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Original Article

Aldosterone downregulates delayed rectifier potassium currents through an angiotensin type 1 receptor-dependent mechanism

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Abstract: We have previously shown that aldosterone downregulates delayed rectifier potassium currents (I_{Ks}) via activation of the mineralocorticoid receptor (MR) in adult guinea pig cardiomyocytes. Here, we investigate whether angiotensin II/angiotensin type 1 receptor (AngII/AT1R) and intracellular calcium also play a role in these effects. Ventricular cardiomyocytes were isolated from adult guinea pigs and incubated with aldosterone (1 μmol·L^{-1}) either alone or in combination with enalapril (1 μmol·L^{-1}), losartan (1 μmol·L^{-1}), nimodipine (1 μmol·L^{-1}), or BAPTA-AM (2.5 μmol·L^{-1}) for 24 h. We used the conventional whole cell patch-clamp technique to record the I_{Ks} component. In addition, we evaluated expression of the I_{Ks} subunits KCNQ1 and KCNE1 using Western blotting. Our results showed that both enalapril and losartan, but not nimodipine or BAPTA-AM, completely reversed the aldosterone-induced inhibition of I_{Ks} and its effects on KCNQ1/KCNE1 protein levels. Furthermore, we found that AngII/AT1R mediates the inhibitory effects of aldosterone on I_{Ks}. Finally, the downregulation of I_{Ks} induced by aldosterone did not occur secondarily to a change in intracellular calcium concentrations. Taken together, our findings demonstrate that crosstalk between MR and AT1R underlies the effects of aldosterone, and provide new insights into the mechanism underlying potassium channels.

Keywords: Arrhythmia, delayed rectifier potassium current, angiotensin type 1 receptor, aldosterone, mineralocorticoid receptor, KCNQ1/KCNE1

Introduction

The pathological remodeling that occurs in cardiac hypertrophy and heart failure (HF) often leads to electrophysiological instability in the heart and eventually to arrhythmias. More than 50% of patients with HF die of sudden cardiac death due to malignant arrhythmia [1]. Action potential duration prolongation is an electrophysiological remodeling that is characteristic of hypertrophy and HF and has been observed in both patients and animal models [2-6]. The accompanying delayed ventricular repolarization often leads to early afterdepolarization and asynchronous repolarization, which can increase the transmural dispersion of ventricular repolarization and ultimately causes reentry. Thus, these pathological electrophysiological changes can encourage the production of tachyarrhythmia.

Previous studies have demonstrated that a reduction in the depolarizing K^+ current is the major cause of action potential duration prolongation [4, 7]. Delayed-rectifier K^+ currents, which include the rapid delayed-rectifier K^+ current (I_{Kr}) and the slow delayed-rectifier K^+ current (I_{Ks}), play a key role in repolarization [8]. The molecular component underlying the I_{Ks} is formed from the assemblage of the potassium voltage-gated channel, KQT-like subfamily, member 1 (KCNQ1) α-subunit with the potassium voltage-gated channel subfamily E, member 1(KCNE1; [mink]) β-subunit. However, the molecular mechanisms underlying the downregulation of I_{Ks} in heart diseases is not fully understood.

The systemic and local renin-angiotensin-aldosterone system plays an important role in regulating normal cardiovascular function, but may also contribute to the progression of certain cardiovascular diseases, including cardiac hypertrophy and HF [9-12]. Our previous work has shown that aldosterone downregulates the I_{Ks} by selectively inhibiting the expression of KCNQ1 and KCNE1, at the level of both mRNA and protein, in adult guinea pig cardiomyocytes [13].

It has been well-established that the mineralocorticoid receptor (MR), also known as the aldosterone receptor, belongs to the ligand-activated steroid receptor superfamily, and that when MR binds to aldosterone it becomes activated and serves as a transcription factor. While inactive MR is primarily located in the cytosol, where it is associated with a multicomponent complex, once MR binds to its ligand it undergoes nuclear translocation, which allows it to regulate gene, and ultimately, protein expression [14, 15]. Importantly, recent research has shown that there is some crosstalk between the MR and other signaling pathways outside the nucleus, such as with the angiotensin type 1 receptor (AT1R) and intracellular calcium signaling pathways [16]. Therefore, the aim of the present study was to investigate this crosstalk and the underlying mechanisms.

Materials and methods

Guinea pig ventricular cardiomyocytes isolation

Male adult guinea pigs, weighing 200-250 g were supplied by the Experimental Animal Center of Hebei Medical University. All of the procedures involving animals were in full compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Single ventricular myocytes were enzymatically dissociated from the hearts of adult guinea pigs as described previously [17]. In brief, the hearts were quickly excised and mounted on a Langendorff apparatus. Retrograde aortic perfusion was performed with Ca^{2+}-free modified Tyrode solution composed of (in mM) NaCl 140; KCl 5.4; $MgCl_2$ 1; HEPES 10; and glucose 10 (pH 7.4 with NaOH). After 5 min of perfusion, the solution was switched to one containing Type II collagenase (Worthington, 0.4 mg·mL^{-1}) and hearts were removed from the perfusion apparatus once ventricular tissue was softened after 10-15 min of perfusion. The left ventricular free wall was cut into small pieces in high K^+ solution, which contained (in mM) KOH 80; KCl 40; KH_2PO_4 25; $MgSO_4$ 3; glutamic acid 50; taurine 20; EGTA 0.5; HEPES 10; and glucose 10 (pH 7.3 with KOH). Cells were then harvested and were used for patch-clamp recordings within 4-6 h after isolation.

Cell culture and treatment

Guinea pig ventricular cardiomyocytes were isolated aseptically by the above method. Cells were concentrated and allowed to settle by gravity. Cardiomyocytes were cultured according to the literature [18]. Cells were reintroduced to calcium by a step-wise method (200, 500, 1000, and 1800 μM). After 2 h, dead cells were removed. Fresh medium (Hyclone M199+Earle'ssaltsandL-glutamine) containing either aldosterone (Sigma-Aldrich) or vehicle in DMSO was then added. Cells were kept in culture for an additional 24 h in an incubator set to 37°C and 5% CO_2. To study the interactions between MR and AT1R or intracellular calcium signals, cells were incubated with aldosterone (1 μmol·L^{-1}) alone or accompanied with enalapril (1 μmol·L^{-1}, an angiotensin converting enzyme inhibitor, ACEI), losartan (1 μmol·L^{-1}, an AT1R blocker), nimodipine (1 μmol·L^{-1}, a blocker of Ca^{2+} channel) or BAPTA-AM (2.5 μmol·L^{-1}, a permanent Ca^{2+} chelator).

Patch-clamp recordings

After 24 h exposure to interventions, cultured cardiomyocytes were washed with Tyrode solution before analysis and were studied within 2-4 h. Borosilicate glass electrodes were used that had tip resistances of 1-3 MΩ when filled with the pipette solution containing (in mM) KCl 140, Mg-ATP 4, $MgCl_2$ 1, EGTA 5, and HEPES 10, with pH adjusted to 7.2 with KOH. The external solution contained (in mM) NaCl 132, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1.2, glucose 5, and HEPES 10; with pH adjusted to 7.4 with NaOH. Nimodipine (1 μM) was added to the external solution to block the L-type Ca^{2+} current. Na^+ and T-type Ca^{2+} currents were inactivated by holding a potential of -40 mV. To record I_{Ks}, E4031 (2 μM) was added to the external solution to block I_{Kr}. All experiments were performed at room temperature (24-25°C) using an Axopatch 700B

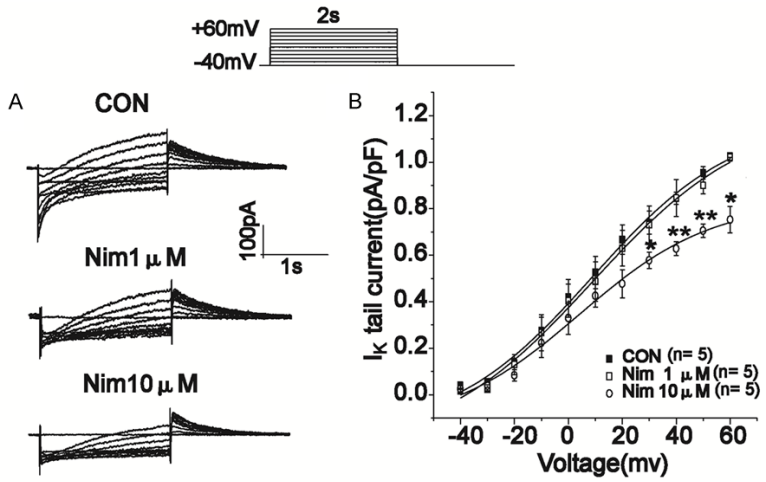

Figure 1. Effects of nimodipine (Nim) on inward and outward ionic currents in adult guinea pig cardiomyocytes. Current traces of I_K were elicited by depolarizing voltage steps between -40 and +60 mV from a holding potential of -40 mV in fresh cardiomyocytes. A. Representative currents traces recorded in freshly isolated left ventricular cardiomyocytes in absence and presence of 1 or 10 μM Nim. B. Effects of Nim on outward delayed-rectifier potassium currents (I_K) at 1 or 10 μM concentration. N indicates number of cardiomyocytes, which were from 3-5 hearts. *P < 0.05, **P < 0.01 *vs.* control (CON) at the same potential.

amplifier (Molecular Devices, Sunnyvale, CA, USA). The electrical signals were sampled at 2.5-10 kHz, filtered at 1 kHz using a low-pass filter and digitized with an A/D converter (Digidata 1322; Axon Instruments, Foster City, CA, USA). The pClamp software (Version 10.2; Molecular Devices) was used to generate voltage-pulse protocols and to acquire and analyze the data.

Western blot analysis

Total protein was isolated from cells after 24-hr of culturing in intervention or vehicle medium. Protein (50 μg) was denatured and fractionated on 10% SDS-PAGE and then transferred electrophoretically to immobilon-P polyvinylidene fluoride membranes. Membranes were blocked with 5% non-fat dry milk for 1 hour and incubated with primary antibodies overnight at 4°C. After washing and reblocking, membranes were incubated with goat anti-rabbit or anti-mouse (1:10000, Rockland, USA) secondary antibodies. Protein bands were analyzed with Odyssey 9120 (LICOR, USA) and denisty analysis softfare. All results were normalized to GAPDH. Antibodies were anti-GAPDH (Proteintech, China), anti-KCNQ1 (Alomone lab, Israel), anti-KCNE1 (Alomone lab, Israel).

Drug preparation and storage

E-4031 (Sigma-Aldrich) was prepared as a 1 mM stock in water and stored at -20°C. Nimodipine (Sigma-Aldrich) was prepared as a 100 mM stock solution in DMSO and stored in the dark. Aldosterone (Sigma-Aldrich) was prepared as a 1 mM stock solution in DMSO and stored at -20°C. Losartan (Tokyo Chemical Industry Co. LTD.), enalapril (Melonepharma) were prepared as a 10 mM stock solution in DMSO and stored at -20°C. BAPTA-AM (Sigma-Aldrich) was prepared as a 25 mM stock solution in DMSO and stored at -20°C. The highest final concentration of DMSO in external solution was 0.1%, a concentration that had no effect on I_{Ks}. Further dilutions were carried out in external solutions to obtain the desired final concentration immediately before each experiment. Control solutions contained the same DMSO concentrations as the test solutions.

Statistical analysis

Data are expressed as means ± SEM. SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA) was used for data analysis. Group comparisons were performed with Student's unpaired t-tests (for single two-group comparisons) and ANOVA with Dunnett's post hoc tests (for multiple-group comparisons). Differences were considered significant if $P < 0.05$.

Results

Determination of the appropriate nimodipine concentration to selectively inhibit L-type calcium channels

Nimodipine is a dihydropyridine calcium antagonist that is often added to extracellular solutions to block L-type calcium channels; however, the concentration of nimodipine used can vary. In addition, high concentrations of nimodipine can inhibit voltage-dependent K^+

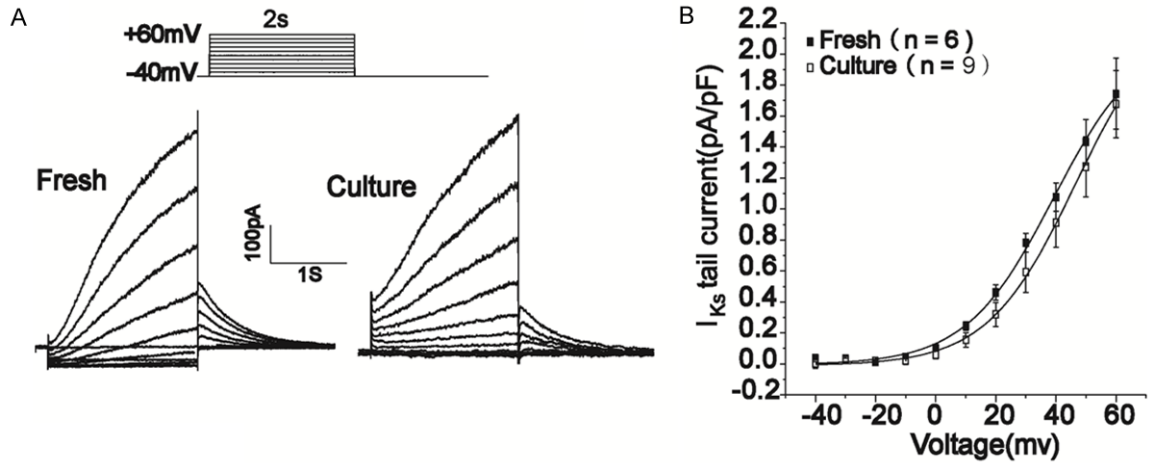

Figure 2. Alteration of I_{Ks} in cultured cardiomyocytes. A. Representative I_{Ks} recordings in freshly isolated (left) and cultured 24 h cells (right). B. Summary data for I_{Ks} tail current density-voltage relationship. N indicates number of cardiomyocytes, which were from 3-5 hearts.

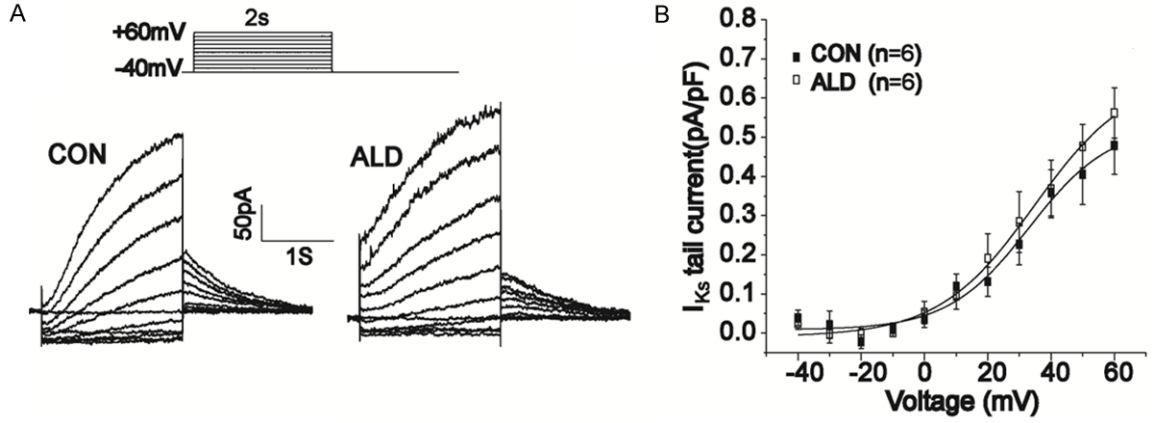

Figure 3. Acute effects of aldosterone on I_{Ks} in adult guinea pig cardiomyocytes. Freshly isolation cells were perfusd by external solution containing 1 μM aldosterone for 30 min. A. Representative I_{Ks} recordings in freshly isolated cells before (CON) and after application of aldosterone (ALD). B. Corresponding I_{Ks} tail current density-voltage relationship. N indicates number of cardiomyocytes, which were from 3-5 hearts.

channels [19]. Therefore, we first tested the effects of multiple nimodipine concentrations (1 μM and 10 μM) on inward (I_{CaL}) and outward (I_K) currents to identify a concentration that would block I_{CaL}, but would not affect I_K. **Figure 1** shows representative, superimposed traces of I_K, which were elicited by performing depolarizing voltage steps (between -40 and +60 mV) from a holding potential of -40 mV in fresh cardiomyocytes. The upper chart (**Figure 1A**) shows representative, superimposed traces of both I_{CaL} and I_K. We found that the I_{CaL}, but not the I_K, was almost completely suppressed by 1 μM of nimodipine after 10 minutes of perfusion (**Figure 1A** and **1B**). Conversely, 10 μM of nimodipine markedly reduced the I_K after 10 minutes (**Figure 1A** and **1B**). These results indicated that 1 μM nimodipine selectively inhibits the I_{CaL} in cardiomyocytes, without affecting the I_K. Thus, we used 1 μM nimodipine to block I_{CaL} in subsequent experiments.

The effect of cardiomyocyte isolation and culturing in vitro on the I_{ks}

Cells cultured *in vitro* have been shown to have prolonged action potential durations and to be

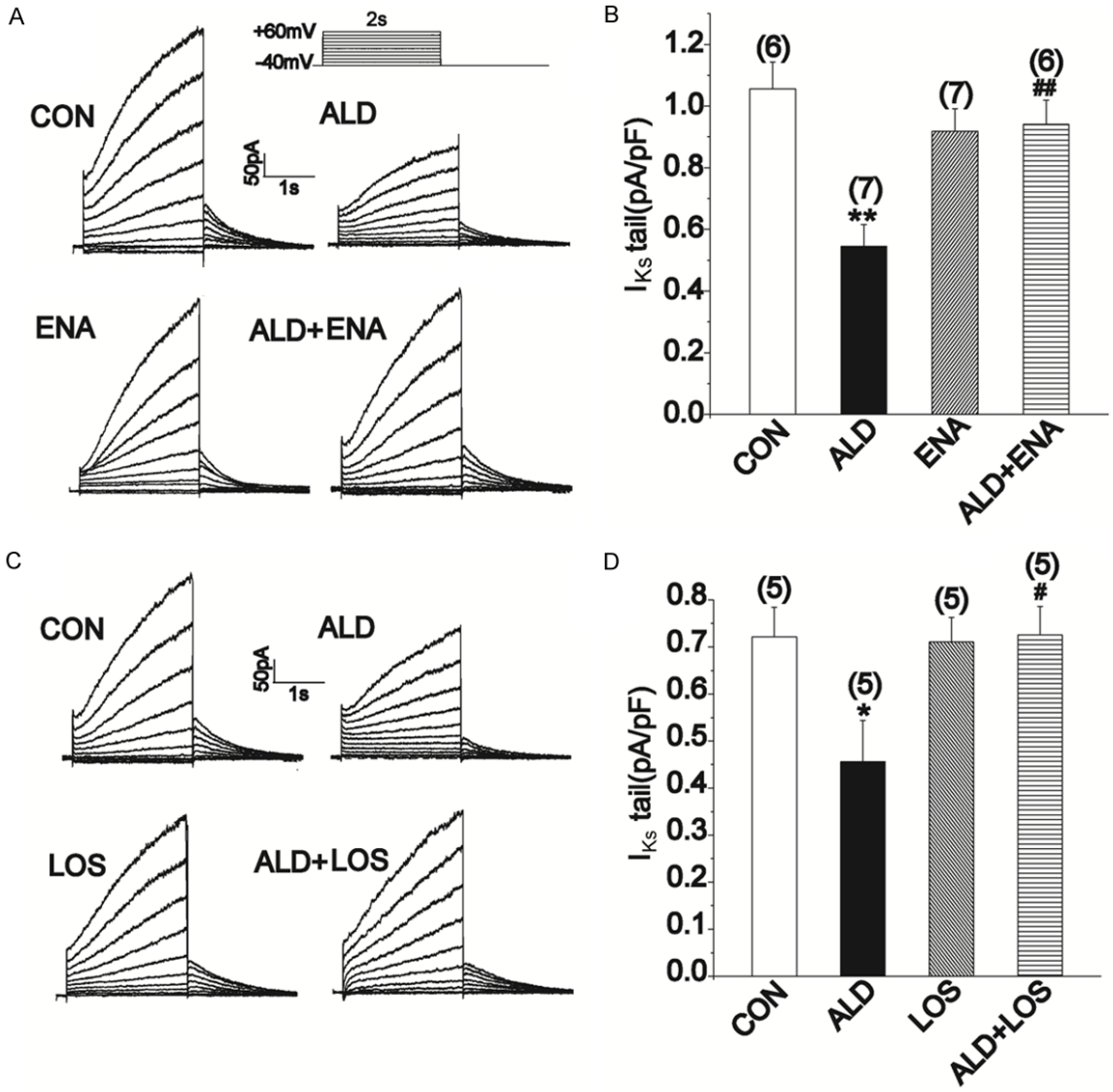

Figure 4. Effects of enalapril (ENA) or losartan (LOS) on the downregulation of I_{Ks} by aldosterone (ALD). Cells were incubated with aldosterone (1 μmol·L^{-1}) alone or accompanied with enalapril (1 μmol·L^{-1}), losartan (1 μmol·L^{-1}) for 24 h. We used conventional whole-cell patch-clamp technique to record I_{Ks}. A. Representative I_{Ks} recording in cells incubated with vehicle control (CON), ALD (1 μM), ENA (1 μM), or ALD+ENA. B. Summary data for I_{Ks} tail current density recorded at +40 mV. C. Representative I_{Ks} recording in cells incubated with CON, ALD (1 μM), LOS (1 μM) or ALD+LOS. D. Summary data for I_{Ks} tail current density recorded at +40 mV. The numbers in brackets represent sample size. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* CON; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* ALD.

slightly depolarized in adult animal cardiomyocytes [18], which indicates that culturing can alter certain ion channels. To this end, we first investigated whether changes in the I_{Ks} would occur following cell isolation and culturing. Our results are shown in **Figure 2**. We found that after culturing for 24 h, cardiomyocytes had no significant changes in either the appearance or tail density of the I_{Ks}. These findings are consistent with previous reports showing that culture of cardiomyocytes for 24 h did not alter the densities of I_{Ks} or the expression of KCNQ1 and KCNE1 [18, 20].

Acute effects of aldosterone on the I_{Ks}

Our previous study revealed that aldosterone binds to the MR and downregulates the I_{Ks} by selectively inhibiting the expression of KCNQ1 and KCNE1 mRNA/protein. To investi-

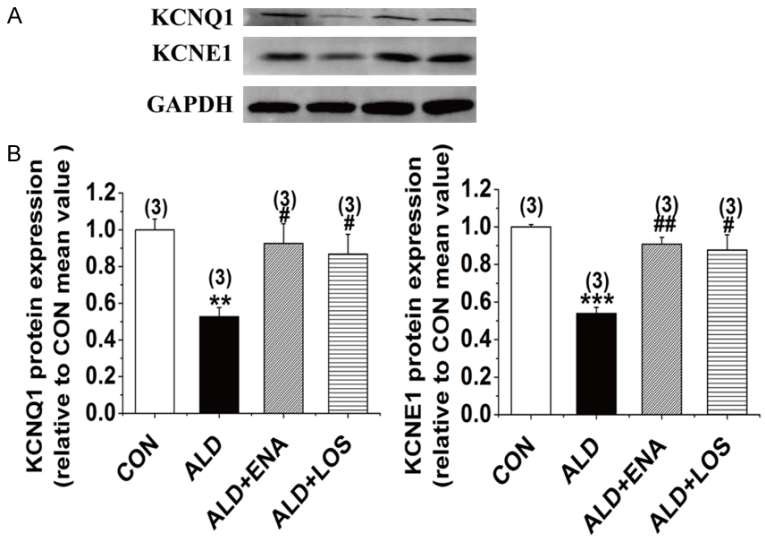

Figure 5. Effects of enalapril (ENA) or losartan (LOS) on the decreased protein expression of I_{Ks} subunits KCNQ1 and KCNE1 by aldosterone (ALD). Cells were incubated with aldosterone (1 μmol·L^{-1}) alone or accompanied with enalapril (1 μmol·L^{-1}), losartan (1 μmol·L^{-1}) for 24 h. We used western blot to assess I_{Ks} subunits KCNQ1 and KCNE1. A. Representative immunoblots for KCNQ1, KCNE1 proteins along with internal standard GAPDH. B. Mean ± SEM expression levels presented as fold change compared to the control group's mean value and the quantification of the band intensities were normalized with GAPDH (n = 3). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. CON; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* ALD.

gate whether aldosterone has a direct influence on the I_{Ks}, we isolated cardiomyocytes, suspended them for 15 min, and then tested the acute effects of aldosterone. As shown in **Figure 3**, perfusion of external solution containing 1 μM of aldosterone for 30 min did not alter the I_{Ks} in fresh cardiomyocytes.

The role of AT1R in the aldosterone-induced downregulation of the I_{Ks}

To test the interaction between the MR and AT1R, cultured cells were incubated with enalapril (1 μmol·L^{-1}) or losartan (1 μmol·L^{-1}) alone or co-incubated with aldosterone (1 μmol·L^{-1}) for 24 h. The results demonstrated that both enalapril and losartan reversed the downregulation of I_{Ks} induced by aldosterone, while treatment with either agent alone had no effect on the I_{Ks} (**Figure 4**).

To further address the role of AT1R in the aldosterone-induced downregulation of the I_{Ks}, we measured expression of the I_{Ks} subunits, KCNQ1, and KCNE1. Whole cell protein extracts were obtained from cardiomyocytes cultured in vehicle- or aldosterone-containing medium. As shown in **Figure 5**, expression of both KCNQ1 and KCNE1 protein was significantly decreased after 24 h of aldosterone exposure (1 μM); however, treatment with enalapril and losartan reversed these effects.

The effects of intracellular calcium on the aldosterone-induced downregulation of the I_{Ks}

Previous reports have shown that aldosterone downregulates transient outward K^+ currents (I_{to}), and that this occurs secondarily to elevations in intracellular calcium concentrations, which are caused by aldosterone-induced enhancements in the I_{CaL} [21]. Therefore, to evaluate the role of intercellular calcium on the aldosterone-induced downregulation of the I_{Ks}, cultured cardiomyocytes were treated with aldosterone either alone or in combination with a Ca^{2+} channel blocker (nimodipine, 1 μmol·L^{-1}) or a permeant Ca^{2+} chelator (BAPTA-AM, 2.5 μmol·L^{-1}). As shown in **Figure 6**, neither nimodipine nor BAPTA-AM treatment reversed the aldosterone-induced reduction in the I_{Ks}. Instead, treatment with nimodipine or BAPTA-AM decreased the I_{Ks}.

Discussion

Our previous study demonstrated that aldosterone downregulates I_{Ks} via chronic stimulation of the MR and by inhibiting the expression of KCNQ1 and KCNE1 in adult guinea pig cardiomyocytes, both *in vitro* and *in vivo* [13]. Here, we further investigated the underlying mechanisms.

Aldosterone increases the expression and activity of angiotensin converting enzyme, while antagonism of MR completely reverses this effect [22, 23]. In addition, aldosterone enhances the actions of AngII on the proliferation of vascular muscle cells by upregulating the expression of AT1R. AT1R is a G-protein-coupled

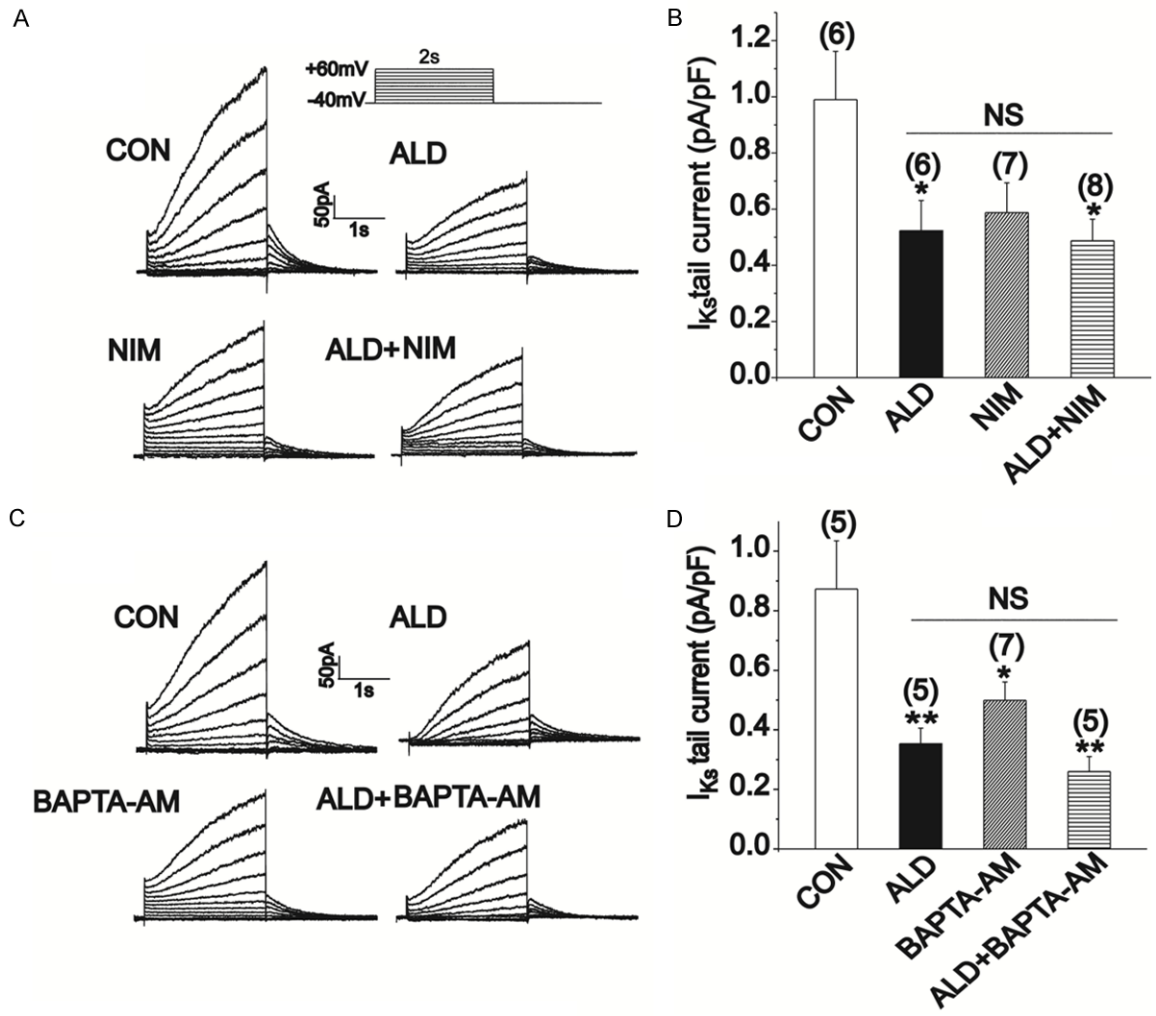

Figure 6. Effects of nimodipine (NIM) or BAPTA-AM on the downregulation of I_{Ks} by aldosterone (ALD). Cells were incubated with aldosterone (1 μmol·L^{-1}) alone or accompanied with nimodipine (1 μmol·L^{-1}) or BAPTA-AM (2.5 μmol·L^{-1}) for 24 h. We used conventional whole-cell patch-clamp technique to record I_{Ks}. A. Representative I_{Ks} recordings in cells incubated with vehicle control (CON), ALD (1 μM), NIM (1 μM) or ALD+NIM. B. Summary data for I_{Ks} tail current density recorded at +40 mV. C. Representative I_{Ks} recording in cells incubation of CON, ALD (1 μM), BAPTA-AM (1 μM) or ALD+BAPTA-AM. D. Summary data for I_{Ks} tail current density recorded at +40 mV. The numbers in brackets represent sample size. *P < 0.05, **P < 0.01 *vs.* CON.

receptor that mediates the effects of AngII on the cardiovascular system. Interactions between MR and AT1R are responsible for mediating injury to the cardiovascular system and kidneys, as well as the proliferation of vascular smooth muscle cells, and involve the production of reactive oxygen species, transactivation of epidermal growth factor receptor, and activation of mitogen-activated protein kinase. Synergy between MR and AT1R accelerates cardiac remodeling, but is reversed by antagonism of MR or AT1R. In addition, in an aldosterone synthase knock-out rat model, AngII was shown to activate MR to induce glomeruli damage and hypertrophy of renal interlobular arteries [24, 25]. Therefore, it is possible that aldosterone participates in AngII production and AT1R upregulation, and interactions between MR and AT1R are involved in pathological changes in the cardiovascular and renal systems, and in vascular smooth muscle. Based on this, we hypothesized that crosstalk between MR and AT1R/AngII may play a role in the effects of aldosterone.

Our results showed that both enalapril (an angiotensin converting enzyme inhibitor) and losartan (an AT1R blocker) reversed the aldo-

sterone-induced downregulation of I_{Ks}. Similarly, both enalapril and losartan reversed the aldosterone-induced decreases in expression of KCNQ1 and KCNE1 protein levels. These results provide evidence that crosstalk between MR and AT1R is involved in the downregulation of I_{Ks} induced by aldosterone in adult guinea pig cardiomyocytes.

It has previously been reported that the aldosterone-induced downregulation of transient outward I_K occurs secondarily to the augmentation of I_{CaL} [21]. This implies that there is a relationship between the modulation of K^+ channels and changes in intercellular calcium signaling. However, our results demonstrated that neither a calcium channel blocker (nimodipine) nor a permeant calcium chelator (BAPTA-AM) affected the aldosterone-induced downregulation of I_{Ks} *in vitro*. Moreover, nimodipine and BAPTA-AM reduced the I_{Ks} in cardiomyocytes. Therefore, our results suggest that the downregulation of I_{Ks} by aldosterone is not dependent on modulation of intracellular calcium. In addition, our results indicate that low intracellular calcium concentrations decrease the I_{Ks} in cardiomyocytes.

In summary, the present study demonstrates that crosstalk between MR and AT1R plays a role in the downregulation of I_{Ks} by aldosterone, and that this does not occur secondarily to an aldosterone-induced elevation in intracellular calcium. These results provide new insight into the mechanisms underlying K^+ channel regulation and its dysfunction in pathological states.

Limitation

This study did not reveal the mechanism by which crosstalk between MR and AT1R occurs during aldosterone-induced downregulation of I_{Ks}. Therefore, our future research will focus on investigating the downstream signaling pathways responsible for these interactions between MR and AT1R.

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Disclosure of conflict of interest

None.

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