Published in final edited form as:
J Mol Cell Cardiol. 2011 January ; 50(1): 128–136. doi:10.1016/j.yjmcc.2010.11.001.

Angiotensin II Induces afterdepolarizations via reactive oxygen species and calmodulin kinase II signaling

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Abstract

Renin-angiotensin system inhibitors significantly reduce the incidence of arrhythmias. However, the underlying mechanism(s) is not well understood. We aim to test the hypothesis that Ang II induces early afterdepolarizations (EADs) and triggered activities (TAs) via the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-ROS-calmodulin kinase II (CaMKII) pathway. ROS production was analyzed in the isolated rabbit myocytes loaded with ROS dye. Ang II (1–2 μM) increased ROS fluorescence in myocytes, which was abolished by Ang II type 1 receptor blocker losartan, NADPH oxidase inhibitor apocynin, and antioxidant MnTMPyP, respectively. Action potentials were recorded using the perforated patch-clamp technique. EADs emerged in 27 out of 41 (66%) cells at 15.8 ± 1.6 min after Ang II (1~2 μM) perfusion. Ang II-induced EADs were eliminated by losartan, apocynin, or trolox. The CaMK II inhibitor KN-93 (n=6) and inhibitory peptide (AIP) (n=4) also suppressed Ang II-induced EADs, whereas the inactive analogue KN-92 did not. Nifedipine, a blocker of L-type Ca current ($I_{Ca,L}$), or ranolazine, an inhibitor of late Na current (I_{Na}), abolished Ang II-induced EADs. The effects of Ang II on major membrane currents were evaluated using voltage clamp. While Ang II at same concentrations had no significant effect on total outward K^+ current, it enhanced $I_{Ca,L}$ and late I_{Na}, which were attenuated by losartan, apocynin, trolox, or KN-93. We conclude that Ang II induces EADs via intracellular ROS production through NADPH oxidase, activation of CaMKII, and enhancement of $I_{Ca,L}$ and late I_{Na}. These results provide evidence supporting a link between renin-angiotensin system and cardiac arrhythmias.

Keywords

Angiotensin II; early afterdepolarizations; triggered activities; reactive oxygen species; CaMKII; L-type calcium channel; sodium channel

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1. Introduction

Reactive oxygen species (ROS), including superoxide anions, hydroxyl radicals, and hydrogen peroxide (H_2O_2), are suggested to be arrhythmogenic factors. Our recent study [1] has shown that exogenous addition of H_2O_2 to ventricular myocytes induces early afterdepolarizations (EADs), delayed afterdepolarizations (DADs) and triggered activities (TAs) via activation of Ca/Calmodulin kinase II (CaMK II), which in turn enhances L-type calcium current ($I_{Ca,L}$) and late sodium current (I_{Na}). However, it remains to be examined whether endogenously generated ROS under certain pathological conditions are sufficient to stimulate CaMKII and generate EADs and DADs.

It has been demonstrated that various membrane receptors (e.g. Angiotensin II type 1 receptor (AT_1R), endothelin type A receptor (ET_AR), and transforming growth factor β receptors (TGFβR) mediate generation of superoxide and H_2O_2 via the reduction of O_2 by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex in non-cardiac cells [2–4]. Recent studies have also found a similar NADPH oxidase system in cardiac myocytes [5–7]. Angiotensin II (Ang II) activates NADPH oxidase, via AT_1R leading to increased generation of ROS and myocardial injury [8–9].

Ang II is an endogenous peptide hormone playing a critical role in the pathophysiological modulation of cardiovascular disorders, such as hypertension, ischemic heart disease, cardiac hypertrophy, and heart failure [10–11]. Recent clinical studies has revealed that angiotensin-converting enzyme (ACE) inhibitors and Ang II receptor blockers are important therapeutic agents in the treatment of atrial and ventricular arrhythmias [12–13], suggesting Ang II is arrhythmogenic under pathophysiological conditions including heart failure and cardiac ischemic-reperfusion. This notion was further supported by experimental data [14–15]. While electrical (ion channels) and structural (fibrosis, dilatation and hypertrophy) remodeling and neurohumoral activation have been suggested to account for the arrhythmogenic effect of Ang II [15–16], little is known about the role of Ang II in inducing arrhythmias via NADPH oxidase-ROS pathway. The present study was designed to assess the hypothesis that Ang II is proarrhythmic by inducing EADs and TAs via NADPH oxidase, ROS generation, activation of CaMK II, and activation of $I_{Ca,L}$ and late sodium current (I_{Na}).

2. Materials and Methods

An expanded Methods section is available in the Online Data Supplement. All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Medicine and Dentistry of New Jersey-New Jersey Medical School. All experiments were performed at 35 to 37°C.

2.1 Cell isolation

Ventricular myocytes were enzymatically isolated from the hearts of New Zealand white rabbits (Male, 2–3 kg) as described previously [1].

2.2. Intracellular calcium ion (Ca_i) measurement

Myocytes were loaded with the Ca^{2+} indicator Fluo-4 AM. The Ca_i fluorescence signals were measured using a charge-coupled device (CCD) camera system.

2.3. Electrophysiological recording

Myocytes were patch-clamped using the perforated patch-clamp technique in the whole-cell configuration. Action potentials (APs) were recorded under current clamp mode, and whole

cell currents ($I_{Ca,L}$, late I_{Na}, total I_K) were recorded under voltage clamp mode with a MultiClamp 700A patch-clamp amplifier controlled by a personal computer using a Digidata 1322A acquisition board driven by pCLAMP 10 software (Molecular Devices, Sunnyvale, CA).

2.4. Detection of intracellular ROS

Myocytes were incubated with 5 μM C-DCDHF-DA-AM. ROS fluorescence was measured by using a charge-coupled device camera.

2.5. CaMKII assay

CaMKII activity was measured using SignaTECT Calcium/Calmodulin-Dependent Protein kinase Assay system (Promega) following manufacturer's instructions.

2.6. Statistical analysis

Data are presented as mean ± SEM. Statistical significance was assessed using paired, unpaired Student's *t* tests or ANOVA analysis, with $P<0.05$ considered significant.

3. Results

3.1. Ang II-induced ROS production in rabbit ventricular myocytes

We first investigated the effect of Ang II on ROS production in isolated rabbit ventricular myocytes by monitoring fluorescence intensity of ROS-sensitive dye C-DCDHF-DA. While H_2DCFDA being widely used to detect hydrogen peroxide [17], it actually detects a broader range of ROS [18]. Thus, we used H_2DCFDA as an indicator of general oxidative stress level. In vehicle-treated myocytes, DCF fluorescence remained stable during the monitoring period (Fig. 1A). The effects of exogenous H_2O_2 at 50 and 200 μM were evaluated as a positive control. A rapid and dramatic increase in DCF fluorescence intensity was observed in the myocytes after H_2O_2 treatment. The F/F_0 of DCF fluorescence intensity reached the value of 2.18 ± 0.24 and 1.51 ± 0.11 at 5~7 min of exposure to 200 and 50 μM H_2O_2, respectively (Fig. 1C). Ang II (1 μM) enhanced DCF fluorescence intensity mildly compared to direct application of H_2O_2. As shown in Fig. 1A, the elevation of intensity initiated at 3–4 min and reached a stable state at 15–20 min after Ang II application. Representative DCF fluorescence images in the presence or absence of Ang II are shown in Fig. 1B, indicating marked fluorescence increases at 12, 20 min after Ang II application. Note that Ang II-induced ROS production appeared higher in some localized cytosolic region (figure 1B). Although we do not have a ready explanation for this phenomenon, it may share a common mechanism of "metabolic sink" accounting for regional polarized mitochondria and high level of ROS during ischemia/reperfusion suggested by O'Rourke's group [19]. The F/F_0 of DCF fluorescence intensity was measured at 18–20 min after Ang II treatment and showed significant increase (1.18 ± 0.03) compared to control group (0.99 ± 0.02) (Fig. 1C). However, Ang II failed to enhance DCF fluorescence intensity in the presence of the AT_1R antagonist losartan (3–5 μM), the NADPH oxidase inhibitor apocynin (1 mM), or manganese (III) tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP, 10 μM) (a SOD/catalase mimetic, which has been considered as a general ROS scavenger [20–21]). Losartan, Apocynin and MnTMPyP had no effect on the DCF fluorescence intensity in the absence of Ang II. These results suggest that Ang II causes ROS production via AT_1R and NADPH oxidase in rabbit ventricular myocytes. Ang II was still able to increase ROS production in the presence of CaMKII inhibitor, KN93 (1 μM), suggesting that CaMKII activation is downstream of ROS production.

Since CaMKII can be activated by oxidation by ROS [8–9], we next determined if Ang II could also activate CaMKII via ROS production. As shown in Fig. 1D, Ang II treatment (1

μM, for ~ 60 min) significantly increased the CaMKII activity in isolated myocytes, however, it failed to activate CaMKII in the presence of apocynin (1mM), Trolox (1 mM), or KN-93 (1 μM).

3.2. Induction of EADs by Ang II in rabbit ventricular myocytes

In the following experiments, we assessed whether Ang II could induce EADs via NADPH oxidase-ROS-CaMKII-$I_{Ca,L}/I_{Na}$ pathway. APs were recorded from isolated rabbit ventricular myocytes using perforated whole-cell patch-clamp technique under current clamp mode. In order to reliably induce EADs, the cells were paced at a PCL of 6 sec based on our previous studies [1,22]. After action potential duration (APD) and morphology reached steady state, cells were perfused with 1 to 2 μM Ang II for up to 40 min or until EADs appeared. As shown in Fig. 2A, the APD was initially prolonged gradually after application of Ang II (e.g. APD was prolonged from 286.3±32.9 to 475.5±95.3 ms (n= 8) at 10 min of exposure to Ang II). EADs were induced in 27 out of 41 cells after an average exposure time of 15.8 ± 1.6 min (n=27). EADs could be irregular, single, or multiple with an oscillating membrane potential before repolarization (see figures 2–6). DADs were also observed in 3 out of 41 cells exposed to 1 μM Ang II (data not shown). EADs were also induced in 3 out of 7 cells by 100 nM Ang II and in 5 out of 9 cell by 50 μM H_2O_2, while control experiments in parallel showed that neither EADs nor DADs occurred in the absence of Ang II up to 40 min (Fig.2-Ab) (n=6),

3.3. Ang II-induced Ca_i abnormality in adult rabbit ventricular myocytes

The effect of Ang II on Ca_i handling correlated to EADs was also investigated. As shown in Fig. 2B, we simultaneously recorded APs (top panel), Ca_i transients (middle panel) and line scan images (bottom panel) in Fluo-4 AM-loaded myocytes. The effect of Ang II (1 μM) on Ca_i transients amplitude was evaluated. Ang II did not cause significant increase in Ca transient amplitude before EAD appeared (F/F_0: 1.89 ± 0.14 at 5 min after Ang II, vs. 1.94 ± 0.11 at control, $P > 0.05$, n = 5), while it enhanced Ca transient amplitude after EADs were induced (F/F_0: 2.04 ± 0.11, n = 5, $P < 0.05$). Increased Ca^{2+} entry though $I_{Ca,L}$ or the reverse mode of NCX has been proposed as the underlying mechanism(s) [23]. During Ang II-induced EADs, Ca_i remained elevated as a plateau (middle panel in Fig. 2B-b) or increased as a second peak (middle panel in Fig. 2B-c). The peak of second EAD in the AP (Fig, 2B-c) constantly preceded the second Ca transient peak following the long elevated state, suggesting an additional SR Ca release attributable to reactivation of $I_{Ca.L}$.

3.4. Cellular signaling pathways involved in Ang II-induced EADs

Ang II interacts with at least two receptors: type 1 Ang II receptor (AT_1R) and type 2 Ang II receptor (AT_2R) [24]. In order to identify the Ang II receptor subtype which mediated the induction of EADs, either AT_1R antagonist losartan (Los, 3–5 μM) or AT_2 receptor antagonist PD-123319 (PD, 2–5 μM) was administrated after EADs were constantly induced by 1 μM Ang II. As shown in Fig. 3A–C, Los suppressed Ang II-induced EADs in a reversible manner. The probability of EAD occurrence was reduced from 68.3 ± 5.6% (Ang II) to 0.7±0.3% (Ang II + Los) (n=6, $P<0.01$), while PD had no effect, suggesting the activation of AT_1R was responsible for the induction of EADs by Ang II. Consistent with its effects on Ang II-elicited ROS generation, apocynin (0.5 or 1 mM), an NADPH oxidase inhibitor, also suppressed the rate of EADs induced by Ang II (from 75.8±4.7% to 0.3 ± 0.2 %, n=4, $P<0.01$) (Fig. 3D & E).

In addition, Ang II-induced EADs were eliminated by antioxidants trolox (0.5 or 1 mM) or MnTMPyP (10 μM). EAD occurrence probability was reduced from 78.3 ± 5.3% to 0% by both regents (n=5, $P<0.01$) (Fig. 4A & B). These results support the hypothesis that the

activation of AT_1R, and in turn the generation of NADPH oxidase-derived ROS are involved in the EAD formation by Ang II.

It has been shown that binding of Ca^{2+}/CaM is required to expose the redox sites in the regulatory CaMKII domain in order for oxidation to persistently activate CaMKII [8,25]. We therefore investigated the Ca^{2+} dependence of Ang II-induced EADs. In myocytes preloaded with BAPTA-AM (4 μM) for 30 min, Ang II did not induce EADs even at a higher concentration (4 μM) in 5 cells, although APDs were markedly prolonged (e.g. the average APD was prolonged from 345.8±38.4 to 673.7±55 ms (n=6) at 10 min of exposure to Ang II in the presence of BAPTA), indicating Ang II-induced EADs were Ca_i dependent, presumably through Ca^{2+}/CaM. Chelating of Ca^{2+} by BAPTA may reduce Ca-sensitive inactivation of $I_{Ca,\ L}$, which may contribute to the prolongation of APD in the presence of BAPTA. Next, we examined the effect of direct CaMKII inhibition on EADs generation by Ang II. As shown in Fig. 4C & D, CaMKII inhibitor KN-93 (1 μM) completely suppressed EADs induced by Ang II (n=4, EAD occurrence probability from 75.8 ± 4.4% to 0%), while KN-92, an inactive analogue of KN-93, had neither preventive nor inhibitory effect on Ang II-induced EADs. To further exclude nonspecific effects of KN-93 [26], we also examined the effect of a membrane permeable CaMKII selective inhibitory peptide, autocamtide-2 related inhibitory peptide (AIP). AIP (1 μM) was perfused after EADs were induced by Ang II (1 μM). As shown in Fig 4-E &F, Ang II-induced EADs were significantly suppressed by AIP; the EAD occurrence probability was suppressed from 68.3 ± 5.6% to 18.8 ± 2.0 % (n=4). These results suggest that Ca-dependent CaMKII activation is responsible for Ang II-induced EADs.

3.5. Contribution of $I_{Ca,L}$ in Ang II-induced EADs

CaMKII modulates multiple sarcolemmal channels [27], among which $I_{Ca.L}$ and late I_{Na} are known to be key factors in ROS-induced EAD formation [1,28–29]. We next assessed the involvement of $I_{Ca.L}$ and late I_{Na} in Ang II-induced EADs. We observed that the amplitude of EADs induced by Ang II depended on their takeoff potentials, i.e. the more negative the takeoff potential, the larger the EAD amplitude (Fig. 5A). This relationship is reminiscent of the voltage dependence of $I_{Ca.L}$ reactivation and the $I_{Ca,L}$ window currents [30], which resembles the effects of H_2O_2 in our previous work. Supporting this notion, Nifedipine (10 μM), a selective $I_{Ca.L}$ blocker abolished EADs (Fig. 5B). To confirm that the activation of $I_{Ca,L}$ plays a key role in Ang II-induced EADs, we directly examined the effects of Ang II on $I_{Ca,L}$ by using voltage clamp recording. As shown in Fig. 5 C–D, Ang II (1 μM) increased the amplitude of $I_{Ca.L}$ at 2–3 min after exposure and reached the steady state at 6–8 min. The peak amplitude of $I_{Ca.L}$ (at 0 mV) was pronouncedly increased by 48.9 ± 5.5 % from 8.3 ±1.4 to 12.2 ± 2.3 pA/pF (n=26, $P < 0.01$). The Ang II-induced increases of $I_{Ca.L}$ were significantly attenuated by 3 μM Los, 1 mM apocynin, 1 mM trolox, or 1 μM KN-93, respectively (as summarized in Fig. 5E). Consistent with previous reports [31–32], we did not observe significant effects of losartan, apocynin, or trolox (at the same concentrations) on basal $I_{Ca,L}$. Although both KN-93 and KN-92 have been reported to suppress basal $I_{Ca,L}$ in a CaMKII-independent manner [26], our results showed only KN-93 eliminated EADs, presumably due to its additional CaMKII inhibitory effect. This conclusion was further supported by the inhibitory effect of the selective CaMKII inhibitory peptide, AIP.

3.6. Contribution of late I_{Na} in Ang II-induced EADs

Although Ang II-induced EAD upstrokes were eliminated by nifedipine, the APD remained prolonged, presumably due to the activation of late I_{Na}, since the selective Na^+ channels blocker tetrodotoxin (TTX, 10 μM) shortened the APD (Fig. 6A). In addition, ranolazine (2 μM), a more selective blocker of late I_{Na}, also suppressed Ang II-induced EADs, suggesting that late I_{Na} also plays a key role in EADs generation by Ang II. Supporting this idea, Fig.

6C and 6D show that late I_{Na} was significantly enhanced from −0.84 ± 0.16 pC to −2.22 ± 0.40 pC (n=20, $P<0.01$) when the increase of late I_{Na} reached the steady state at 10–15 min after application of Ang II (1 μM). Ang II-induced enhancement of late I_{Na} was significantly attenuated by 1 mM apocynin, (to −1.14±0.33 pC, n=6, $P<0.01$), 1 mM trolox (to −0.88±0.3 pC, n =6, $P<0.01$) or 1 μM KN-93 (to −1.37±0.29 pC, n=7, $P<0.05$), respectively (Fig. 6D).

3.7. Less involvement of outward K^+ currents in Ang II-induced EADs

Since outward K^+ currents may also play roles in APD alternation and EAD genesis, we next evaluated the effects of Ang II on total outward K^+ currents. As shown in Fig. 7, neither peak ($I_{K,\ peak}$) (ctl: 10.5 ± 1.1 vs. Ang II: 11.2 ± 1.7 pA/pF, n = 14; $P > 0.05$) nor steady state ($I_{K,ss}$) (ctl: 1.2 ± 0.2 vs. Ang II: 1.4 ± 0.3 pA/pF, n = 14; $P > 0.05$) outward potassium currents were significantly altered by Ang II at the same concentration that induced EADs under current clamp condition. We also tested the effects of aforementioned regulatory agents and found that none of losartan, Apocinin, trolox, and KN-93 affected total I_K under our experimental condition, excluding the possibility that these agents may suppress Ang II-induced EADs via alternation in I_K.

4. Discussion

We have recently shown that exogenously applied ROS (H_2O_2) induced EADs, DADs and TAs via CaMKII signaling pathway, which may cause arrhythmias under pathological conditions such as heart failure and ischemia-reperfusion. The activation of the rennin-angiotensin system (RAS) has been implicated in arrhythmias associated with heart failure and ischemia-reperfusion since inhibitors of this system reduce the incidence of sudden death. However, direct evidence linking AT_1R to NADPH oxidase-dependent ROS production and induction of EADs is missing. In the present study, we found that: 1) Ang II induces EADs in isolated ventricular myocytes; 2) the activation of AT_1R, and NADPH oxidase causes ROS production which is responsible for the generation of EADs; 3) CaMKII activation via ROS plays a key role in Ang II-induced EADs; 4) the activation of $I_{Ca.L}$ and late I_{Na} by Ang II contributes to the inward currents accounting for the EAD generation. In summary, the present study provides direct evidence that endogenous ROS, derived via the NADPH oxidase, mediate generation of arrhythmogenic EADs in response to angiotensin II. These results also provide mechanistic basis for the clinical approaches for treatment of arrhythmias by angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers, or NADPH oxidase inhibitors [13,33].

Activation of various membrane receptors (such as AT_1R, ET_AR, and TGFβR) have been shown to trigger a common signaling cascade [3]. Thus, it is very likely that NADPH oxidase-ROS-CaMKII pathway could represent a common mechanism accounting for arrhythmias induced by the agonists of above receptors. Supporting this assumption, endothelin-1 has been reported to cause ventricular tachyarrhythmias via prolongation of APD and formation of EADs [34–36], promote mitochondrial ROS production triggered by NADPH oxidase [37], and activates $I_{Ca,L}$ by CaMKII [38]. Further studies are needed to provide direct link between these individual steps.

4.1. Ang II-induced endogenous generation of ROS in myocytes via AT_1R and NADPH oxidase

The NADPH oxidase family plays a central role in generation of ROS in cardiovascular disorders [39–40]. It catalyzes the formation of superoxide oxygen, which in turn is reduced to H_2O_2 by superoxide dismutases. In the present study, we have clearly demonstrated the correlation between ROS production by NADPH oxidase and EAD induction by Ang II via AT_1R. It is obvious that Ang II elevated the intracellular ROS level in a slower time course

and to a lesser extent compared to exogenous application of H_2O_2 (50 or 200 μM) (Fig. 1), suggesting Ang II-induced endogenous ROS production involves intracellular signal pathways and enzyme reactions so that the ROS increase in a mild and slow manner. In addition to the ROS generation directly via NADPH oxidase, the ROS-induced ROS release from mitochondria may be involved in Ang II-induced ROS formation in cardiac myocytes [41–42].

4.2. Afterdepolarizations induced by endogenously generated ROS via Ang II: comparison with exogenously administrated H_2O_2

Although the effects of Ang II on membrane ion currents and AP have been well investigated, it is not known if Ang II can induce EADs via NADPH oxidase-ROS pathway in the cardiac myocytes. In our current study, we observed EADs occurred with a slow time course (at 15.8 ± 1.6 min) after Ang II perfusion, which is consistent with that for ROS production by Ang II. It should be noticed that the incidence rate of EAD or DAD was lower and the perfusion time to induce EADs was longer by Ang II than by direct H_2O_2 treatment. These results suggest that endogenously generated ROS by receptor stimulation (AT_1R in our case) showed lower inducibility of EADs, consistent with the relatively mild increase of intracellular ROS level by Ang II compared to H_2O_2 as measured with ROS fluorescence dye (Fig. 1C).

4.3. Downstream of ROS — Similar cellular and ionic mechanisms underlying Ang II- and exogenous H_2O_2-induced EADs

Our results demonstrate that Ang II and exogenous H_2O_2 share the same downstream targets including CaMKII, $I_{Ca,L}$ and I_{Na}. CaMKII is a multifunctional protein kinase expressed abundantly in the heart. Previous studies have implicated that ROS-activated CaMKII may be arrhythmogenic due to alternation of cardiac repolarization and Ca^{2+} handling and induction of EADs and DADs [1,8,43]. Our present finding that the CaMKII inhibitors AIP and KN-93 (but not its inactive analogue KN-92) suppressed Ang II-induced EADs suggests that CaMKII activation is the common mechanism for both Ang II- and H_2O_2- induced EADs. CaMKII further activates $I_{Ca,L}$ and late I_{Na}, which account for the inward membrane current causing EADs. However, the overall contribution of K currents to Ang II-induced EADs is neglectable under our experimental conditions. It has been reported that Ang II promotes Ca influx by activating the reverse mode of Na-Ca exchange current (NCX) [44–45]. However, this effect seemed unlikely to contribute to the EAD induction, since the reverse mode of NCX would generate an outward current.

We have shown in our previous study that direct application of exogenous H_2O_2 causes Ca^{2+} overload and generates spontaneous Ca^{2+} release and Ca^{2+} waves, which in turn induces DADs via Na^+-Ca^{2+} exchange current [1]. These effects may result either from direct modification of Ca_i-cycling proteins, including ryanodine receptors [46] and SERCA2a [47] by ROS, or secondarily from APD prolongation [1]. It should be noticed that Ang II treatment seldom induced DADs (only in 3/41 cells), suggesting that endogenously generated ROS cause less intracellular Ca^{2+} overload compared to exogenous ROS (H_2O_2) application.

4.4. Possible involvement of other signal transduction pathways

Ang II receptors couple to various signal transduction pathways that involve G-proteins, intracellular second messengers, and protein kinases. [24,48], [49]. The detailed mechanism(s) of NADPH activation has not been completely understood yet. Some studies have shown that NADPH oxidase activation requires upstream PKC in various tissues, such as brain, vascular smooth muscle and mesangial cells [4,50]. PKC may also activate membrane ion channels, including $I_{Ca,L}$, via direct phosphorylation [51]. However, we did

not observe an inhibitory effect of selective PKC blockers (chelerythrine or GF 109203X) on Ang II-induced EADs, suggesting PKC may not be involved in current signaling pathway. In fact, a novel signal transduction pathway of Ang II-induced ROS production in cardiomyocytes was reported by Nishida et al, who demonstrated that AT_1R stimulation by Ang II activates $G_{\alpha 12/13}$ proteins, which in turn cause Rho/ROCK-mediated Rac1 activation. Rac, one of the small GTP-binding proteins, promotes the production of ROS by activating NADPH oxidase [52].

4.5. Arrhythmogenesis by Ang II and clinical relevance

Our results underscore the propensity for Ang II to induce EADs and cause cardiac arrhythmias via triggered activity mechanism. The possible role of renin-angiotensin system activation in the genesis of atrial and ventricular arrhythmias suggests that ACE inhibitors and Ang II receptor blockers may serve as effective therapeutic agents in the prevention and treatment of arrhythmias [13]. This postulation is supported by both experimental and clinical studies [12,16]. The anti-arrhythmic effects of ACE inhibitors and AT_1R blockers may be attributed to the suppression of both long-term structural remodeling (e.g prevention of fibrosis) and short-term electrical remodeling (direct modulation of ion-channel function) [16].

Similar to H_2O_2 [1,22], Ang II-induced EADs were slow-rate-dependent and were observed at PCL > 2 sec. Accordingly, Ang II-induced ventricular arrhythmias may more readily manifest in the clinical setting of bradycardia [53], such as sinus pauses (sinus-node dysfunction), atrialventricular conduction disturbances, atrial fibrillation or long QT syndromes.

Ang II can be produced locally in the myocardium as an autocrine regulator. Ang II levels in cardiac tissue are much higher than those in plasma and can reach up to ~20 nM [54]. It is also well known that Ang II levels can increase under pathological condition such as hypertrophy, heart failure and ischemic heart disease [10]. For example, the Ang II concentration in the medium of serum-deprived cardiomyocytes increases near 100-fold upon stretch [55]. In addition, the density of Ang II receptors is also upregulated during pathological conditions such as ischemia-reperfusion [56–57]. Therefore the cardiomyocytes in vivo may be challenged by a wide range of Ang II level, and the Ang II concentrations (0.1–2 μM) used in the present study are likely within *pathophysiologically* relevant range.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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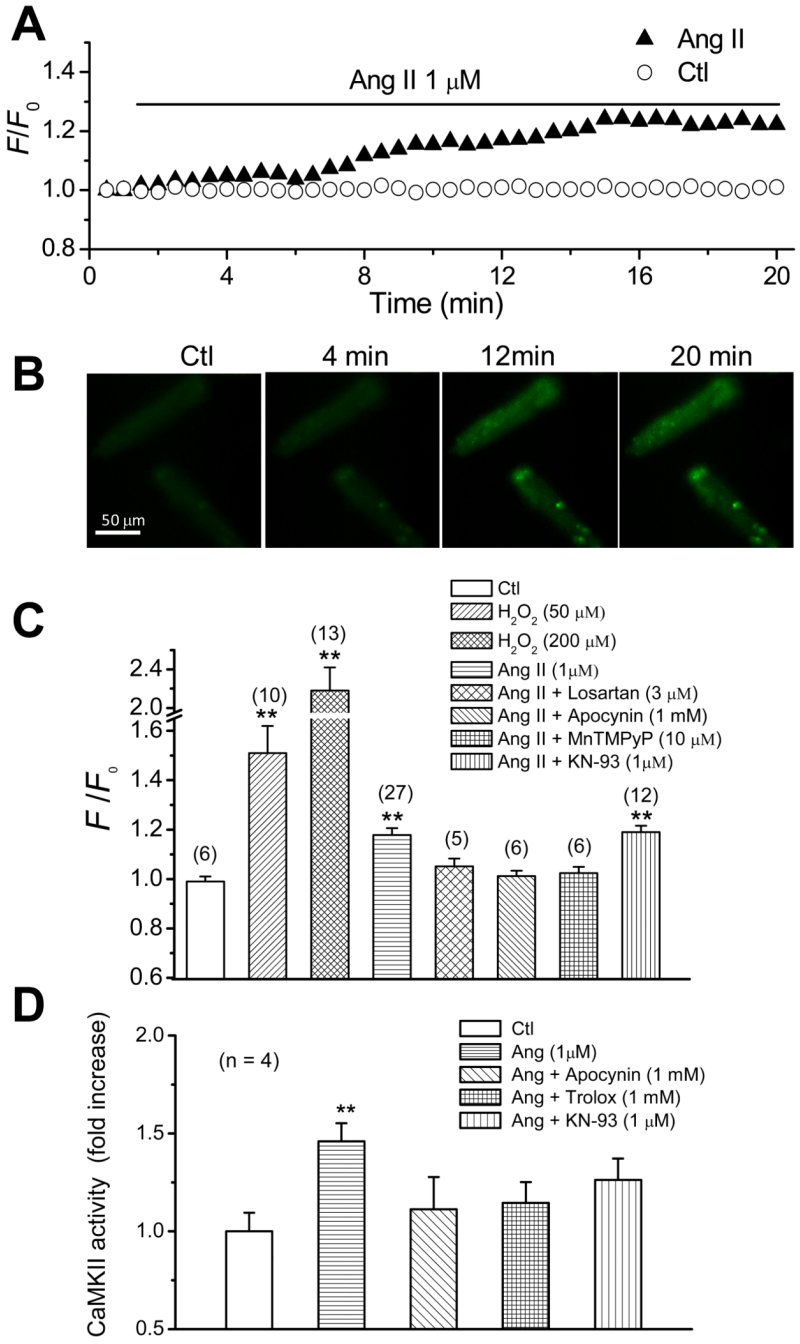

Fig. 1.

Ang-II induced ROS production in isolated rabbit ventricular myocytes. (A). Time course of DCF intensity in cells treated with vehicle (open circles) or 1 μM Ang II (filled triangles). (B). Images showing DCF intensity of myocytes at control, and 4, 12, 20 mins after treatment with 1 μM Ang II. (C). Histograms illustrating the effect of 1 μM Ang II treatment (18–20 min) on DCF fluorescence intensity in the absence and presence of losartan, apocynin, MnTMPyP, and KN-93 (measured at 2–5 min after application). H_2O_2 (50 and 200 μM)-induced increase of DCF intensity is shown as a positive control. Note the break on the Y-axis. (D). Histograms illustrating the effect of Ang II on CaMKII activity in the

absence and presence of apocynin, Trolox, or KN-93. **$P < 0.01$ compared to control. Numbers in parentheses indicate the number of cells in each group.

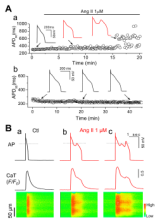

Fig. 2.

Early afterdepolarizations (EADs) and intracellular calcium (Ca_i) alteration induced by Ang II. (A-a). APs recorded under perforated whole-cell configuration before and during Ang II perfusion. Values of consecutive APD_{90} are plotted over time. EADs were induced at 15.8 ± 1.6 min after exposure to Ang II. (A-b). A representative AP recording from a cell perfused with control perfusate for > 40 min. No EADs were observed. (B). Ca_i Transients recorded under control and during Ang II-Induced EADs. a. AP, whole-cell Ca_i transient, and a line-scan image along the long axis of the myocyte before Ang II treatment. b & c. Same following exposure to 1μM Ang II for ~20 min. EADs result in persistent elevation (b) and additional release in Ca_i (c).

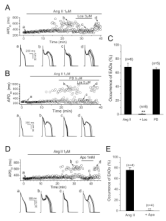

Fig. 3.

Ang II-induced EADs are mediated by Ang II type 1 receptor (AT_1R) and NADPH oxidase. (A). Time course of APD_{90} in a myocyte treated with Ang II and AT_1R blocker Losartan (Los). Five consecutive APs recorded at points a–d are displayed underneath. (B). Time course of APD_{90} in a myocyte treated with Ang II and AT_2R blocker PD-123319 (PD) and AT_1R blocker Losartan (Los). Five consecutive APs recorded at points a–d are displayed underneath. (C). Summarized histogram shows probabilities of EAD occurrence in the presence of 1 μM Ang II, 1 μM Ang II + 3 μM Los, and 1 μM Ang II + 5 μM PD. (D). Time course of APD_{90} in a myocyte treated with Ang II and NADPH oxidase inhibitor apocynin (Apo). Five consecutive APs recorded at points a–d each are shown beneath. (E). Summarized histogram shows probabilities of EAD occurrence in the presence of 1 μM Ang II and Ang II+ 1 μM Apo, respectively. .

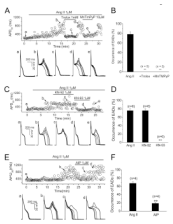

Fig. 4.

Suppression of Ang II-induced EADs by antioxidant and CaMKII inhibitor. (A). Time course of APD_{90} in a myocyte treated with Ang II and antioxidants trolox (1 mM) and MnTMPyP (10 μM). Five consecutive APs recorded at points a–e are shown underneath. (B). Summarized histogram shows probabilities of EAD occurrence in the presence of 1 μM Ang II, 1 μM Ang II+ 1mM trolox, and 1 μM Ang II +10 μM MnTMPyP, respectively. (C). Time course of APD_{90} in a myocyte treated with Ang II, KN-93 (CaMK II inhibitor), and KN-92 (an inactive analog of KN-93). Five consecutive APs recorded at points a–e each are shown underneath. (D). Summarized histogram shows probabilities of EAD occurrence in the presence of 1 μM Ang II, 1 μM Ang II+1 μM KN-92, and 1 μM Ang II+1 μM KN-93, respectively. (E). Time course of APD_{90} in a myocyte treated with Ang II, and AIP. Five consecutive APs recorded at points a–d each are shown underneath. (F). Summarized histogram shows probabilities of EAD occurrence in the presence of 1 μM Ang II, and 1 μM Ang II+1 μM AIP, respectively.

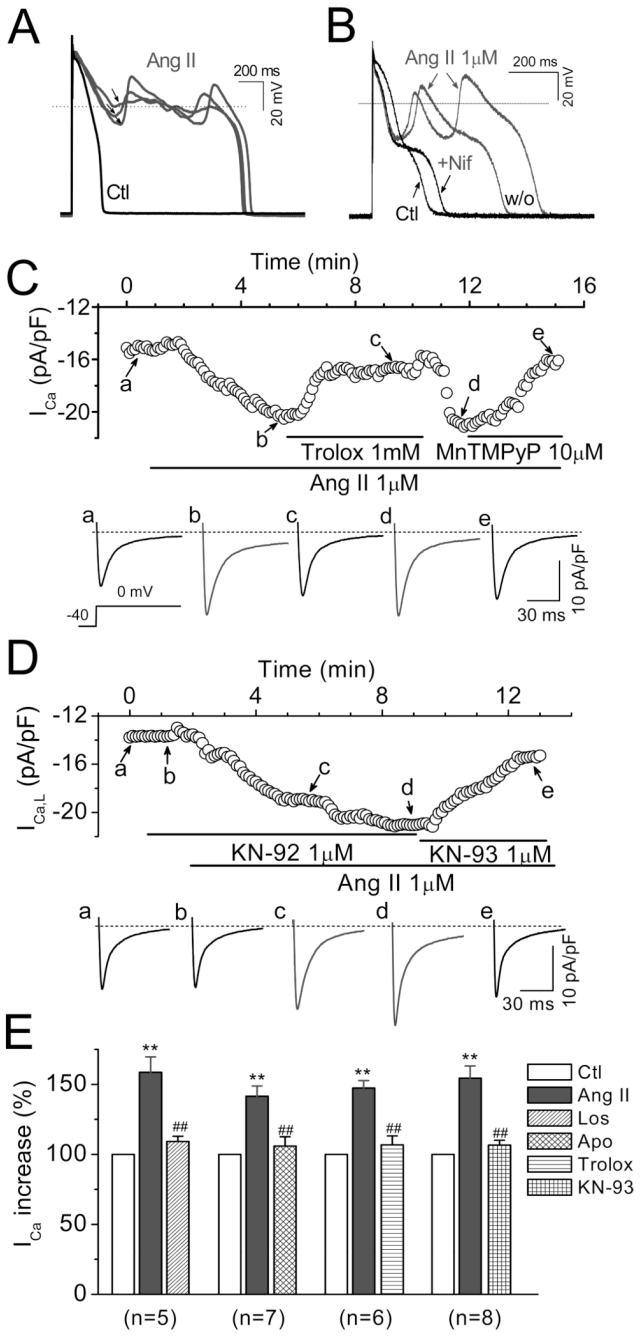

Fig. 5.

Involvement of $I_{Ca,L}$ in Ang II-induced EADs. (A). APs recorded under control condition (Ctl) and after EADs were induced by Ang II perfusion. The amplitude of Ang II-induced EADs depended on their take-off potentials (arrows). (B). The $I_{Ca,L}$ blocker nifedipine (Nif, 10 μM) reversibly suppressed the EADs upstroke induced by Ang II. (C). Time course of peak of $I_{Ca,L}$ in a myocyte treated with Ang II, and antioxidants trolox or MnTMPyP (MnT). Representative traces of $I_{Ca,L}$ corresponding to points a–e are shown. (D). Time course of peak $I_{Ca,L}$ in a myocyte treated with Ang II, and KN-92 or KN-93. (E). Summary of the activation of peak $I_{Ca,L}$ by Ang II (1 μM), and antagonistic effect of losartan (5 μM),

apocynin (1 mM), trolox (1mM), and KN-93 (1 μM). **P<0.01 vs. control; $^{\#\#}P$< 0.05 vs. Ang II. .

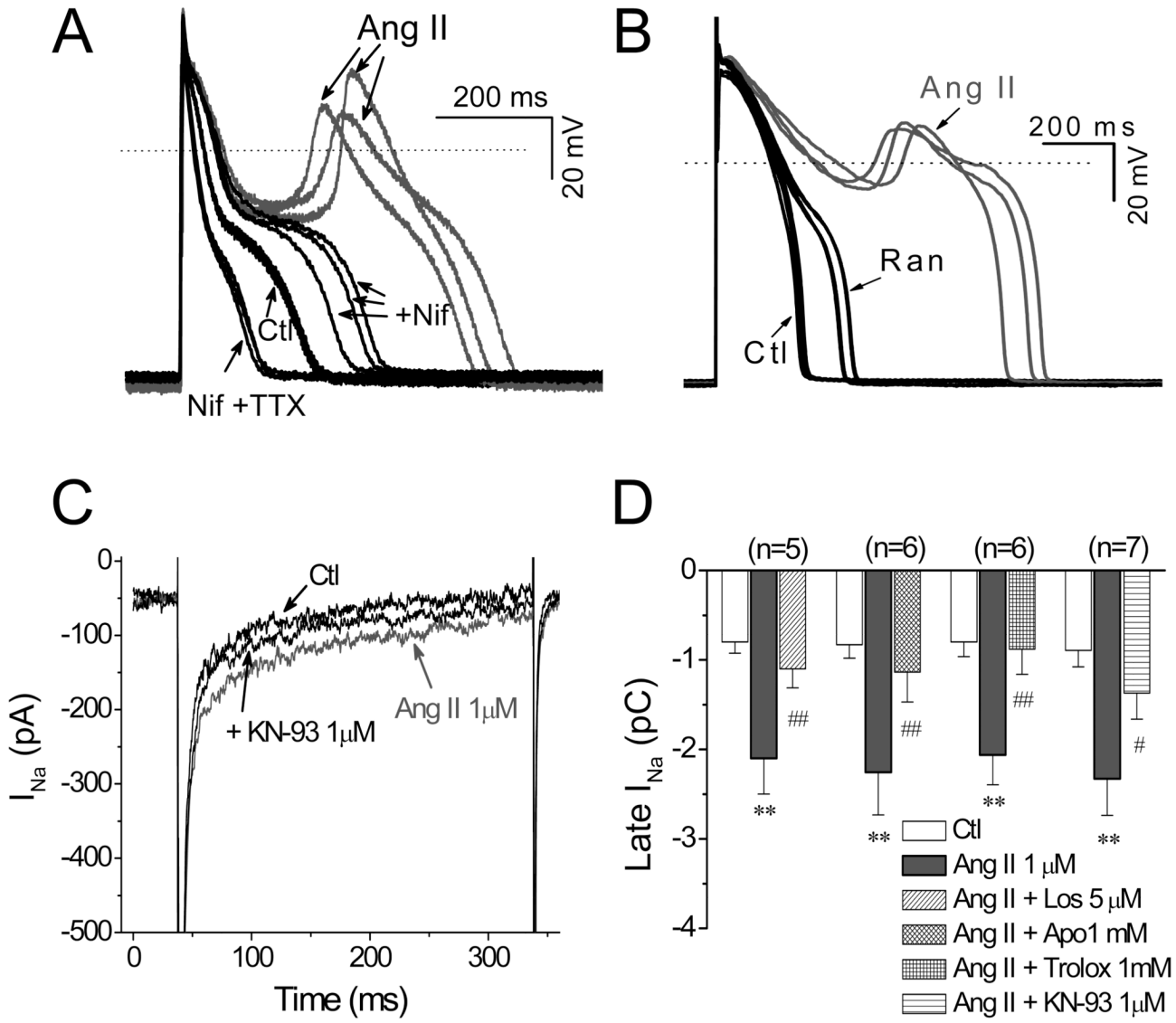

Fig. 6.

Involvement of late I_{Na} in Ang II-induced EADs. (A). The specific I_{Na} blocker TTX (10 μM) further shortened APD after EADs were eliminated by nifedipine. (B). The selective inhibitor of late I_{Na}, ranolazine (Ran), also abolished EADs induced by Ang II. (C). Representative I_{Na} traces under control condition (Ctl), in the presence of Ang II, and Ang II + KN-93. (D). A bar graph summarizing 1 μM Ang II-induced increase of late I_{Na}, which is significantly suppressed by 1mM trolox, 1 μM apocynin, or 1 μM KN-93, respectively.

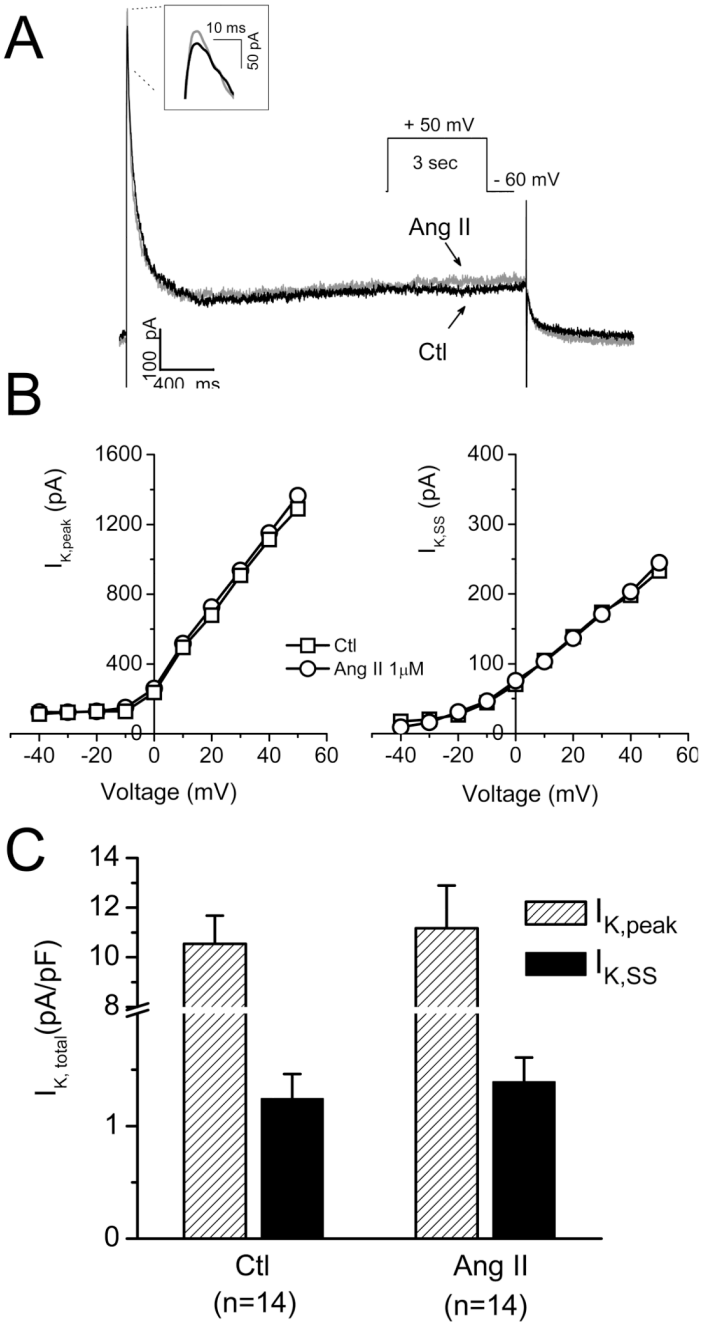

Fig. 7.

Less effect of Ang II on total outward K^+ current. (A). Representative traces of the total K^+ current in the absence (Ctl) and presence of Ang II (1μM). (B). Current-voltage curves of the total peak currents ($I_{K,peak}$, left) and stead-state currents ($I_{K,SS}$, right). (C). Summarized data showing no significant effects of 1 μM Ang II on the total outward $I_{K,peak}$ and $I_{K,SS}$.