

# Beta-adrenergic stimulation reverses the $I_{K1}$ – $I_{Ks}$ dominant pattern during cardiac action potential

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**Abstract**  $\beta$ -Adrenergic stimulation differentially modulates different  $K^+$  channels and thus fine-tunes cardiac action potential (AP) repolarization. However, it remains unclear how the proportion of  $I_{Ks}$ ,  $I_{Kr}$ , and  $I_{K1}$  currents in the same cell would be altered by  $\beta$ -adrenergic stimulation, which would change the relative contribution of individual  $K^+$  current to the total repolarization reserve. In this study, we used an innovative AP-clamp sequential dissection technique to directly record the dynamic  $I_{Ks}$ ,  $I_{Kr}$ , and  $I_{K1}$  currents during the AP in guinea pig ventricular myocytes under physiologically relevant conditions. Our data provide quantitative measures of the magnitude and time course of  $I_{Ks}$ ,  $I_{Kr}$ , and  $I_{K1}$  currents in the same cell under its own steady-state AP, in a physiological milieu, and with preserved  $Ca^{2+}$  homeostasis. We found that isoproterenol treatment significantly enhanced  $I_{Ks}$ , moderately increased  $I_{K1}$ , but slightly decreased  $I_{Kr}$  in a dose-dependent manner. The dominance pattern of the  $K^+$  currents was  $I_{Kr} > I_{K1} > I_{Ks}$  at the control condition, but reversed to  $I_{Kr} < I_{K1} < I_{Ks}$  following  $\beta$ -adrenergic stimulation. We systematically

determined the changes in the relative contribution of  $I_{Ks}$ ,  $I_{Kr}$ , and  $I_{K1}$  to cardiac repolarization during AP at different adrenergic states. In conclusion, the  $\beta$ -adrenergic stimulation fine-tunes the cardiac AP morphology by shifting the power of different  $K^+$  currents in a dose-dependent manner. This knowledge is important for designing antiarrhythmic drug strategies to treat hearts exposed to various sympathetic tones.

**Keywords** Cardiac · Myocyte · Potassium channel · Beta-adrenergic · Calcium · Action potential

## Introduction

Cardiac action potential (AP) is *fine-tuned* by adrenergic tone. Extensive studies have shown that  $K^+$  channels essential for the cardiac AP repolarization are intricately regulated by  $\beta$ -adrenergic stimulation, and different  $K^+$  channels— $I_{Ks}$ ,  $I_{Kr}$ , and  $I_{K1}$ —show different sensitivities to  $\beta$ -adrenergic stimulation [10, 25, 26, 28]. In all previous studies, however,  $I_{Ks}$ ,  $I_{Kr}$ , and  $I_{K1}$  were each recorded from different cells and using different V-clamp conditions (i.e., voltage protocol, ionic composition,  $Ca^{2+}$  buffering). Thus, it remains unknown how  $\beta$ -adrenergic stimulation *coordinately* regulates all these  $K^+$  currents in the same cell during the AP, nor is it clear how various  $\beta$ -adrenergic states may change the relative contribution of each  $K^+$  channel to the total repolarization reserve. Yet, such knowledge is essential for designing antiarrhythmic strategies using specific  $K^+$  channel blockers. Recently, we have developed an innovative AP-clamp sequential dissection (called “onion-peeling”) method that gives us unprecedented ability to measure multiple ionic currents during the AP in the single myocyte [1, 4]. The onion-peeling data enable us, for the first time, to analyze the proportion of different currents flowing in the same cell during the AP under physiologically relevant conditions. The first goal of this study is to determine the relative contribution of  $I_{Ks}$ ,  $I_{Kr}$ , and  $I_{K1}$  to the

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AP repolarization in response to various extent of  $\beta$ -adrenergic stimulation; such in-depth knowledge is important for understanding how cardiac APs are altered under various sympathetic tones during exercise, stress, or diseases.

$\beta$ -Adrenergic stimulation can affect the  $K^+$  channels directly and indirectly. Downstream from  $\beta$ -adrenergic stimulation, activation of the cyclic AMP-dependent protein kinase A (PKA) causes phosphorylation of many ion channels and  $Ca^{2+}$  handling proteins. PKA phosphorylation of  $K^+$  channels directly modifies the magnitude and the kinetics of  $K^+$  currents [10]. Meanwhile, PKA phosphorylation of  $Ca^{2+}$  handling proteins such as the ryanodine receptor, the sarcoplasmic reticulum  $Ca^{2+}$  pump, and the  $Ca^{2+}$ -calmodulin-dependent protein kinase II (CaMKII) can alter the  $Ca^{2+}$  homeostasis of cardiac myocytes [9]. Altered  $Ca^{2+}$  homeostasis can exert a secondary effect to alter the  $K^+$  currents because  $K^+$  channels are sensitive to  $Ca^{2+}$ /CaMKII modification. In order to understand the full impact of  $\beta$ -adrenergic stimulation on modulating the  $K^+$  currents and AP repolarization, we need to maintain physiologic  $Ca^{2+}$  cycling during the AP. However, most of our current knowledge on  $\beta$ -adrenergic modulation of  $K^+$  currents is based on the V-clamp data obtained when the intracellular  $Ca^{2+}$  was buffered by exogenous  $Ca^{2+}$  buffers (ethylene glycol tetraacetic acid—EGTA or 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid—BAPTA). In 1998, Zaza et al. [30] conducted an elegant study to show that  $Ca^{2+}$  can reduce  $I_{K1}$  during the AP in ventricular myocytes. Since then, many studies found  $Ca^{2+}$  sensitivity of other  $K^+$  currents [8, 10, 22]. The data from these studies suggest that the  $Ca^{2+}$  transient during the AP can significantly modify the  $K^+$  currents. Nonetheless, the early experiments still used 1 mM EGTA in the pipette solution, with the assumption that low EGTA concentration might not interfere with  $Ca^{2+}$  homeostasis [22, 30]. Contrary to this assumption, we found that EGTA at 1 mM almost eliminated the  $Ca^{2+}$  transient during AP; hence, the data from the previous studies need to be reinterpreted. The second goal of this study is to determine the full impact of  $\beta$ -adrenergic stimulation on modulating the  $K^+$  currents during the AP with normal cycling  $Ca^{2+}$  under physiologically relevant condition. The overarching goal is to systematically determine the changes in the relative contribution of  $I_{Ks}$ ,  $I_{Kr}$ , and  $I_{K1}$  to cardiac repolarization during AP at different adrenergic states under physiologically relevant conditions.

This knowledge is important for designing effective and safe therapeutic strategies using K channel inhibitors (Singh–Williams class III antiarrhythmic drugs) to treat hearts exposed to various sympathetic tones.

## Methods

All laboratory procedures in this study conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health, the Guide for the Care and

Use of Laboratory Animals laid out by Animal Care Committee of the University of California (UC). The animal use was approved by the UC Davis Institutional Animal Care and Use Committee (IACUC, protocol #16914).

## Cell isolation

Hartley guinea pigs (male, 3–4 months old, purchased from Charles River Laboratories USA) were first injected with heparin (800 U, i.p.) and then anesthetized with nembutal (100 mg/kg, i.p.). After achieving deep anesthesia to suppress spinal cord reflexes, a standard enzymatic technique was used to isolate ventricular myocytes [3].

## Electrophysiology

Cells were continuously superfused with a modified Tyrode solution supplemented with bicarbonate (BTy) containing (in mmol/L) NaCl 120, KCl 5,  $CaCl_2$  2,  $MgCl_2$  1, HEPES 10,  $NaHCO_3$  25, and glucose 10. pH was set to 7.3. BTy was kept in glass flasks with airtight cap and used within 6 h after preparation; previous tests confirmed that there was no pH shift within this period. The pipette solution contained (in mmol/L) K-aspartate 115, KCl 45, Mg-ATP 3, HEPES 5, and cAMP 0.1; pH was set to 7.25 using KOH. Borosilicate glass pipettes were fabricated with Sutter (Sutter Instrument Company, Novato CA, USA) laser puller having resistance of 1.8–2.5 M $\Omega$  after filling with pipette solution. Experiments were recorded using Axopatch 200B Amplifier, DigiData 1440A Analog/Digital Converter, and pClamp10 software (Molecular Devices, Sunnyvale CA, USA). Series resistance of the pipette and input resistance of the cell were fully compensated. Cell capacitance compensation was 80 %. The access resistance was continuously monitored during the experiment and only cells having constant access resistance (< 5 MOhm) were used for analysis.

The self-AP-clamp sequential dissection (called “onion-peeling” from here on) experiments were conducted as described in our previous publication [1]. Briefly, after establishing the ruptured patch whole-cell clamp configuration, the cell was paced at 1 Hz frequency under I-clamp mode to reach the steady-state action potential. The cell’s steady-state AP was recorded. After switching to V-clamp mode, this AP waveform was applied as voltage command onto the same cell at 1 Hz frequency. After recording “zero current,” specific ion channel blockers were applied sequentially and compensation current recorded. The  $K^+$  currents were obtained using the following specific inhibitors: 1  $\mu$ M chromanol 293B was used to obtain  $I_{Ks}$ ; 1  $\mu$ M E4031 for  $I_{Kr}$ ; and 50  $\mu$ M  $Ba^{2+}$  for  $I_{K1}$ , respectively. A number of earlier studies have shown that each blocker is highly specific at the concentrations used. For studying the dose-dependent  $\beta$ -adrenergic stimulation effects, the cells were exposed to isoproterenol at the given concentration throughout the entire onion-peeling experiments.

[Ca<sup>2+</sup>]<sub>i</sub> measurement

The intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) was measured using Fura-2 ratiometric method [3]. Briefly, Fura-2 K<sup>+</sup> salt was added into the pipette solution at a concentration of 20 μM and diffused into the cytosol through the ruptured patch during paced cell contraction to reach steady state. The IonOptix system (IonOptix Inc. USA) with dual excitation at 340 and 380 nm and single emission >510 nm (through emission filter 510–645 nm) was used to measure the Fura-2 fluorescence ratio. The IonOptix system was synchronized with the electrophysiology setup to simultaneously measure the [Ca<sup>2+</sup>]<sub>i</sub> and the electric signals.

## Statistical analysis

The numerical values are calculated for the mean value, the standard deviation (SD), and the standard error of mean (SEM). The mean±SEM values are shown in the bar charts

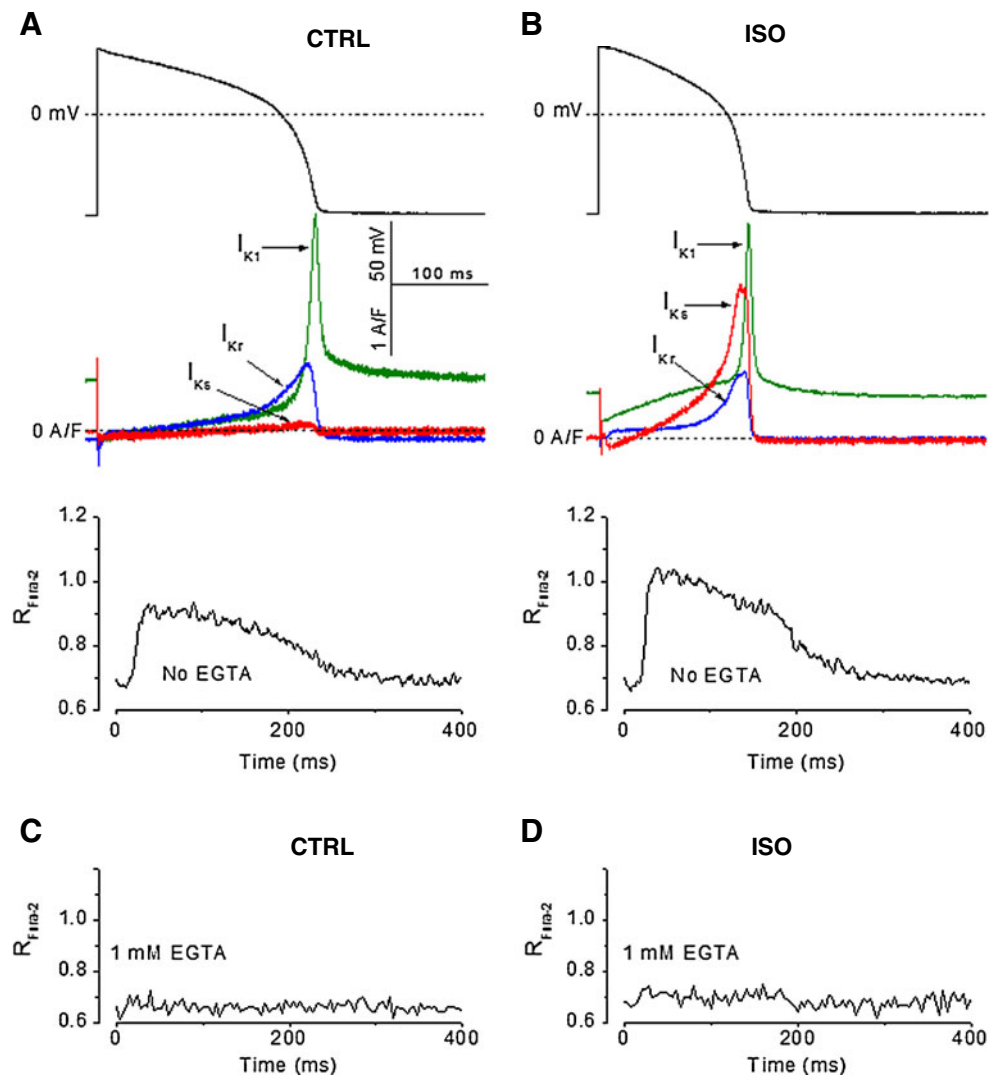
in the figures. The mean±SD values are reported in the text. The number of cells and the number of animals in each experimental group were reported in the legends. Statistical significance of the difference between different groups was evaluated using Student's *t* test and deemed significant if *p*<0.05.

## Results

Recording of three major K<sup>+</sup> currents during the AP with Ca<sup>2+</sup> cycling in the single myocyte

We used the onion-peeling technique to record the ion currents that are naturally flowing during the AP in the guinea pig ventricular myocyte when the cell is undergoing normal excitation–contraction coupling under physiological condition and following β-adrenergic stimulation. Figure 1a demonstrates a typical onion-peeling experiment in which we

**Fig. 1** β-Adrenergic stimulation effects on the AP, the three K<sup>+</sup> currents, and the Ca<sup>2+</sup> transient. **a**, **b** AP-clamp sequential dissection experiments to directly record the steady-state AP (*upper panel*) at 1 Hz pacing rate, the three K<sup>+</sup> currents (*mid panel*) in the same cell, and the Ca<sup>2+</sup> transients (*lower panel*) under physiological condition (CTRL) and following 30 nM isoproterenol (ISO) treatment. Note that the Ca<sup>2+</sup> transient during the AP is preserved by having the endogenous Ca<sup>2+</sup> buffers and without adding any exogenous Ca<sup>2+</sup> buffer (no EGTA) in pipette solution. **c**, **d** Ca<sup>2+</sup> transients were largely eliminated by using 1 mM EGTA in the pipette solution under both the control condition and following ISO treatment



recorded three major  $K^+$  currents— $I_{Ks}$ ,  $I_{Kr}$ , and  $I_{K1}$ —during the AP in the same cell. The steady-state AP (upper panel) was recorded under I-clamp mode, with 1 Hz pacing frequency, at body temperature ( $36\pm0.3^\circ\text{C}$ ). Then this AP waveform was used as the voltage command under V-clamp mode to record the ion currents flowing under the AP. The  $I_{Ks}$ ,  $I_{Kr}$ , and  $I_{K1}$  currents (middle panel) were pharmacologically dissected out one-by-one from the same cell by sequentially adding chromanol 293B 1  $\mu\text{M}$ , E4031 1  $\mu\text{M}$ , and  $\text{Ba}^{2+}$  50  $\mu\text{M}$ . The data show that the intracellular  $\text{Ca}^{2+}$  transient during the AP cycle (lower panel) was preserved under our experimental conditions.

Next, we studied the effects of  $\beta$ -adrenergic stimulation on fine-tuning the three  $K^+$  channels by using isoproterenol at 3, 10, and 30 nM concentrations. As shown in Fig. 1b, isoproterenol shortened the AP duration (upper panel) and differentially modified the profiles of the  $I_{Ks}$ ,  $I_{Kr}$ , and  $I_{K1}$  currents during the AP (middle panel, see below for detailed analysis). Isoproterenol also slightly increased the amplitude of the  $\text{Ca}^{2+}$  transient (lower panel), which should contribute to the  $\text{Ca}^{2+}$ -dependent changes in the  $K^+$  currents during the AP with  $\text{Ca}^{2+}$  cycling.

#### Preserving $\text{Ca}^{2+}$ homeostasis during self-AP-clamp by eliminating exogenous $\text{Ca}^{2+}$ buffer

In order to understand the full impact of  $\beta$ -adrenergic stimulation on altering the  $K^+$  currents during AP through PKA phosphorylation and  $\text{Ca}^{2+}$ /CaMKII signaling, we designed experimental conditions to preserve the  $\text{Ca}^{2+}$  transient during the AP cycle by eliminating exogenous  $\text{Ca}^{2+}$  buffer. In literature, pioneering studies aimed at measuring  $K^+$  currents while preserving  $\text{Ca}^{2+}$  signaling still used 0.5–1.0 mM EGTA in the pipette solution, assuming that low concentrations of EGTA would not interfere with  $\text{Ca}^{2+}$  transients [22, 30]; however, the actual  $\text{Ca}^{2+}$  concentration was not measured in those early experiments. We conducted experiments to simultaneously record the  $\text{Ca}^{2+}$  transient and ion currents during sAP-clamp. The result shows that having 0.5–1 mM EGTA in the pipette solution largely eliminated the  $\text{Ca}^{2+}$  transient after pacing at 1 Hz to reach steady state (Fig. 1c); the same result was seen in the presence of 30 nM isoproterenol (Fig. 1d). We also observed that the  $\text{Ca}^{2+}$  transient during AP was high at the beginning of pacing, but gradually declined during pacing, and then diminished after reaching steady state (shown in Fig. 1c, d). Furthermore, our earlier experiments [1] using 10 mM EGTA in the pipette solution caused the  $\text{Ca}^{2+}$  transient to rapidly decline during pacing. Retrospectively, this is not surprising because EGTA should diffuse into the cell and gradually buffer the cytosolic  $\text{Ca}^{2+}$  while the cell is being paced; the speed of  $\text{Ca}^{2+}$  buffering is slowed with low EGTA concentration, but eliminates the  $\text{Ca}^{2+}$  transient at steady state nonetheless. It is noteworthy that the  $\text{Ca}^{2+}$  transient is preserved in our sAP-clamp experiments by eliminating exogenous  $\text{Ca}^{2+}$  buffer in the pipette solution. We surmise that since the myocyte cell

membrane (size  $\sim 150\times 40\times 30\ \mu\text{m}$ ) is substantially larger than the pipette tip (diameter  $\sim 1\ \mu\text{m}$ ) and the ion channels and transporters are functioning normally while being paced under the cell's own steady-state AP, the myocyte should maintain its ionic homeostasis in our sAP-clamp experiments. The fact that the myocyte experiences its natural state of excitation–contraction coupling (with AP,  $\text{Ca}^{2+}$  transient, and contraction) distinguishes our sAP-clamp experiments from the traditional V-clamp experiments using simplified conditions (i.e., rectangular voltage waveform, ion substitution, exogenous  $\text{Ca}^{2+}$  buffer) that disrupt the ionic homeostasis and  $\text{Ca}^{2+}$  transient.

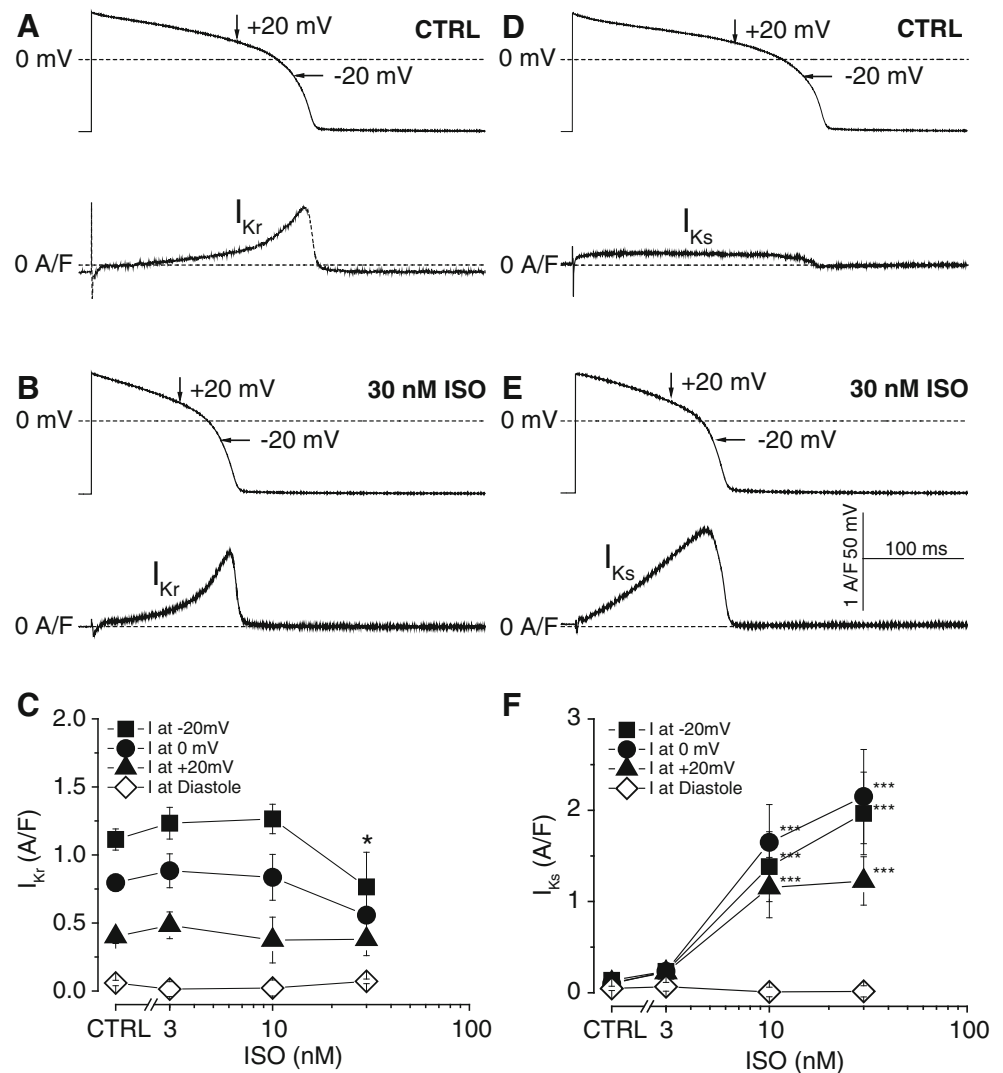
#### $\beta$ -Adrenergic stimulation effect on $I_{Kr}$ during the AP with $\text{Ca}^{2+}$ cycling

Figure 2a shows the profile of E4031-sensitive  $I_{Kr}$  current during the AP with  $\text{Ca}^{2+}$  cycling. The current density (normalized to the cell capacitance) of  $I_{Kr}$  was 0 during diastole, remained small during the AP phases 1 and 2, increased rapidly during the AP phase 3, peaked at the end of phase 3, and then declined rapidly back to the diastolic level. The effect of  $\beta$ -adrenergic stimulation on the  $I_{Kr}$  current was subtle and only seen at high isoproterenol concentration, albeit a faster time course in correspondence to a shorter AP duration (Fig. 2b). Neither the peak current density nor the profile of  $I_{Kr}$  during AP was altered by isoproterenol at low concentrations of 3–10 nM. Isoproterenol at 30 nM did not significantly alter the  $I_{Kr}$  current density during the plateau phase at +20 mV, but caused a reduction of  $I_{Kr}$  during the repolarizing phase as seen at 0 and –20 mV membrane potentials (Fig. 2c). Isoproterenol concentration higher than 30 nM routinely evoked afterdepolarizations in the myocytes and therefore was not suitable for conducting AP-clamp experiments. Our data reveal that the  $I_{Kr}$  current during the AP was largely insensitive to isoproterenol at physiological concentrations of 3–30 nM. In comparison, many previous V-clamp studies used maximal concentrations of isoproterenol ranging from 100 nM to 10  $\mu\text{M}$ .

#### Dose-dependent $\beta$ -adrenergic tuning of $I_{Ks}$ during the AP with $\text{Ca}^{2+}$ cycling

In the absence of  $\beta$ -adrenergic stimulation, the chromanol 293B-sensitive  $I_{Ks}$  was seen as a tiny and slow current throughout the AP in the guinea pig ventricular myocyte (Fig. 2d). The  $I_{Ks}$  current was 0 during diastole, built up slowly during the AP phases 1 and 2, reached a peak value at the end of phase 2 (near 0 mV membrane potential), and then declined rapidly during phase 3 in correspondence to AP repolarization. Isoproterenol treatment caused significant changes in  $I_{Ks}$  throughout the AP (Fig. 2e). The magnitude of  $I_{Ks}$  was augmented by isoproterenol in a dose-dependent manner, with a slight increase at 3 nM isoproterenol, and a substantial increase from the control value of  $0.152\pm0.027$

**Fig. 2** Dose-dependent  $\beta$ -adrenergic tuning of delayed rectifier  $K^+$  currents during the AP. **a, b**  $I_{Kr}$  recorded during the cell's own AP before and after  $\beta$ -adrenergic stimulation using 30 nM ISO. In each panel, the upper trace shows the AP and the lower trace shows the corresponding current. ISO had a little effect on the amplitude of  $I_{Kr}$  at lower concentrations but slightly reduced  $I_{Kr}$  at 30 nM (**c**). **d, e** AP and  $I_{Ks}$  current traces before and after 30 nM ISO treatment, respectively. ISO increased  $I_{Ks}$  during AP in a dose-dependent manner (**f**). Each point in **c** and **f** represents averaged current values and standard error from 7 to 15 cells isolated from seven hearts. Student's *t* test *p* values: \**p*<0.05, \*\**p*<0.01, \*\*\**p*<0.001



A/F to  $2.067 \pm 0.223$  A/F in 30 nM isoproterenol (Fig. 2f). Importantly, the profile of  $I_{Ks}$  during AP following  $\beta$ -adrenergic stimulation (Fig. 2e) became similar to that of  $I_{Kr}$  (Fig. 2b) and even surpassed  $I_{Kr}$  in magnitude. The peak of the current shifted from mid plateau to phase 3 of AP. The substantial alterations in the  $I_{Ks}$  current magnitude and time course indicate a strong  $\beta$ -adrenergic control of this channel.

#### Dose-dependent $\beta$ -adrenergic tuning of $I_{K1}$ during the AP with $Ca^{2+}$ cycling

The profile of  $Ba^{2+}$ -sensitive  $I_{K1}$  current during the AP with  $Ca^{2+}$  cycling is shown in Fig. 3a. During diastole,  $I_{K1}$  was present as a sustained outward current. At the upstroke of AP, the  $I_{K1}$  had an instant reduction of the current density which is characteristic of inward rectification. During the AP phases 1 and 2, the  $I_{K1}$  current remained very small, but then shot up sharply during phase 3, reached the peak value at the end of phase 3, and then declined rapidly to return to the diastolic level.

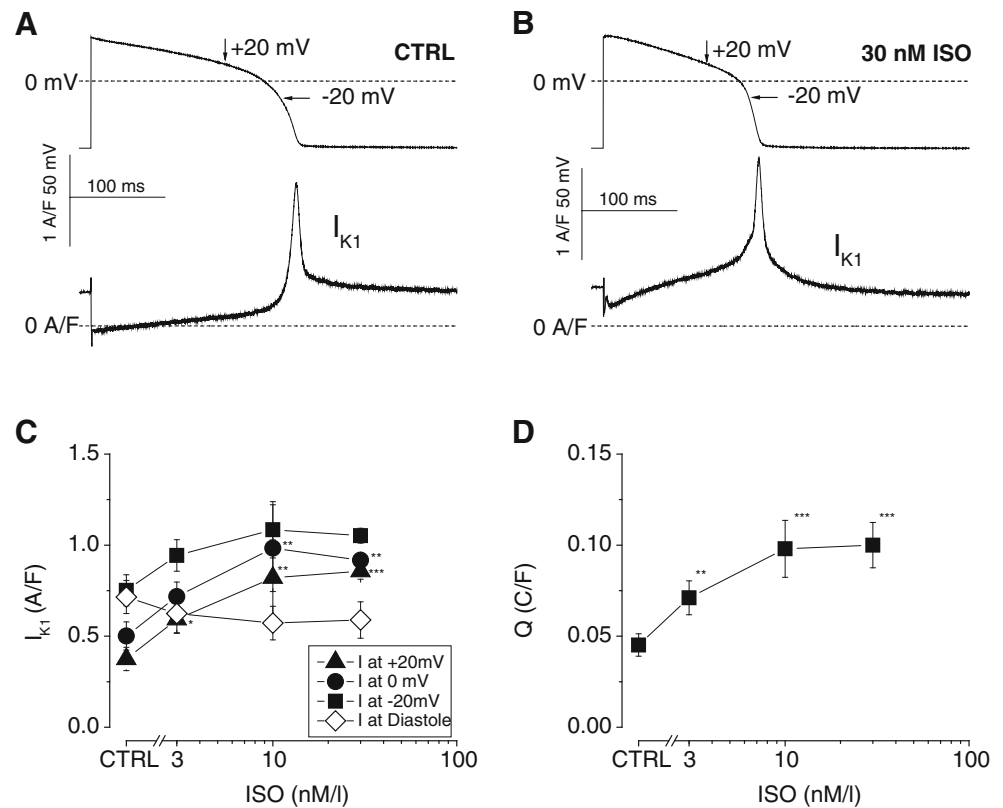
The isoproterenol effect on the  $I_{K1}$  profile seemed subtle at first glance, but quantitative analysis reveal considerable changes in several features (Fig. 3b). The average diastolic current density was not changed by isoproterenol, but the inward rectification became less obvious. Isoproterenol increased the  $I_{K1}$  current during phase 2 in a dose-dependent manner, as determined at the membrane potential of +20, 0, and -20 mV (Fig. 3c). Consequently, isoproterenol treatment significantly increased the total charge carried by  $I_{K1}$  during the AP (Fig. 3d).

$\beta$ -Adrenergic stimulation shifts the relative contribution of individual  $K^+$  currents to the total repolarization reserve

The onion-peeling method allows recording of all three  $K^+$  currents in the same cell. This enables, for the first time, analysis on how each  $K^+$  current contribute to the total repolarization current within a single cell, without the confounding effect of cell-to-cell variations. First, we calculated the sum of all three  $K^+$  currents which make up the repolarization current,



**Fig. 3** Dose-dependent  $\beta$ -adrenergic tuning of  $I_{K1}$  current. Representative traces were recorded in the absence (a) and presence (b) of 30 nM ISO. Dose-response curves for current values at different membrane potentials and total charge movement during AP are shown in c and d, respectively. ISO increased  $I_{K1}$  during AP plateau, but left diastolic current value unaltered.  $n=7-15$  cells from seven animals



and then, we calculated the proportion of each individual  $K^+$  current to the total repolarization current (or repolarization reserve) at different phases of AP. Figure 4a, b show the analysis result at +20 and -20 mV membrane potentials, respectively. These points were chosen to give representative values for the plateau and the repolarization phases of AP.

Isoproterenol treatment shifted the relative contribution of each  $K^+$  current to the repolarization reserve in a dose-dependent manner. The most striking change is a reversal of the dominance of  $I_{Kr}$  and  $I_{Ks}$ . Under the control condition in the absence of isoproterenol,  $I_{Kr}$  presents the most powerful repolarizing power, whereas  $I_{Ks}$  contributes very little, as measured at both +20 and -20 mV. With 3 nM isoproterenol, the relative contribution of  $I_{Ks}$  increases and that of  $I_{Kr}$  declines. The two become equal at 10 nM isoproterenol, and then,  $I_{Ks}$  surpassed  $I_{Kr}$  at 30 nM isoproterenol. The relative contribution of  $I_{K1}$  to the total repolarizing current did not show a significant change.

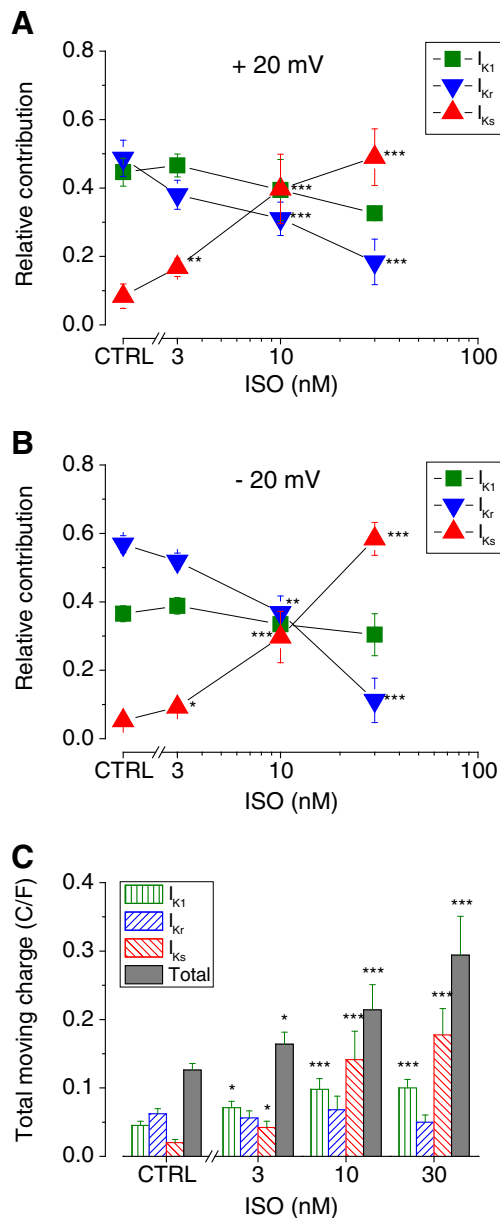
Consistent with the changes in the currents, the total charges carried by the  $K^+$  currents were also altered by isoproterenol treatment. As shown in Fig. 4c, the total  $K^+$  charge movement during the AP was increased by isoproterenol in a concentration-dependent manner. This charge increase resulted from increased  $I_{Ks}$  and  $I_{K1}$  while  $I_{Kr}$  was unaltered.

Consequently, the total outward  $K^+$  charge movement going through the three  $K^+$  currents was significantly increased

by isoproterenol treatment (Fig. 4c). Importantly, the relative contributions of  $I_{Ks}$ ,  $I_{Kr}$ , and  $I_{K1}$  change significantly with increasing isoproterenol concentration, due to different sensitivities of the individual  $K^+$  currents to  $\beta$ -adrenergic stimulation. This shift of the relative strength between the currents has profound implications on how each individual  $K^+$  channel might contribute to arrhythmogenesis at different  $\beta$  adrenergic states and how to design effective antiarrhythmia drug therapies for various pathological conditions.

$\beta$ -Adrenergic stimulation alters the effects of  $K^+$  channel blockers (class III antiarrhythmia drugs) on modifying cardiac AP

Since different  $K^+$  channels have different sensitivities to isoproterenol, it is plausible that the  $\beta$ -adrenergic state of the heart may modify the effect of  $K^+$  channel blockers on modulating the AP. To test this, we studied the effects of specific  $K^+$  channel blockers on modifying the AP duration in the absence and presence of 30 nM isoproterenol. Figure 5a, b shows that blocking  $I_{Ks}$  using 1  $\mu$ M chromanol 293B caused a moderate lengthening of action potential duration (APD) under control condition, but drastically lengthened APD in the presence of isoproterenol. In comparison, blocking  $I_{Kr}$  using 1  $\mu$ M E4031 also caused a moderate lengthening of APD in the absence of isoproterenol (Fig. 5c); however, the APD lengthening remained small in the presence of isoproterenol



**Fig. 4**  $\beta$ -Adrenergic stimulation shifts the relative contribution of individual  $K^+$  currents to the total repolarizing reserve. The three  $K^+$  currents measured in the same cell were summed, and then each current was normalized to this sum. ISO dose-response curves for normalized values measured at +20 and -20 mV are shown in **a** and **b**, respectively. **c** Total amount of  $K^+$  charge movement for the individual  $K^+$  current and the sum of the currents during the AP.  $n=7-15$  cells from seven animals

(Fig. 5d). The above difference in the  $I_{Ks}$  versus  $I_{Kr}$  blocker effect on APD is consistent with the differential regulation of  $I_{Ks}$  versus  $I_{Kr}$  by  $\beta$ -adrenergic stimulation.

## Discussion

The main goal of this study is to determine the relative contributions of three major  $K^+$  currents— $I_{Ks}$ ,  $I_{Kr}$ , and  $I_{K1}$ —

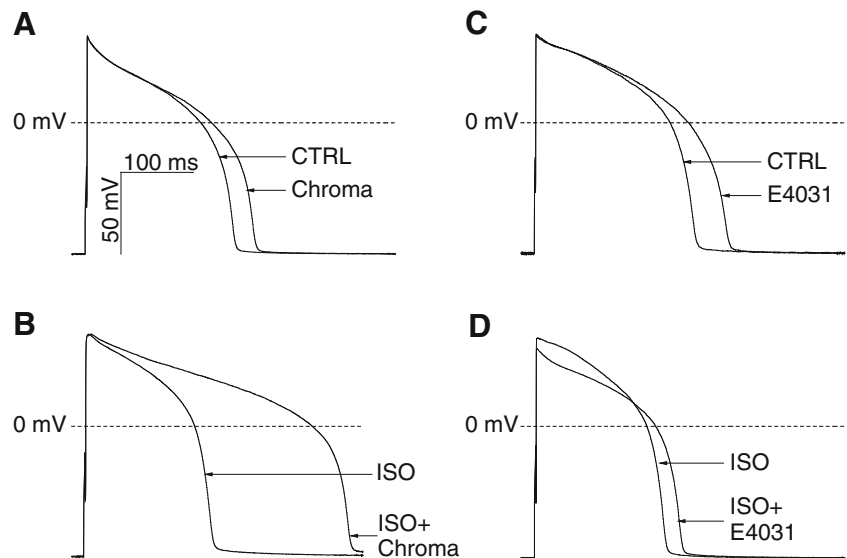
to the AP repolarization in response to various degrees of  $\beta$ -adrenergic stimulation. By our best knowledge, this is the first time these three  $K^+$  currents have been measured from the same cell and during the cardiac AP with  $Ca^{2+}$  cycling. Most previous studies used conventional V-clamp experiments to characterize the biophysical properties of  $K^+$  channels under simplified conditions; the data were then used in mathematical modeling to predict the dynamic profile of the current during AP. However, because of the simplifications used in experimental conditions and also in model assumptions, the model predictions might deviate from the physiological reality. Therefore, it is critically important to compare the model predictions with direct experimental recording of the dynamic ion currents during AP. The present study provides such experimental data for evaluating model predictions and for improving the models.

Furthermore, we systematically characterized the concentration-dependent effects of isoproterenol on modulating the three major  $K^+$  currents during cardiac AP. Our data show that isoproterenol treatment facilitates  $I_{K1}$  during the AP plateau phase, significantly increases the magnitude of  $I_{Ks}$ , but has little effect on  $I_{Kr}$ . Consequently, isoproterenol increases the contribution of  $I_{Ks}$  but decreases the contribution of  $I_{Kr}$  to the total repolarization reserve, leading to a reversal of the dominance of  $I_{Ks}$  versus  $I_{Kr}$  in repolarizing the AP (Fig. 4). Therefore, the dominant  $K^+$  current switches from  $I_{Kr}$  under the control condition to  $I_{Ks}$  under  $\beta$ -adrenergic stimulation with 30 nM isoproterenol. Such a reversal of dominance pattern has a significant implication for using specific  $K^+$  channel blockers, to treat cardiac arrhythmias.

### Effects of $\beta$ -adrenergic stimulation on $I_{Kr}$

The effects of  $\beta$ -adrenergic stimulation on  $I_{Kr}$  have been controversial in literature. Harmati et al. [10] and Heath et al. [11] reported facilitation of  $I_{Kr}$  by isoproterenol via PKA and PKC pathways in canine and guinea pig ventricular myocytes. Karle et al. [13] reported a reduction of  $I_{Kr}$  current amplitude following isoproterenol application in guinea pig ventricular myocytes. Sanguinetti et al. [23] reported no measurable isoproterenol-induced change of  $I_{Kr}$ . All of these experiments used standard V-clamp technique to measure the  $I_{Kr}$  as the tail current elicited with long square pulses in conjunction with blocking the  $I_{Ks}$  component. In addition, most previous studies used high isoproterenol concentration (1–10  $\mu$ M), whereas we used isoproterenol in the range of 3–30 nM (closer to physiological  $\beta$ -adrenergic stimulation range) because higher isoproterenol induced afterdepolarizations. In this study, we directly recorded the  $I_{Kr}$  current during the AP with  $Ca^{2+}$  cycling. The  $I_{Kr}$  profile we recorded is largely consistent with the previous model simulations of the current under the control condition [20, 31], although some quantitative differences exist. Our data

**Fig. 5**  $\beta$ -Adrenergic state alters the effects of specific  $K^+$  channel inhibitors on modifying the AP. The AP lengthening effect of using 1  $\mu$ M chromanol 293B to block  $I_{Ks}$  is moderate under control condition (**a**) but became prominent after 30 nM ISO treatment (**b**). The AP lengthening effect of using 1  $\mu$ M E-4031 to block  $I_{Kr}$  is similar under control conditions (**c**) and after ISO treatment (**d**)



demonstrate that isoproterenol did not significantly alter  $I_{Kr}$  in the concentrations below 30 nM and caused only moderate reduction of  $I_{Kr}$  at 30 nM. Our data provide the first experimental measures on the isoproterenol dose–response of  $I_{Kr}$  in the presence of cytosolic  $Ca^{2+}$  transient; these data can be used to fine-tune the quantitative models.

#### Effects of $\beta$ -adrenergic stimulation on $I_{Ks}$

$I_{Ks}$  is known to be facilitated by  $\beta$ -adrenergic stimulation according to previous V-clamp studies [10, 17, 23, 28]. Our data largely agree with the previous findings. The novel findings from our experiments is that  $\beta$ -adrenergic stimulation changes the profile of  $I_{Ks}$  during the AP (Fig. 2d, e). Under the control condition, the profile of  $I_{Ks}$  displays a small and flat current throughout the AP (Fig. 2d), similar to that seen by Rocchetti et al. [21] in their pioneering AP-clamp study. However, the isoproterenol effect on altering the  $I_{Ks}$  profile is much greater in our experiments than that seen in Rocchetti et al. [21]. The peak  $I_{Ks}$  current density we measured was  $2.15 \pm 0.52$  A/F, about four times larger than their measured value between 0.5 and 0.7 A/F. This apparent discrepancy may arise from methodological difference. One major difference is in the pipette solution design. Rocchetti et al. used 1 mM EGTA which would buffer the intracellular  $Ca^{2+}$ , whereas in our onion-peeling experiments, the  $Ca^{2+}$  transient during AP was preserved. In our earlier work when  $I_{Ks}$  was recorded in the presence of 10 mM EGTA, the peak amplitude was found lower in the range of 0.4–0.6 A/F [1]. Given that  $I_{Ks}$  is sensitive to  $Ca^{2+}$  [2, 19], differences in these experimental data would be expected. Another major difference is that Rocchetti et al. used the AP waveform recorded before isoproterenol application as the voltage command in their AP-clamp experiment, and then, the  $I_{Ks}$  current was dissected out as the isoproterenol-induced current. In comparison, we used

the AP waveform recorded after the isoproterenol application that resulted in a higher plateau and a steeper phase 3 repolarization. This difference in the AP-clamp command voltage should result in a larger  $I_{Ks}$  current seen in our data, since  $I_{Ks}$  is highly voltage sensitive in the range of the AP plateau [12]. Because we used the AP at the new adrenergic state, the  $I_{Ks}$  currents recorded with the onion-peeling method provide an accurate measure of the  $\beta$ -adrenergic stimulatory effect on  $I_{Ks}$  under increased sympathetic tone. Interestingly, following  $\beta$ -adrenergic stimulation, the peak of  $I_{Ks}$  shifted from mid plateau to phase 3 of AP, similar to that of  $I_{Kr}$ , but the magnitude of  $I_{Ks}$  even surpassed that of  $I_{Kr}$ . The observation that  $I_{Ks}$  is facilitated by  $\beta$ -adrenergic stimulation to a much larger extent than  $I_{Kr}$  was reported earlier [10, 11, 23]. Nevertheless, this is the first time when changes in the profile of  $I_{Ks}$  during AP following  $\beta$ -adrenergic stimulation were experimentally recorded and quantitatively measured.

Increased adrenergic tone also increases heart rate; hence,  $\beta$ -adrenergic-related increase of  $I_{Ks}$  current should help to shorten the AP duration in support of faster heartbeats. However, when the  $I_{Ks}$  channel is defective in long QT1 syndrome, the lack of a significant adrenergic-related increase of  $I_{Ks}$  could be a relevant substrate for arrhythmias. An example of such case is seen in a KCNE1 knockout mouse model in which tachycardia-induced heterogeneity blunts the QT adaptation to heart rate variations [5]. Hence, long QT1 patients with defective  $I_{Ks}$  have a greater susceptibility to arrhythmias.

#### Effects of $\beta$ -adrenergic stimulation on $I_{K1}$

The profile of  $I_{K1}$  during the AP with  $Ca^{2+}$  cycling shows a sustained outward current during diastole. At the AP upstroke,  $I_{K1}$  rapidly decreased due to inward rectification. During phases 2 and 3,  $I_{K1}$  remained small, then sharply increased with fast repolarization at the end of phase 3, and then rapidly



declined back to the diastolic level. Isoproterenol caused a slight increase of  $I_{K1}$  during the AP but did not change the diastolic  $I_{K1}$  current density (Fig. 3). Previous studies of the  $\beta$ -adrenergic stimulation effects on  $I_{K1}$  have reported controversial results. Facilitation of  $I_{K1}$  by isoproterenol treatment was reported by Tromba and Cohen [26], Gadsby [7], and Scherer et al. [24], whereas reduction of  $I_{K1}$  by isoproterenol was reported by Koumi et al. [14], Wischmeyer et al. [29], and Fauconnier et al. [6]. The above experiments were all conducted using the traditional V-clamp technique. Using the AP-clamp method, Zaza et al. [30] provided data to suggest that isoproterenol might reduce  $I_{K1}$  during the plateau phase. In contrast, our data show that isoproterenol facilitated the  $I_{K1}$  during the AP. This apparent discrepancy may result from differences in the experimental methods used to measure  $I_{K1}$ . We used  $\text{Ba}^{2+}$  (50  $\mu\text{M}$ )-sensitive current to estimate  $I_{K1}$ , whereas Zaza et al. used the  $I_{0K}$  current which was dissected out by removing  $\text{K}^+$  from the extracellular solution. The  $I_{0K}$  obtained this way is a composite current containing all  $\text{K}^+$  currents including  $I_{K1}$ ,  $I_{Kr}$ , and  $I_{Ks}$ . The difference between the  $\text{Ba}^{2+}$ -sensitive current and  $I_{0K}$  is expected to change with the isoproterenol treatment, since our data show that both  $I_{K1}$  and  $I_{Ks}$  were significantly increased by isoproterenol. The second major difference is that Zaza et al. used the AP waveform recorded before isoproterenol application as the AP-clamp voltage command, and then added isoproterenol to obtain the isoproterenol-sensitive  $I_{0K}$  current. In comparison, we used the AP waveform recorded after the isoproterenol application that resulted in a higher plateau and a steeper repolarization. The third major difference is in the  $\text{Ca}^{2+}$  buffering condition. Zaza et al. [30] used 1 mM EGTA in their pipette solution which would have buffered the intracellular  $\text{Ca}^{2+}$  and eliminate the  $\text{Ca}^{2+}$  transient (Fig. 1a, b). Instead, the  $\text{Ca}^{2+}$  transient during AP was preserved in our experiments. Given the above differences in the experimental methods, it is difficult to compare the data obtained by us with those reported in Zaza et al. [30]. Since the  $\text{Ca}^{2+}$  transient during AP is preserved in our experiments, we assume that our data more closely reflect the  $I_{K1}$  flowing in the cell in vivo.

Isoproterenol shifts the relative contribution of individual  $\text{K}^+$  current to the AP repolarization

The onion-peeling recording of three  $\text{K}^+$  currents from the same cell enables, for the first time, analysis of the relative contribution of each  $\text{K}^+$  current to the repolarization of AP in a single cardiac myocyte. We found that, under the control condition,  $I_{Kr}$  and  $I_{K1}$  are the major repolarizing currents while the contribution of  $I_{Ks}$  is minor. But isoproterenol treatment greatly increased  $I_{Ks}$  in a dose-dependent manner, ultimately making it to the most powerful repolarizing current. Meanwhile,  $I_{Kr}$  did not change with isoproterenol treatment, so its relative contribution to the total  $\text{K}^+$  current was significantly

reduced. At the same time,  $I_{K1}$  current magnitude was slightly increased, but resulted in no change in its relative contribution. With 30 nM isoproterenol treatment,  $I_{Ks}$  became the largest contributor to the total  $\text{K}^+$  current, surpassing the  $I_{Kr}$  contribution by 4–5 folds. This striking reversal of the relative contribution by  $I_{Kr}$  and  $I_{Ks}$  to the AP repolarization has significant implications.

The contributions of different  $\text{K}^+$  currents to the AP repolarization have been a subject of debate.  $I_{K1}$  and  $I_{Kr}$  are generally considered important repolarizing currents, but the role of  $I_{Ks}$  has been controversial. Some suggested that  $I_{Ks}$  is crucial for repolarization [16, 18], others found  $I_{Ks}$  contributed very little to normal repolarization [15, 27]. Our data clearly demonstrate that the relative contributions of  $I_{Ks}$ ,  $I_{Kr}$ , and  $I_{K1}$  should change with different extent of  $\beta$ -adrenergic stimulation. This finding helps to resolve the apparent contradiction reported in previous studies. Since catecholamine levels are subject to changes during daily activity, exercise, stress, or diseases, our new observations have high clinical relevance. Our results suggest that the efficacy of class III antiarrhythmic drugs targeting various  $\text{K}^+$  channels may change according to the sympathetic tone. This new mechanistic insight is confirmed by the differential effects of chromanol 293B and E-4031 on lengthening APD in the absence and presence of isoproterenol (Fig. 5). Therefore, in the design of new therapeutic strategies targeting specific  $\text{K}^+$  channels, the reversal of the dominance pattern of  $I_{Kr}$  and  $I_{Ks}$  with adrenergic stimulation must be taken into account. Our data provide accurate experimental measures of the three major K currents during the AP under physiologically relevant conditions, which contribute to important quantitative understanding of the adrenergic effects on AP repolarization and arrhythmogenesis.

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**Conflict of interest** None

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