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Zacopride selectively activates the Kir2.1 channel via a PKA signaling pathway in rat cardiomyocytes

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We recently reported that zacopride is a selective inward rectifier potassium current (I_{K1}) channel agonist, suppressing ventricular arrhythmias without affecting atrial arrhythmias. The present study aimed to investigate the unique pharmacological properties of zacopride. The whole-cell patch-clamp technique was used to study I_{K1} currents in rat atrial myocytes and Kir2.x currents in human embryonic kidney (HEK)-293 cells transfected with inward rectifier potassium channel (Kir)2.1, Kir2.2, Kir2.3, or mutated Kir2.1 (at phosphorylation site S425L). Western immunoblots were performed to estimate the relative protein expression levels of Kir2.x in rat atria and ventricles. Results showed that zacopride did not affect the I_{K1} and transmembrane potential of atrial myocytes. In HEK293 cells, zacopride increased Kir2.1 homomeric channels by 40.7%±9.7% at −50 mV, but did not affect Kir2.2 and Kir2.3 homomeric channels, and Kir2.1-Kir2.2, Kir2.1-Kir2.3 and Kir2.2-Kir2.3 heteromeric channels. Western immunoblots showed that similar levels of Kir2.3 protein were expressed in rat atria and ventricles, but atrial Kir2.1 protein level was only 25% of that measured in the ventricle. In addition, 5-hydroxytryptamine (5-HT)$_3$ receptor was undetectable, whereas 5-HT$_4$ receptor was weakly expressed in HEK293 cells. The Kir2.1-activating effect of zacopride in these cells was abolished by inhibition of protein kinase A (PKA), but not PKC or PKG. Furthermore, zacopride did not activate the mutant Kir2.1 channel in HEK293 cells but selectively activated the Kir2.1 homomeric channel via a PKA-dependent pathway, independent to that of the 5-HT receptor.

zacopride, inward rectifier potassium channel, 5-HT receptor, protein kinase

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Past studies have gone to great efforts in the search for selective inward rectifier potassium current/channel (I_{K1}) channel agonists for their anti-ventricular arrhythmia potential [1]. However, non-selectivity of agonists for multiple ion channels and/or receptors may thus induce differential effects on arrhythmogenesis in the atria and ventricles. Zacopride, a potent 5-hydroxytryptamine (5-HT)$_3$ receptor antagonist and 5-HT$_4$ receptor agonist has been experimentally used as a gastrointestinal prokinetic agent [2]. We have recently shown that zacopride is also a selective I_{K1} channel agonist in rat ventricular myocytes, moderately increasing the I_{K1} current, hyperpolarizing the resting membrane potential (RMP), and shortening the action potential duration (APD) without affecting other channels, transporters, and

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pumps [3]. Furthermore, zacopride suppressed the trigger of ventricular arrhythmias, but did not affect the electrophysiology of the atria [3]. To our knowledge, this is the first discovered agonist with high selectivity for the I_{K1} channel, thus the advantage of an anti-ventricular arrhythmia effect but with only minimal adverse effects on the atria.

Although zacopride was shown to exert an antiarrhythmic action in the ventricles via I_{K1} channels [3], its potential adverse effect on atrial electrophysiology should be taken into account. Activation of 5-HT_4 receptors by zacopride in human atria may result in atrial fibrillation (AF) due to calcium overload and activation of the pacemaker current (I_f) [4–6]. Up-regulation of I_{K1} in atrial myocytes may also lead to AF [7–9]. Despite its selectivity for both 5-HT_4 and the I_{K1} channel, zacopride does not induce atrial arrhythmia for treatment against triggered ventricular arrhythmia in the rat [3] or rabbit (unpublished data by our group). The mechanism underlying this lack of effect is poorly understood.

Inward rectifier potassium channel (Kir)2.1 (KCNJ2), Kir2.2 (KCNJ12) and Kir2.3 (KCNJ4) channels assemble as homotetramers or heterotetramers, and are the molecular basis of the native pore-forming subunit of I_{K1} channels in the heart [10,11]. Tissue- and species-specific expression profiles of Kir2.x isoforms determine the biophysical and physiological characteristics of I_{K1} channels in the heart. Moreover, all of the Kir2.x channels, including Kir2.1, Kir2.2 and Kir2.3, are the substrates of protein kinase A (PKA) and protein kinase C (PKC). Phosphorylation of these channel pore-forming proteins may thus play important regulatory roles in I_{K1} function [12]. We therefore hypothesized that zacopride may exert differential effects on atrial and ventricular I_{K1} through selective actions on different Kir2.x channels via phosphorylation, rather than the 5-HT receptor. This signaling pathway may serve as a mechanism by which zacopride suppresses ventricular arrhythmias without inducing atrial arrhythmias [3]. Therefore, the present study aimed to investigate the agonist properties of zacopride on the I_{K1} channels.

In the current study we investigated the effects of zacopride on I_{K1} channels in rat atrial myocytes. Furthermore, to determine the Kir2.x channel and the signaling pathway that is selectively activated by zacopride, we observed the effects of this agonist on the homotetramer or heterotetrameric channels of Kir2.1, Kir2.2, Kir2.3, or a mutant Kir2.1 channel transfected in human embryonic kidney (HEK)-293 cells. Our findings showed that zacopride increased I_{K1} in ventricular but not atrial myocytes, and this specific action was due to the selective activation of Kir2.1 via PKA, but not PKC, protein kinase G (PKG), and the 5-HT receptor.

1 Materials and methods

1.1 Animals

Male Sprague Dawley (SD) rats (220–250 g) were provided by the Experimental Animal Center (Hebei, China). Animal care, surgery, and handling procedures were approved by the Bioethical Committee of Shanxi Medical University, and performed according to the guidelines for the Care and Use of Laboratory Animals (National Institutes of Health No. 85-23, revised 1996). Rats were heparinized (1000 U kg^{-1}, intraperitoneally (i.p.)) and anesthetized with sodium pentobarbital (65 mg kg^{-1}, i.p.) in all experiments.

1.2 Isolation of cardiomyocytes

Isolated atrial myocytes were used for electrophysiological studies. They were isolated from rat heart by collagenase digestion [13,14]. Briefly, the heart was harvested under anesthesia and perfused with oxygenated calcium-free Tyrode's solution (135 mmol L^{-1} NaCl, 5.4 mmol L^{-1} KCl, 1.8 mmol L^{-1} $CaCl_2$, 1 mmol L^{-1} $MgCl_2$, 0.33 mmol L^{-1} NaH_2PO_4, 10 mmol L^{-1} HEPES, 10 mmol L^{-1} glucose 10; pH 7.3–7.4) at 37°C for approximately 7–8 min, followed by a 15 min perfusion with Tyrode's solution containing 100 mg L^{-1} collagenase P (Boehringer, Mannheim, Germany). Afterward, the atrium was separated, minced in KB solution (85 mmol L^{-1} KOH, 50 mmol L^{-1} L-glutamic acid, 30 mmol L^{-1} KCl, 1 mmol L^{-1} $MgCl_2$, 30 mmol L^{-1} KH_2PO_4, 10 mmol L^{-1} glucose, 20 mmol L^{-1} taurine, 10 mmol L^{-1} HEPES, 0.5 mmol L^{-1} EGTA; pH 7.4) and filtrated.

1.3 Cell transfection of Kir2.x

Respective rat cardiac orthologs of Kir2.1, Kir2.2 and Kir2.3 were cloned by reverse transcriptase polymerase chain reaction. S425L mutation of the Kir2.1-encoding gene, KCNJ2, was introduced by site-directed mutagenesis. Respective cDNAs encoding Kir2.1, Kir2.2, Kir2.3 and a Kir2.1 mutant (S425L) [12,15] were subcloned into the eukaryotic expression vector, pEGFP-N1. HEK293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Expression plasmids were then transfected into confluent HEK293 cells using the Lipofectamine 2000 kit (Invitrogen, USA).

1.4 Electrophysiology

Whole-cell recordings were performed using the Axopatch 200B amplifier (Axon Instrument, USA) with a sampling rate of 10 kHz, and filtered at 2 kHz. The electrodes were filled with pipette solution, and electrode resistance maintained at 2–5 MΩ. All experiments were conducted at room temperature (23–25°C). Transfected HEK293 cells expressing enhanced green fluorescent protein (EGFP) were viewed under a xenon arc lamp (488/530 nm; excitation/emission, respectively), and selected for the patch clamp study. The standard bath solution used in this experi-

ment was 136 mmol L^{-1} NaCl, 5 mmol L^{-1} KCl, 1.8 mmol L^{-1} $CaCl_2$, 1 mmol L^{-1} $MgCl_2$, 10 mmol L^{-1} glucose, and 10 mmol L^{-1} HEPES (pH 7.4). The standard pipette solution used in this study was 40 mmol L^{-1} KCl, 80 mmol L^{-1} K-aspartate, 10 mmol L^{-1} KH_2PO_4, 3 mmol L^{-1} phosphocreatine, 5 mmol L^{-1} EGTA, 5 mmol L^{-1} HEPES, and 5 mmol L^{-1} ATP-Mg (pH 7.2). To record native atrial I_{K1}, the extracellular solution was similar to that used for transfected cells, except that 0.2 mmol L^{-1} $CdCl_2$ was added to block L-Ca^+ channel. For native atrial I_{K1} recordings, pipette solution (5 mmol L^{-1} EGTA, 150 mmol L^{-1} KCl, 3 mmol L^{-1} K_2-ATP, 5 mmol L^{-1} HEPES, 5 mmol L^{-1} 4-AP, 1 mmol L^{-1} $MgCl_2$, 1 mmol L^{-1} Mg-ATP; pH 7.3) was used.

Zacopride, RS23597-190 (5-HT_4 receptor antagonist), PBT (selective 5-HT_4 receptor agonist), KT5720 (PKA inhibitor), KT5823 (PKG inhibitor), and forskolin (adenylate cyclase activator) were purchased from Tocris Cookson Inc. (UK). GF109203X (PKC inhibitor) and 8-bromo-cAMP were from Sigma-Aldrich (USA). 2-1-(4-Piperonyl) piperazinylbenzothiazole (PBT), a known selective 5-HT_4 receptor agonist was also purchased from Sigma-Aldrich.

1.5 Western immunoblotting

The following primary antibodies were used: monoclonal Anti-KCNJ2 antibody produced in mouse, monoclonal Anti-KCNJ12 antibody produced in mouse and monoclonal Anti-KCNJ4 antibody produced in mouse. They were all from Sigma-Aldrich (USA). Anti-5HT3A receptor antibody and anti-5HT4 receptor antibody were purchased from Abcam (UK).

Rat brain or heart protein were lysed by solution containing l mmol L^{-1} phenylmethanesulfonyl fluoride (pH 7.4) and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 10% polyacrylamide-Tris gels (Sigma-Aldrich, USA), and then transferred electrophoretically (2 mA cm^{-2}, 20 min) to polyvinylidene fluoride membranes (Sigma-Aldrich, USA) with 120 V voltage for 2 h. Membranes were blocked by solution containing 5% (w/v) skim milk powder and 0.1% Tween 20 in Tris-buffered saline for 2 h at room temperature and then incubated (4°C, overnight) with the primary antibodies (1∶1000 dilution). Membranes were then incubated (2 h, at room temperature) with horseradish peroxidase-conjugated secondary antibodies (1∶1000 dilution, from Santa Cruz Biotechnology, USA), and bands were visualized using enhanced chemiluminescence kit (Beyotime Institute of Biotechnology, China).

1.6 Statistical analyses

Data are expressed as mean±SEM, and were analyzed by the paired *t* test, or one-way ANOVA followed by least-significant difference test. Significance was reached at values of $P<0.05$ or $P<0.01$.

2 Results

2.1 Zacopride does not affect the I_{K1} of rat atrial myocytes

Original I_{K1} current tracings (Figure 1A) and the respective current-voltage (*I-V*) curves (Figure 1B) were obtained from isolated atrial myocytes with or without 1 μmol L^{-1} zacopride. Zacopride did not significantly modify the inward (at −110 mV; $n=6$) or outward (at −50 mV; $n=6$) components of the I_{K1} current. Application of zacopride did not affect the RMP or APD ($n=5$) (Figure 1C). These results indicate that zacopride does not affect the atrial I_{K1} and action potential.

2.2 Kir2.1 and Kir2.3 are the major Kir2.x isoforms in rat ventricles and atria

Western immunoblots and densitometric analysis revealed that despite the expression of all Kir2.x isoforms in the ventricles and atria, Kir2.1 was the isoform predominantly expressed in the ventricles, whereas Kir2.3 was the major isoform in the atria (Figure 1D). Kir2.3 was similarly expressed in both the atria and ventricles. However, level of Kir2.1 in the atria was only 25% of that in the ventricle.

2.3 Zacopride activates Kir2.1 but not Kir2.2 or Kir2.3 channels in HEK293 cells

Because zacopride affects I_{K1} only in the ventricles, and the compositions of Kir2.x tetramers are different between atria and ventricles [10,11], we thus hypothesized that zacopride may be selective for one of the Kir2.x channels. Results indicated that zacopride significantly ($P<0.01$) increased the outward component of $I_{Kir2.1}$ by 40.7%±9.7% at −50 mV compared with baseline ($n=6$) (Figure 2A), but did not affect the inward component (9.6%±4.2% at −110 mV; $n=6$) (Figures 2B). The half maximal effective concentration was determined as 30.7 μmol L^{-1}.

Despite its significant ($P<0.01$) agonist effect on Kir2.1 channels (Figure 3A), zacopride did not affect the currents of Kir2.2 (3.6%±6.4% at −50 mV; $n=5$) (Figure 3B) or Kir2.3 (1.9%±0.5% at −50 mV; $n=4$) (Figure 3F). Several reports have concluded that both heteromeric and homomeric assembly of Kir2.x channels are the basis of native I_{K1} [16–18]. We have previously observed the effects of zacopride on cells co-transfected with heteromers of Kir2.1/Kir2.2, Kir2.2/Kir2.3, or Kir2.1/Kir2.3 [19]. Our results showed that zacopride did not induce an effect on the inward or outward components of the heteromeric channels, Kir2.1/Kir2.2, Kir2.2/Kir2.3, or Kir2.1/Kir2.3 (Figures 3D–F, respectively). These results suggested that zacopride exerted a significant agonist effect on only Kir2.1 homomeric channels.

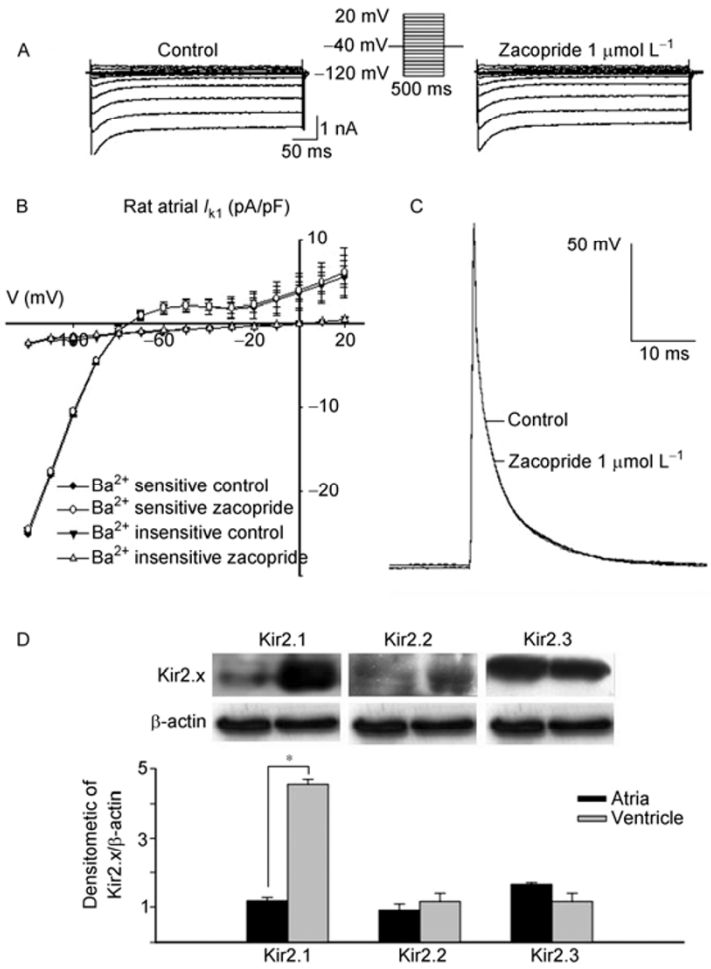

Figure 1 Whole cell recordings of I_{K1} currents and the expression profile of Kir2.x in rat atria and ventricles. A, Atrial I_{K1} current traces recorded at a holding potential of −40 mV and 500 ms voltage steps ranging from −120 to 20 mV with or without zacopride (1 μmol L^{-1}). B, *I-V* curves for atrial I_{K1} before and after addition of zacopride and $BaCl_2$ (1 μmol L^{-1}). All currents were normalized for cell capacitance. C, Effect of zacopride on the AP of isolated rat atrial myocytes. D, Western immunoblots and densitometric analysis of Kir2.x proteins in rat atria and ventricles. *, P<0.05 vs. atria.

2.4 Zacopride regulates the I_{K1} channel via a 5-HT receptor-independent pathway

We next investigated the possibility that zacopride affects the Kir2.1 channel independently from native 5-HT_3 and 5-HT_4 receptors in the HEK293 cells. Western immunoblots revealed relatively low protein expression of 5-HT_4, whereas 5-HT_3 was undetectable (however, both receptors were expressed in rat brain and heart) (Figure 4).

We also examined the effect of a direct activation of 5-HT_4 receptors using PBT (selective 5-HT_4 receptor agonist) on the activation of Kir2.1 channels in HEK293 cells. Compared with baseline, 100 μmol L^{-1} PBT did not show an effect on the outward component of $I_{Kir2.1}$ at −50 mV (10.5%±4.2%; n=4) (Figure 5A). However, PBT significantly (P<0.05) decreased the inward $I_{Kir2.1}$ by 31.4%±8.6% at −110 mV (n=4) (Figure 5B), indicating that zacopride may not activate Kir2.1 via the 5-HT_4 receptor. Considering that the 5-HT_3 receptor was undetectable in HEK293 cells (Figure 4A), the above observations strongly support the selectivity of zacopride for the Kir2.1 channel, independent of the 5-HT receptor.

We next sought to determine whether 5-HT_4 receptors influenced the I_{K1} response mediated by zacopride, using RS23597-190 (5-HT_4 receptor antagonist). A mean *I-V* relationship for $I_{Kir2.1}$ was recorded in Kir2.1-transfected HEK293 cells with or without a 5 min exposure of RS23597-190, alone or with zacopride. RS23597-190 alone did not affect the $I_{Kir2.1}$ response compared with control (Figure 5C). Furthermore, RS23597-190 did not affect the enhanced response of $I_{Kir2.1}$ by zacopride (Figure 5C).

2.5 Zacopride increases Kir2.1 currents via the PKA-mediated signaling pathway

To elucidate the key signaling pathway(s) by which zacopride activates Kir2.1 channels, we investigated the effects of various protein kinase inhibitors on the zacopride-mediated responses via Kir2.1 in HEK293 cells. HEK293 cells were pretreated with each of these protein kinase in-

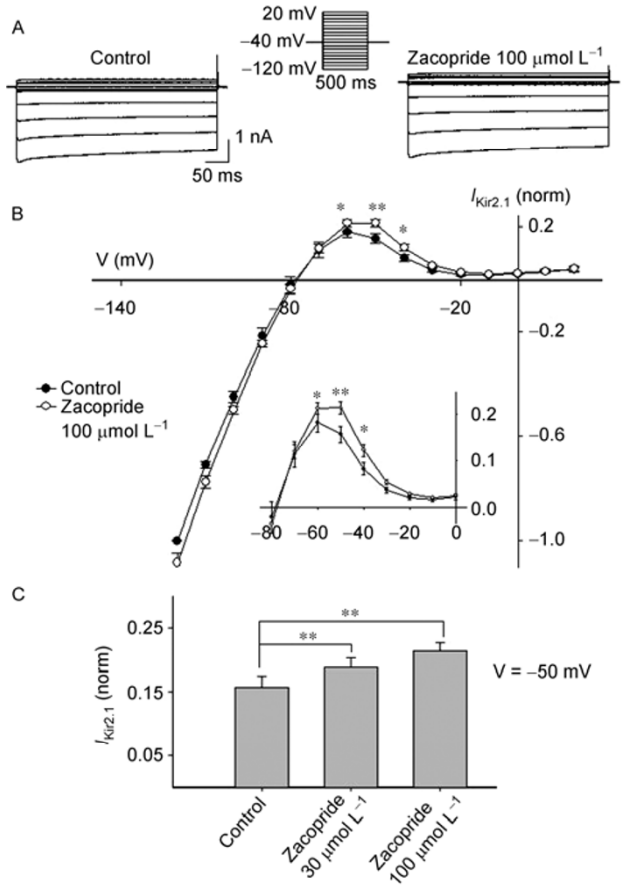

Figure 2 Zacopride increases Kir2.1 currents in transiently transfected HEK293 cells. A, Kir2.1 current traces recorded at a holding potential of −40 mV and 500 ms voltage steps ranging from −120 to 20 mV with or without zacopride. B, *I-V* curves for Kir2.1 before and after addition of zacopride, and data at potentials positive to E_K in an expanded scale (inset graph). C, Changes of $I_{Kir2.1}$ at different concentrations of zacopride measured at −50 mV. *, P<0.05; **, P<0.01 vs. control.

hibitors for 5 min before applying zacopride. Compared with baseline, zacopride alone significantly (P<0.05) increased the outward Kir2.1 current by 40.7%±9.7% at −50 mV (n=6) (Figures 2B and C). However, in the presence of KT5720, this response was diminished (Figures 2B, C and 6A) (P>0.05). In contrast, GF109203X or KT5823 did not significantly alter zacopride-mediated activation of Kir2.1 (Figures 6B and C). These results indicated that inhibition PKA activation, but not that of PKC or PKG, abolished the agonist-mediated effect of zacopride on Kir2.1 current.

We next aimed to determine the effect of adenylyl cyclase activation on the activity of Kir2.1 channels expressed in HEK293 cells. Compared with baseline, forskolin (Figure 7A) or 8-bromo-cAMP (Figure 7B) significantly enhanced Kir2.1 currents at −50 mV, by 24.1%± 5.9% (P<0.01) or 22.1%±1.9% (P<0.05), respectively. These data support our hypothesis that zacopride activates the Kir2.1 channel via a PKA-dependent pathway.

2.6 PKA-mediated phosphorylation of Kir2.1 is involved in zacopride-induced activation of Kir2.1 channels

Based on the finding that zacopride activated Kir2.1 channels via a PKA-dependent pathway, we thus hypothesized that it may induce the phosphorylation of Kir2.1 via PKA. In mutant Kir2.1 (Kir2.1(S425L)) HEK293 cells, zacopride did not significantly increase $I_{Kir2.1(S425L)}$ at −50 mV compared with control (Figure 7C). This result indicated that inhibition of the PKA phosphorylation site almost completely abolished the agonist effect of zacopride on Kir2.1. Thus, the selective activation of Kir2.1 channel by

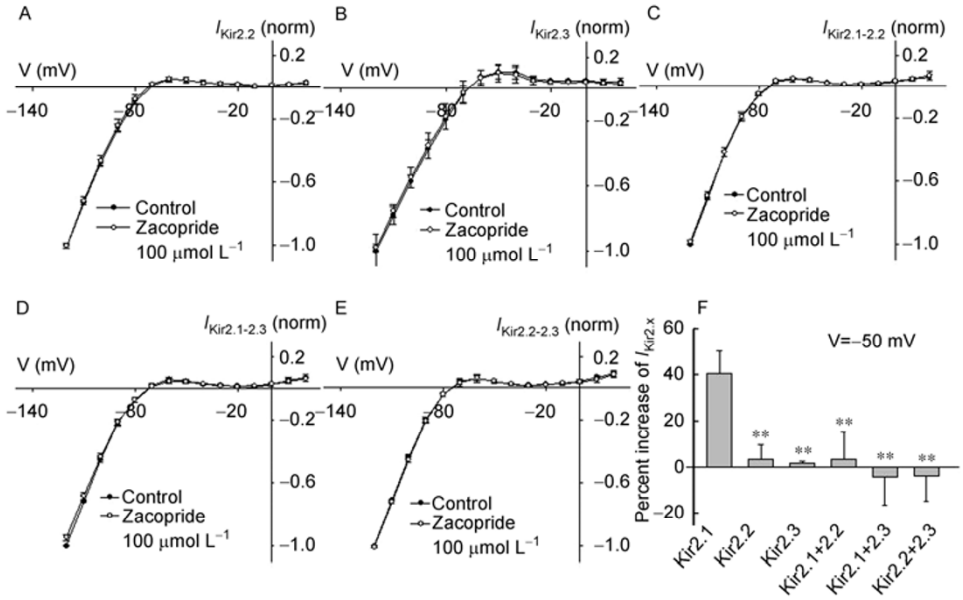

Figure 3 Zacopride does not affect Kir2.2 and Kir2.3 currents in transiently transfected HEK293 cells. *I-V* curves for the (A) Kir2.2 or (B) Kir2.3 homomeric channels, and the (C) Kir2.1-Kir2.2, (D) Kir2.1-Kir2.3, or (E) Kir2.2-Kir2.3 heteromeric channels, with or without zacopride. F, Bar graphs showing the differential effects of zacopride on Kir2.x currents measured at −50 mV (data for Kir2.1 are from Figure 2). **, P<0.01 vs. $I_{Kir2.1}$.

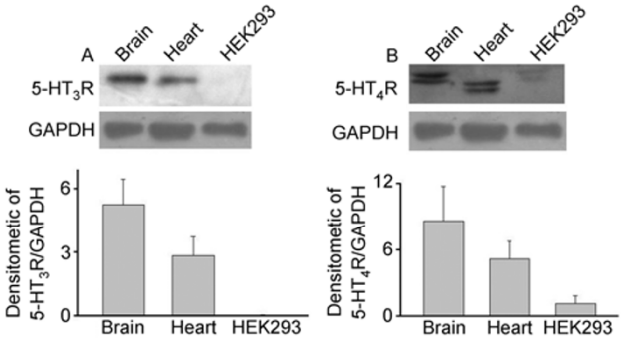

Figure 4 Protein expression profile 5-HT receptors in different tissues. Western immunoblot analysis of (A) 5-HT_3 receptor and (B) 5-HT_4 receptor in rat brain, heart, and HEK293 cells.

zacopride may occur primarily via PKA-mediated phosphorylation of this channel.

3 Discussion

In the present study, we demonstrate for the first time that zacopride, a recently approved selective I_{K1} channel agonist, increases I_{K1} current in myocytes of ventricles but not of atria. This finding is corroborated by further results showing that zacopride selectively activates the Kir2.1 channel, which was found to be the predominant isoform of I_{K1} channel in the ventricles but not atria. We also show that the agonist action of zacopride on Kir2.1 channels is via a PKA-dependent but 5-HT receptor-independent pathway, and that its induced activation of the Kir2.1 channel is predominantly via the PKA-mediated phosphorylation of Kir2.1. Overall, zacopride suppresses triggered ventricular arrhythmias while exhibiting no adverse effects on atrial electrophysiology. These findings may thus provide a deeper insight into the molecular basis underlying the unique properties of zacopride as a myocardial I_{K1} agonist.

Our group has recently demonstrated that zacopride prevents or eliminates aconitine (toxin)-induced ventricular arrhythmias in rat, because of the enhancement of I_{K1} [3]. Activation of atrial I_{K1} current is well known to be a potential cause of AF [7–9]. Therefore, potential adverse effects on atrial electrophysiology should be considered during zacopride treatment for triggered ventricular arrhythmias. However, the present study showed that in contrast to its significant effects on the RP and APD in ventricular myocytes, zacopride did not modify the RP and APD of rat atrial myocytes. Tissue-specific activation of I_{K1} by zacopride may thus account for the above differential actions on ventricles and atria, and this may further explain why zacopride did not induce AF in our previous study [3]. Tissue and species-specific expression profiles of Kir2.x isoforms have determined the biophysical and regulatory properties of I_{K1} in the heart [10,11]. Western immunoblots of the present study showed that all Kir2x isoforms were expressed in the rat heart, with Kir2.1 and Kir2.3 the predominant isoform expressed in the ventricle and atria, respectively. However, atrial Kir2.1 protein level was only 25% of that measured in the ventricle. Several reports have concluded that both heteromeric and homomeric assemblies of Kir2.x channels are the basis of native I_{K1} [16–18]. In the present study, zacopride selectively activated Kir2.1 but not Kir2.2 or

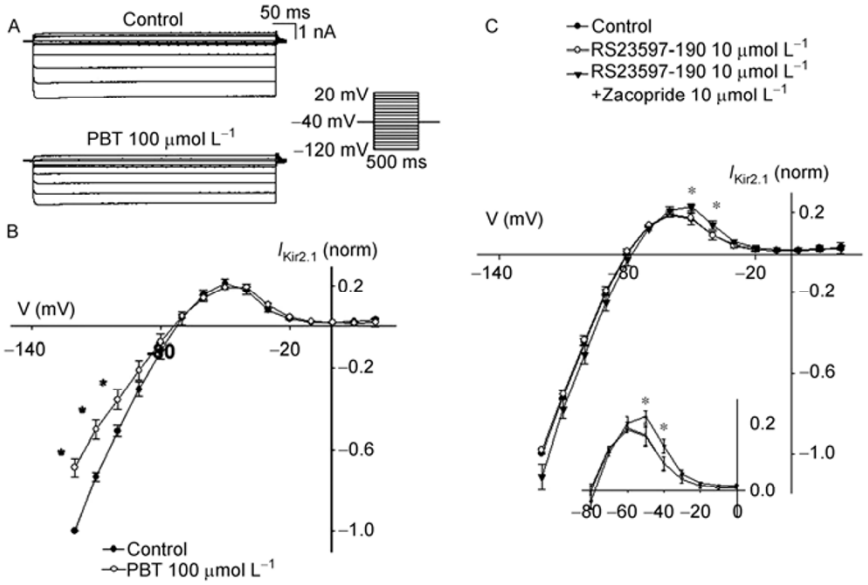

Figure 5 Function of 5-HT receptors in the I_{K1}-agonist effect by zacopride in HEK293 cells. A, Kir2.1 current traces recorded in transiently transfected HEK293 cells at –40 mV and 500 ms voltage steps ranging from –120 to 20 mV with or without PBT (100 μmol L^{-1}). B and C, Respective *I-V* curves for $I_{Kir2.1}$ for 5-HT_4 receptor agonist, 5-HT_3 receptor antagonist and zacopride treatments, and data at potentials positive to E_K in an expanded scale (inset graph). *, $P<0.05$ vs. control.

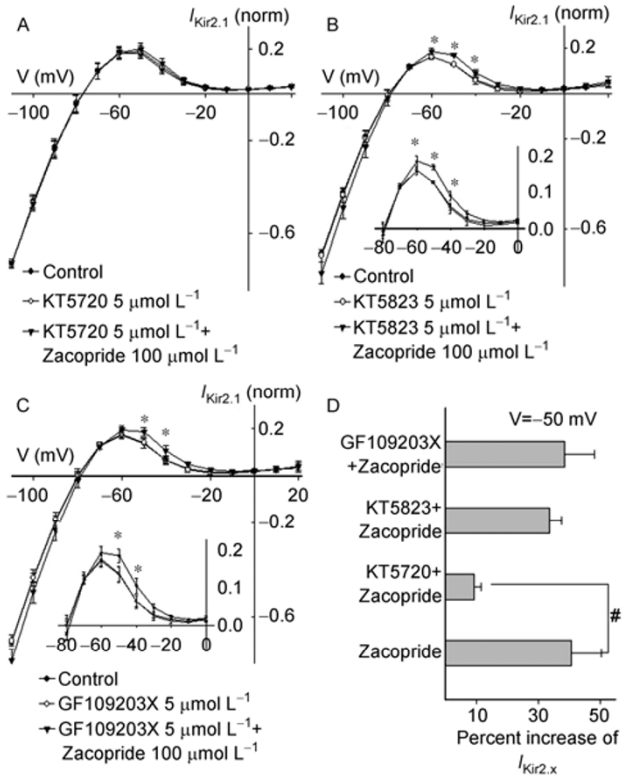

Figure 6 Zacopride increases Kir2.1 currents via a PKA-mediated signaling pathway. *I-V* curves for $I_{Kir2.1}$ recorded in HEK293 cells before and after a 5 min exposure to (A) KT5720 or KT5720 (both 5 μmol L^{-1}) with zacopride, (B) KT5823 or KT5823 (both 5 μmol L^{-1}) with zacopride, or (C) GF109203X or GF109203X (both 5 μmol L^{-1}) with zacopride. Inset graph, data at potentials positive to E_K in an expanded scale. D, Outward increment of $I_{Kir2.1}$ following application of protein kinase inhibitors with zacopride. *, P<0.05 vs. protein kinase inhibitors alone; #, P<0.05 vs. zacopride alone.

Kir2.3, and this effect was only found in Kir2.1 homotetrameric channels. Therefore, zacopride-mediated effect on I_{K1} may only occur in those species and/or tissues in which I_{K1} is mainly generated by Kir2.1 homotetrameric channels. Our present pharmacological findings suggest that rat atrial I_{K1} is mainly generated by Kir2.3 homotetrameric channels and/or Kir2.x (including Kir2.1) heterotetrameric channels. Furthermore, these channels are predominantly unaffected by zacopride, and thus may explain the increase of I_{K1} in only ventricles.

Our previous study using zacopride revealed that 1 μmol L^{-1} was the optimal concentration to increase both the inward and outward components of the I_{K1} in rat ventricular myocytes [3]. However, in the present study, the effect of zacopride on Kir2.1 was different to the I_{K1} of native Kir2x in cardiomyocytes. First, the concentration of zacopride to achieve a maximal efficacy for the activation of Kir2.1 was much higher in HEK293 cells (100 μmol L^{-1}) than in ventricular myocytes (1 μmol L^{-1}) [3]. Second, our current findings showed that zacopride enhanced only the outward $I_{Kir2.1}$ in HEK293 cells, whereas it enhances both inward and outward components of I_{K1} in native ventricular myocytes [3]. One possibility for this phenomenon is that the heterologous expression system may not reproduce the sophisticated cellular environment of native cells [20], including the accessory subunits and regulatory proteins of the I_{K1} channel that are linked to the intracellular cytoskeleton and to the extracellular matrix. Although the effects of these accessory proteins do not completely change the direct action of drugs, care must be taken when extrapolating the data obtained from heterologous expression systems to native cells [21]. A previous study suggests that Kir2.1 interacts with A kinase-anchoring protein 79 (AKAP79), and the presence of AKAP79 enhances the response of Kir2.1 to elevated intracellular cyclic adenosine monophosphate (cAMP) [22]. Therefore, in the present study, AKAP79 protein expression (via Western immunoblotting) was examined in HEK293 cells and rat heart. Results showed that the expression level of AKAP79 was 4-fold higher in the rat ventricle than in HEK293 cells (data not shown). This result may help explain the higher sensitivity of zacopride to native (i.e., ventricular) cells than HEK293 cells in I_{K1} channel-PKA signaling.

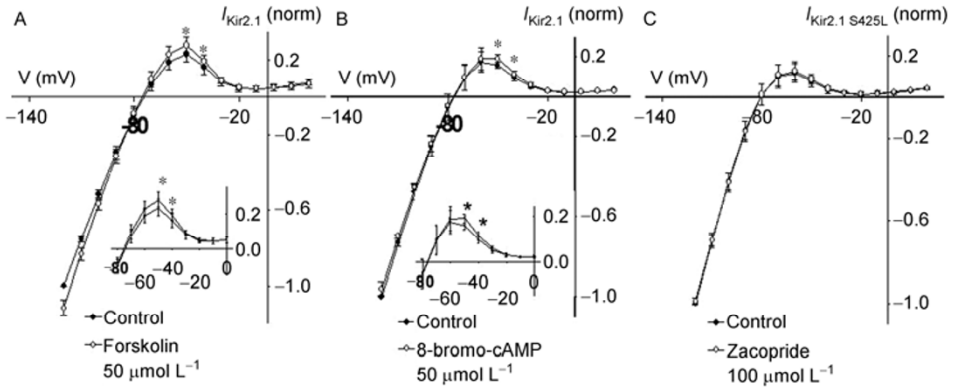

Figure 7 PKA-mediated phosphorylation of Kir2.1 is involved in zacopride-induced activation of Kir2.1 channels. *I-V* curves for $I_{Kir2.1}$ before and after addition of (A) forskolin (50 μmol L^{-1}), or (B) 8-bromo-cAMP (50 μmol L^{-1}). Inset graph, data at potentials positive to E_K in an expanded scale. C, *I-V* curves for $I_{Kir2.1\ S425L}$ before and after addition of zacopride. *, P<0.05 vs. control.

Zacopride may enhance Kir2.1 currents by blocking 5-HT_3 receptors or activating 5-HT_4 receptors via its antagonist or agonist effects, respectively. However, the present study has shown that in HEK293 cells, endogenous 5-HT_3 receptors are not expressed, while endogenous 5-HT_4 is expressed at very low levels. Our findings also indicated that PBT (5-HT_4 receptor agonist) did not affect the outward Kir2.1 component but inhibited the inward Kir2.1 current. In addition, RS23597-190 (5-HT_4 receptor antagonist), did not affect zacopride-mediated activation of Kir2.1. These findings strongly suggest that zacopride regulates I_{K1} via a 5-HT receptor-independent pathway.

The Kir2.1 channel has been shown to be differentially regulated by protein kinases, including PKA and PKC [12]. Furthermore, the Kir2.1 channel is rather sensitive to PKA-dependent regulation [10,12,15]. In the present study, blocking PKA signaling inhibited zacopride-mediated enhancement of the Kir2.1 current, and PKC or PKG inhibition did not have an effect. Therefore, the results suggested that enhancement of Kir2.1 current by zacopride may be PKA-dependent. Recent studies have provided inconsistent results regarding the effect of PKA on modulating the activity of the Kir2.1 channel. For example, cAMP was shown to increase Kir2.1 currents in CHO cells [22], and activation of PKA resulted in an inhibitory effect on Kir2.1 and homomeric Kir2.1 currents, in xenopus oocytes [23] and COS-7 cells [15], respectively. Our current findings revealed that forskolin or 8-bromo-cAMP activated Kir2.1 in HEK293 cells. Overall, these discrepancies may, at least in part, result from different heterologous expression systems used, because numerous model lines may differ in their expression of proteins or in the cross-talk of signaling pathways.

Modulation of ion channel activity through protein phosphorylation is an important physiological control mechanism. In Kir2.1, Kir2.2 and Kir2.3 subunits, the PKA phosphorylation motif is present at the serine residue near the COOH terminus [12,24]. The present study revealed that the activation of the $Kir2.1_{S425L}$ channel by zacopride was lost, thereby providing further solid evidence that the $I_{Kir2.1}$-enhancing effect of zacopride was dependent on the phosphorylation of Kir2.1 at the single PKA consensus site (ser-425). However, this study was not able to detect the membrane receptor of zacopride. Future studies may help resolve this issue.

In conclusion, the present study demonstrates that zacopride selectively activates the homomeric Kir2.1 channel via a PKA-mediated signaling pathway. This finding may suggest an important mechanism by which zacopride exerts an I_{K1} agonist effect in the ventricle but not atrium. This unique feature of zacopride suggests that its potential risk to induce AF may be overestimated when used as a drug against ventricular arrhythmia. Therefore, this study may shed light on the development of I_{K1} channel agonists as anti-arrhythmia drugs.

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