0.6 μmol/l) was added before application of Ang II. D: Effects of various agents on inhibition of* I_{K1} *by Ang II (slope conductance at −120 mV). Each bar represents the mean±SEM of more than 5 experiments.* $^*p < 0.05$.

quent administration of Ba^{2+} (3 mmol/l) produced a further reduction of this current (b–c in Fig. 2B). Subtraction of the Ba^{2+}-insensitive fraction indicated that Ang II (100 nmol/l) reduced inward I_{K1} by 40% at −120 mV and outward I_{K1} by 44% at 0 mV (a–c to b–c in Fig. 2C). Similar effects of Ang II were obtained using voltage step pulses: Ang II (100 nmol/l) reduced inward I_{K1} by 40% at −120 mV and outward I_{K1} by 40% at −40 mV (Fig. 2D). The reduction of the I_{K1} slope conductance by Ang II was concentration-dependent (*29*) with an IC_{50} of 46 nmol/l and a Hill coefficient of 1.1, and the effect was saturated at concentrations higher than 1 μmol/l (Fig. 3A).

To determine the receptor subtype responsible for the Ang II effects, we used the AT_1 antagonist RNH6270 and the AT_2 antagonist PD123319. The inhibition of I_{K1} by Ang II was abolished by RNH6270 but not at all by PD123319, indicating that AT_1 is responsible for the inhibition of I_{K1}.

To determine G protein subtypes coupled with AT_1 in these cells, we examined the effects of Ang II on the cAMP concentration and $[Ca^{2+}]_i$. Ang II (100 nmol/l) reduced cAMP contents by 65%, and this effect was abolished by pretreatment of the cells with PTX (Fig. 3B). This inhibition was also abolished by RNH6270 (data not shown). Ang II (100 nmol/l) caused a transient increase in $[Ca^{2+}]_i$, which was completely

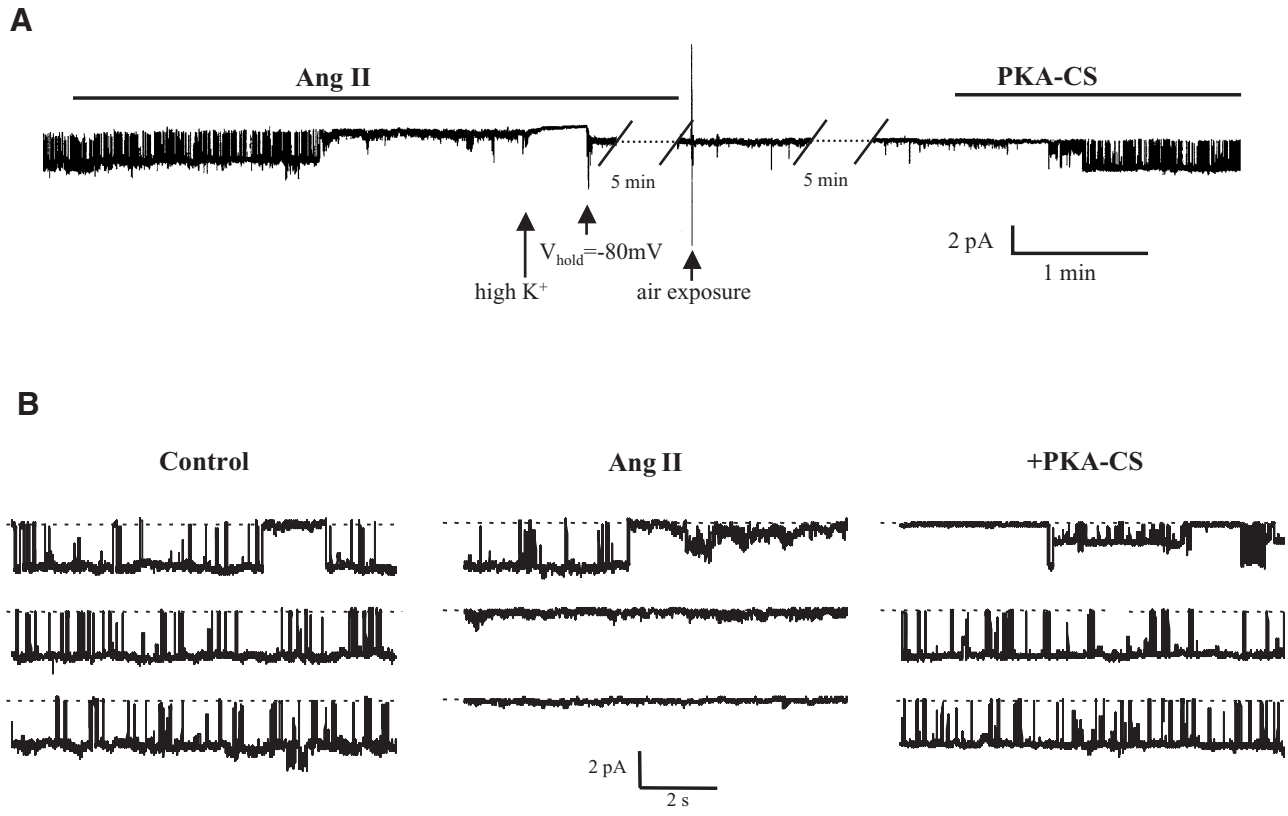

Fig. 4. *Reactivating effects of PKA catalytic subunits on the atrial* I_{K1} *channel inactivated by Ang II in control cells. A: Activity of a single* I_{K1} *channel current during applications of Ang II and PKA catalytic subunits (PKA-CS). Before air exposure, the inward* I_{K1} *current appeared under the cell-attached mode at the resting potential and was inactivated by subsequent bath application of Ang II (100 nmol/l). After air exposure, the* I_{K1} *channel current was reactivated by application of PKA-CS (10 U/ml) intracellularly, after formation of the inside-out patch configuration at voltage clamp at −80 mV. B: Expanded current records before application of Ang II (left), just before and after the inactivation of the current by Ang II (middle), and just before and after the reactivation by PKA-CS (right). The same results were obtained from 4 independent experiments.*

abolished by the cell permeable PLC inhibitor U-73122 (*30*) (Fig. 3C). This effect on $[Ca^{2+}]_i$ was also abolished by RNH6270 (data not shown). These data indicated that AT_1 coupled with the $G_{i\alpha}$ and $G_{q\alpha}$ proteins in these cells.

To determine the G protein subtype responsible for inhibition of I_{K1}, we examined the effects of PTX and an EC-blocking peptide against $G_{i\alpha\text{-}3}$ proteins and U-73122. The Ang II effect was abolished when cells were pretreated with PTX or the EC peptide, indicating the involvement of PTX-sensitive $G_{i\alpha}$ proteins. In contrast, U-73122 failed to effect the blocking of I_{K1} by Ang II (Fig. 3D). To confirm the role of cAMP/PKA, we tested whether forskolin or dibutyryl-cAMP could counteract Ang II and found that both drugs abolished the Ang II effects. Neither isoproterenol, forskolin or dibutyryl-cAMP (data not shown), when applied alone, affected the slope conductance of I_{K1}. Taken together, these results indicate that $G_{i\alpha}$, but not $G_{q\alpha}$, is responsible for the inhibition of I_{K1}.

To characterize the mode of action of Ang II, we performed single channel recordings using cell-attached and inside-out patches. In the cell-attached patch, extracellular Ang II (100 nmol/l) reduced I_{K1} channel activities (Fig. 4A, before air exposure), by increasing interburst intervals but not unit amplitudes (Fig. 4B, left and middle). The single channel conductances before and during administration of Ang II were 35±3 pS and 33±6 pS, respectively, which were consistent with the characteristics of I_{K1} (*20*). In the inside-out patch, Ang II inhibited the I_{K1} channel activities and the activities were restored by application of PKA catalytic subunits on the intracellular side (10 U/ml) (Fig. 4A, after air exposure and Fig. 4B, middle and right). This was consistent with the finding in the whole cell clamp that the bath application of dibutyryl-cAMP restored the I_{K1} (data not shown).

Lack of Ang II Effects on I_{K1} in Cells from SHR

Given the previous report on alterations of I_{K1} in ventricular myocytes isolated from SHR (*14*), we compared I_{K1} between

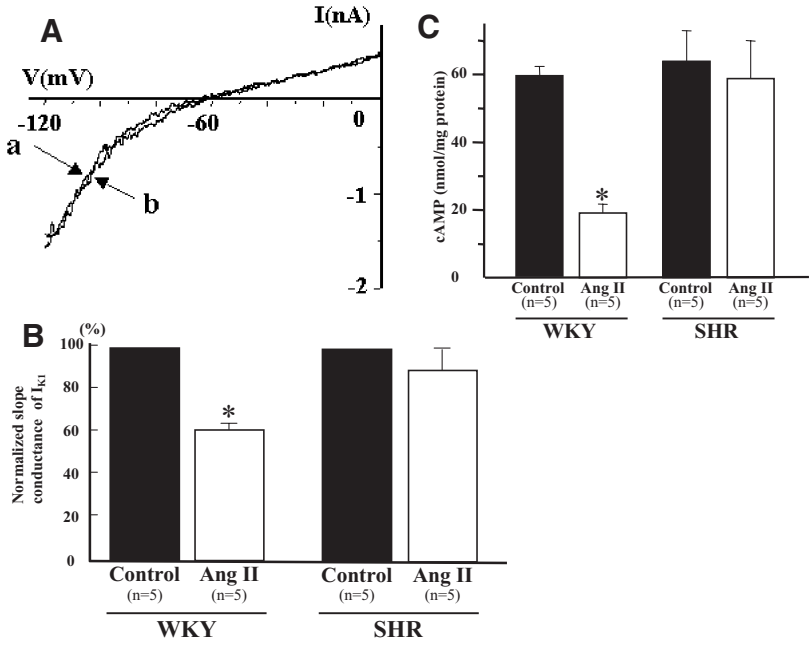

Fig. 5. *Effects of Ang II on* I_{K1} *and cAMP production in atrial myocytes. A: Effect of Ang II on the atrial* I_{K1} *of SHR.* Ba^{2+}*-sensitive currents were measured by ramp pulses before (a) and during (b) application of Ang II. B: Effect of Ang II on the slope conductance of* I_{K1}*. C: Effect of Ang II on cAMP production in atrial myocytes.*

cells from control rats and SHR. We found little difference either in the inward or outward portions of the steady-state currents: as shown in Fig. 5A, the slope conductance of I_{K1} (inward portion at −120 mV) in cells from SHR (15.0±4.9 nS) was close to that in cells from normal rats (11.1± 5.2 nS).

In spite of the increased binding capacities for [^{125}I]Ang II in the atrial membrane preparations (Fig. 1), Ang II (100 nmol/l) failed to inhibit I_{K1} in cells from SHR (Fig. 5A, B). Ang II failed to decrease cAMP contents in SHR cells (Fig. 5C), suggesting an impaired signal transduction from AT_1 to AC. These findings were reproduced in the presence of PD123319, indicating the negative contribution of AT_2 (data not shown). We then examined the protein levels of G_i proteins in SHR atrial extracts by Western blotting and their association with AT_1 by immunoprecipitation. There were no significant differences in the expression levels of AT_1, $G_{i\text{-}2\alpha}$ and $G_{i\text{-}3\alpha}$ proteins in the extracts between control and SHR extracts (Fig. 6A, B), although the ventricular AT_1 expressions in SHR were higher than those in WKY rats (Fig. 6A), indicating the left ventricular remodeling of SHR.

AT_1 immunoprecipitation, however, showed that the amounts of $G_{i\text{-}2\alpha}$ and $G_{i\text{-}3\alpha}$ proteins associated with this receptor were reduced in SHR (Fig. 6C). The data obtained from the 6 different experiments indicated that the fraction of densities of immunoprecipitated $G_{i\text{-}2\alpha}$ and $G_{i\text{-}3\alpha}$ proteins with this receptor were significantly reduced in the atrium of SHR (to 52±12% and 54±9%, respectively, of the levels in normal rats, Fig. 6D).

Discussion

We found that in rat atrial myocytes, Ang II inhibited I_{K1} and that this inhibition was due to activation of AT_1 coupled with PTX-sensitive G_i proteins. The finding that the effects of Ang II were suppressed by forskolin, dibutyl-cAMP and the PKA catalytic subunit argues that the decreased cAMP levels and hence the decreased PKA activity resulted in inhibition of I_{K1}. Neither isoproterenol, forskolin or dibutyl-cAMP affected I_{K1}, indicating that the channel activity did not respond to an increase in the cAMP/PKA activity. One possible explanation for this lack of response is that the channels may be fully phosphorylated by PKA at the steady state: in this case, the increase in PKA activity would not cause any increase in the phosphorylation of the channels, whereas the decrease in the activity could result in reduced phosphorylation. Direct assessment of the phosphorylation state of the channel proteins is required to test this assumption. As for the regulation of I_{K1} by PKA, our findings are contradictory to a previous report by Koumi *et al*. (*11*), who found that PKA activation decreased I_{K1} in guinea-pig ventricular myocytes. This discrepancy is most likely explained by the differences in the molecular nature of the channel proteins, and thus further studies will be needed to characterize the channel proteins responsible for I_{K1} in individual cell types.

Our findings are also contradictory to those of Morita *et al*. (*8*), who found that Ang II increased I_{K1} in rabbit ventricular

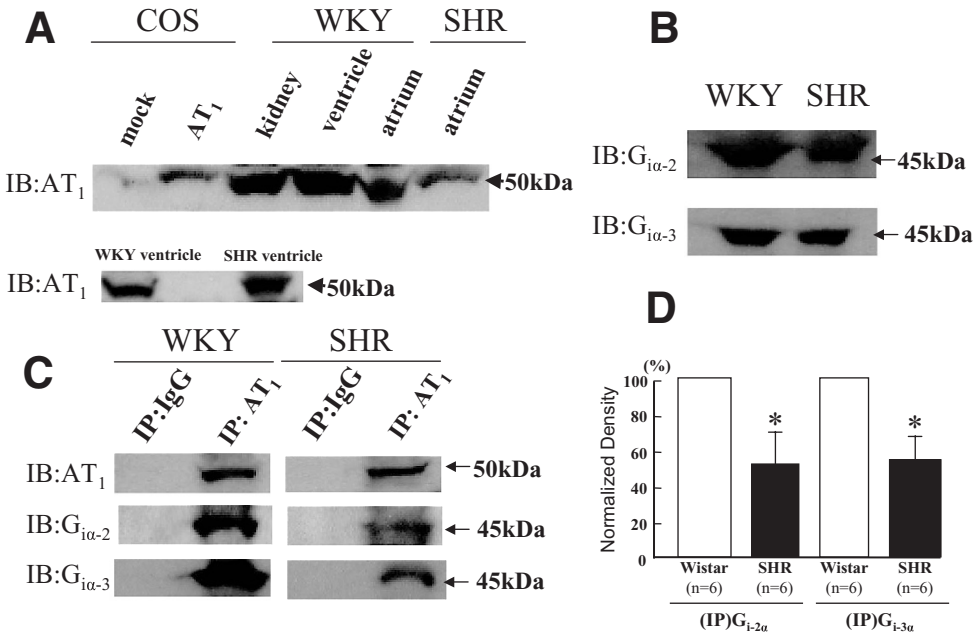

Fig. 6. *Co-precipitation of $G_{i\alpha\text{-}2}$ and $G_{i\alpha\text{-}3}$ proteins with AT_1 of atrium. A: Anti-AT_1 Western blotting. Cell extracts were prepared from COS7 cells transfected with an empty vector or pcDNA3/AT_1. Tissue extracts were prepared as described in Methods. B: Anti–G_i protein Western blotting of atrial extracts. C: Co-precipitation of G_i proteins with AT_1 from atrial extracts. Anti-AT_1 immunoprecipitates were analyzed by immunoblotting with indicated antibodies. D: The fraction of densities of the immunoprecipitated $G_{i\alpha\text{-}2}$ and $G_{i\alpha\text{-}3}$ protein of SHR atrium were normalized to those of the normotensive Wistar Kyoto rat atrium.* $^*p < 0.05$.

cells. This discrepancy may be due either to species/tissue-selective activation of signaling pathways or to the differential nature of the channel proteins themselves. Preliminary results from our laboratory showed that the amounts of G_i proteins co-precipitated with AT_1 were considerably less in rat ventricular extracts than in atrial extracts in both WKY rats and SHR (data not shown), suggesting tissue-selective activation of AT_1/G_i signaling pathways by Ang II.

We could not see any differences in the electrophysiological characteristics of atrial I_{K1} between normotensive rats and SHR, in contrast to the observation by Li and Jiang (*15*), who found that the inward portion of ventricular I_{K1} was significantly smaller in SHR than that in normotensive rats. Although there was not any significant difference of I_{K1} between SHR and WKY rats in the present study, the message level among the two strains might be different. Since in general the channel level is determined by not only the transcriptional level but also the posttranslational modification, it remains possible that the increases in mRNA of the atrial I_{K1} in SHR could be canceled by the excess degradation of I_{K1} channel proteins. Neither could we detect any differences in the expression levels of G_i proteins in atrial extracts, in contrast to the observation by Thibault and Anand-Srivastava (*31*), who found an increased expression of $G_{i\text{-}\alpha}$ proteins in the ventricles of SHR compared with normotensive rats. Although the precise reason for these discrepancies remains uncertain, they may reflect differential responses to chronic hypertension between atrial and ventricular myocytes.

There was a discrepancy between [^{125}I]-SarIle-Ang II–binding capacities and the protein levels in the Western blot analysis. Although the precise reason for this discrepancy remains uncertain, this phenomenon is not specific to AT_1 (*32*). Similar discrepancies between ligand-binding capacities and protein levels detected with anti-receptor antibodies have been described for various GPCRs, such as endothelin receptor type A (*33*), platelet-activation factor receptors (*34*) and opioid receptors (*35*). Recently, it has been reported that alternative splicing of AT_1 results in differential protein levels (*36*). Since the protein levels of AT_1 were unaltered on the Western blot, it is unlikely that the alternative splicing resulted in the difference in binding capacities. Alternatively, the difference of Ang II–binding capacities and AT_1 expression in the atrium of SHR might be explained by an increase in the affinity of AT_1 to compensate for the uncoupling of AT_1 to G_i, which should be a subject for future study.

Despite the increase in the [^{125}I]Ang II–binding capacities and the unaltered expression levels of G_i proteins, Ang II failed to activate G_i proteins or to inhibit I_{K1} in SHR atrial cells. Results from AT_1 immunoprecipitation experiments suggested that this failure was due to uncoupling of AT_1 with G_i proteins. The activation of AT_1 produces oxidative stress (*37*, *38*). The coupling state of signaling proteins can be altered in SHR. For example, it has been reported that the elevated oxidative stress in SHR induced uncoupling of the

dopamine receptor from the G_i protein in renal proximal tubules (*39*). We also confirmed that ventricular AT_1 was increased in SHR in the present study, indicating that the SHR underwent left ventricular remodeling. Therefore, this coupling status may be at least partly the consequence of hypertensive cardiac remodeling involving oxidative stress, although we did not measure the oxidative stress in the atrium in the present study.

However, it remains possible that it is a phenomenon specific to the SHR strain. Therefore, the effect of the hypertensive state on the heart should be tested using another animal model, such as Dahl rats with or without salt loading. The precise mechanism responsible for this uncoupling of receptors from G protein is a worthy subject for future study.

The plasma concentration of Ang II is between 5 and 50 pmol/l in humans (*40*), while in the present study Ang II effectively inhibited I_{K1} with IC_{50} values of 46 nmol/l. Ang II secretion from podocytes is elevated in various disease states with increased mechanical stress (*41*) and the tissue concentrations of Ang II, such as in the skeletal muscle and heart, can be higher than those in plasma (*42*), suggesting that the level of local Ang II might not be very different from the concentration used in the present study. The finding that a higher concentration of Ang II was required to block I_{K1} casts doubt on the physiological relevance of the action of Ang II. However, most studies estimating the effects of Ang II on the ion channels have been conducted within the concentration range of 1–100 nmol/l, and thus the concentrations of Ang II used in the present study can be considered acceptable for electrophysiological experiments.

Previous experimental and clinical studies (*43–47*) have reported that activation of the atrial renin-angiotensin system may contribute to the occurrence of atrial arrhythmias. Hypertension is the most common cardiovascular antecedent cause of atrial fibrillation (*45*). Goette *et al*. (*44*) have reported that the activation of AT_1 receptor in the atrium could promote Ang II–dependent progressive atrial fibrosis. Nakashima *et al*. (*45*) have demonstrated in the canine atrium that AT_1 blockers prevent shortening of the effective refractory period during high rate pacing, suggesting that the activation of the AT_1 receptor would shorten the atrial action potential duration (APD), which in turn causes atrial arrhythmias. We recently reported that Ang II prolonged the atrial action potential duration in WKY rats (*48*), indicating its antiarrythmic action on the atrium; however, in another study Ang II shortened the atrial action potential duration in SHR (*49*), indicating its proarrhythmic action on the atrium in hypertensives. Thus, reduction of I_{K1} could lead to prolongation of the action potential duration, which may underlie the antiarrhythmic action of Ang II in normotensive rats (*20*). In SHR, the lack of I_{K1} inhibition by Ang II may lead to attenuation of its antiarrythmic action. There may be a link between the lack of I_{K1} inhibition by Ang II in SHR and the incidence of arrythmias in human hypertensive patients.

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