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

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Abstract

β-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} current in the same cell would be altered by β-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}, 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}, 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 β-adrenergic stimulation. We systematically determined the changes in the relative contribution of I_{Ks}, I_{Kr}, I_{K1} to cardiac repolarization during AP at different adrenergic states. In conclusion, the β-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 anti-arrhythmic drug strategies to treat the hearts exposed to various sympathetic tones.

Keywords

Cardiac; myocyte; potassium channel; beta-adrenergic; calcium; action potential

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Conflict of Interest: none.

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 β-adrenergic stimulation, and different K^+ channel I_{Ks}, I_{Kr}, I_{K1} shows different sensitivity to β-adrenergic stimulation. [28] [26] [25] [10] In all previous studies, however, I_{Ks}, I_{Kr}, I_{K1} was 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 β-adrenergic stimulation *coordinately* regulates all these K^+ currents in the same cell during the AP, nor is it clear how various β-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 AP repolarization in response to various extent of β-adrenergic stimulation; such in-depth knowledge is important for understanding how cardiac APs are altered under various sympathetic tones during exercise, stress, or diseases.

β-adrenergic stimulation can affect the K^+ channels directly and indirectly. Downstream from β-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 β-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 β-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 (EGTA or 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. [10] [22] [8] 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. [30] [22] 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 β-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}, 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 anti-arrhythmic drugs) to treat the 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 #15347).

Cell isolation

Male Hartley guinea pigs (male, 3–4 months old, purchased from Charles River Laboratories USA) were first injected with heparin (800u, 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, Glucose 10. pH was set to 7.3. BTy was kept in glass flasks with airtight cap and used within 6 hours after preparation; previous tests confirmed 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, 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 MΩ 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 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 μM Chromanol -293B was used to obtain I_{Ks}; 1 μM E4031 for I_{Kr}; and 50 μM 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 β-adrenergic stimulation effects, the cells were exposed to isoproterenol at the given concentration throughout the entire onion-peeling experiments.

$[Ca^{2+}]_i$ measurement

The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured using Fura-2 and the ratiometric method [3]. Briefly, Fura-2 K^+ 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. 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 $[Ca^{2+}]_i$ 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 figures. The Mean ± SD values are reported in 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^+ currents during the AP with Ca^{2+} 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 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±0.3°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 μM, E4031 1 μM, and Ba^{2+} 50 μM. The data show that the intracellular Ca^{2+} transient during the AP cycle (lower panel) was preserved under our experimental conditions.

Next, we studied the effects of β-adrenergic stimulation on fine-tuning the three K^+ channels by using isoproterenol at 3 nM, 10 nM and 30 nM concentrations. As shown in Figure-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 Ca^{2+} transient (lower panel), which should contribute to the Ca^{2+}-dependent changes in the K^+ currents during the AP with Ca^{2+} cycling.

Preserving Ca^{2+} homeostasis during self-AP–clamp by eliminating exogenous Ca^{2+} buffer

In order to understand the full impact of β-adrenergic stimulation on altering the K^+ currents during AP through PKA phosphorylation and Ca^{2+}/CaMKII signaling, we designed experimental conditions to preserve the Ca^{2+} transient during the AP cycle by eliminating exogenous Ca^{2+} buffer. In literature, pioneering studies aimed at measuring K^+ currents while preserving 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 Ca^{2+} transients [30] [22]; however, the actual Ca^{2+} concentration was not measured in those early experiments. We conducted experiments to simultaneously record the 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 Ca^{2+} transient after pacing at 1 Hz to reach steady-state (Figure-1C); the same result was seen in the presence of 30 nM isoproterenol (Figure-1D). We also observed that the 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 Figure-1C and 1D). Furthermore, our earlier experiments [1] using 10 mM EGTA in the pipette solution caused the 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 Ca^{2+} while the cell is being paced; the speed of Ca^{2+} buffering is slowed with low EGTA concentration, but eliminates the Ca^{2+} transient at steady state nonetheless. It is noteworthy that the Ca^{2+} transient is preserved in our sAP-clamp experiments by eliminating exogenous Ca^{2+} buffer in the pipette solution. We surmise that since the myocyte cell membrane (size ~150x40x30 μm) is substantially larger than the pipette tip (diameter ~1 μm) 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, 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 Ca^{2+} buffer) that disrupt the ionic homeostasis and Ca^{2+} transient.

β-adrenergic stimulation effect on I_{Kr} during the AP with Ca^{2+} cycling

Figure 2A shows the profile of E4031 sensitive I_{Kr} current during the AP with Ca^{2+} cycling. The current density (normalized to the cell capacitance) of I_{Kr} was zero during diastole, remained small during the AP phase-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 β-adrenergic stimulation on the I_{Kr} current was subtle and only seen at high isoproterenol concentration, albeit a faster time course in corresponding to a shorter AP duration (Figure 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 mV and −20 mV membrane potentials (Figure 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 to10 μM.

Dose-dependent β-adrenergic tuning of I_{Ks} during the AP with Ca^{2+} cycling

In the absence of β-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 (Figure-2D). The I_{Ks} current was zero during diastole, built up slowly during the AP phase-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 corresponding to AP repolarization. Isoproterenol treatment caused significant changes in I_{Ks} throughout the AP (Figure-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 ±0.027 A/F to 2.067 ±0.223 A/F in 30 nM isoproterenol (Figure 2F). Importantly, the profile of I_{Ks} during AP following β-adrenergic stimulation became similar to that of I_{Kr} (Figure-2D, 2E), and even surpassed I_{Kr} in magnitude. The peak fo the current shifted from mid plateau to the phase-3 of AP. The substantial alterations in the I_{Ks} current magnitude and time course indicate a strong β-adrenergic control of this channel.

Dose-dependent β-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 Figure 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 phase-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 (Figure-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 (Figure-3C). Consequently, isoproterenol treatment significantly increased the total charge carried by I_{K1} during the AP (Figure-3D).

β-adrenergic stimulation shifts the relative contribution of individual K^+ currents to the total repolarization reserve

The Onion-Peeling method gave us unprecedented ability to record 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; 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 and 4B show the analysis result at +20 mV 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 absence of isoproterenol, I_{Kr} presents the most powerful repolarizing power whereas I_{Ks} contributes very little, as measured at both +20 mV 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} by 4–5 folds at 30 nM isoproterenol. The relative contribution of I_{K1} to the total repolarizing current did not show significant change.

Consistent with changes in the currents, the total charges carried by the K^+ currents were also altered by isoproterenol treatment. As shown in Figure-3C, the total K^+ charge movement during the AP was increased by isoproterenol in 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 isoproterenol treatment (Figure-4C). Importantly, the relative contributions of I_{Ks}, I_{Kr}, and I_{K1} change significantly with increasing isoproterenol concentration, due to different sensitivity of the individual K^+ current to β-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 β adrenergic state and how to design effective anti-arrhythmia drug therapies for various pathological conditions.

β-adrenergic stimulation alters the effects of K^+ channel inhibitors (Class III anti-arrhythmia drugs) on modifying cardiac AP

Since different K^+ channels have different sensitivity to isoproterenol, it is plausible that the β-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 and 5B show that blocking I_{Ks} using 1 μM Chromanol-293B caused a moderate lengthening of APD under control condition, but isoproterenol treatment drastically lengthened APD. In comparison, blocking I_{Kr} using 1 μM E4031 also caused a moderate lengthening of APD in the absence of isoproterenol (Figure-5C); however, the APD lengthening remained small in the presence of isoproterenol (Figure-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 β-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 β-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 (Figure-4). Therefore, the dominant K^+ current switches from I_{Kr} under the control condition to I_{Ks} under β-adrenergic stimulation with 30 nM isoproterenol. Such a reversal of dominance pattern has significant implication for using specific K^+ channel blockers, which belong to the Class-II antiarrhythmic drugs, to treat cardiac arrhythmias.

Effects of β-adrenergic stimulation on I_{Kr}

The effects of β-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 currents elicited with long square pulses in conjunction with blocking the I_{Ks} component. In addition, most previous studies used high isoproterenol concentration (1 μM –10 μM), whereas we used isoproterenol in the range of 3–30 nM (closer to physiological β-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 [31] [20], although some quantitative differences exist. Our data 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 cycling cytosolic calcium; these data can be used to fine tune the quantitative models.

Effects of β-adrenergic stimulation on I_{Ks}

I_{Ks} is known to be facilitated by β-adrenergic stimulation according to previous V-clamp studies [17] [10] [23] [28]. Our data largely agree with the previous findings. The novel findings from our experiments is that β-adrenergic stimulation changes the profile of I_{Ks} during the AP (Figure-2D, 2E). Under the control condition, the profile of I_{Ks} displays a small and flat current throughout the AP (Figure-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±0.52 A/F, about 4 times larger than their

measured value between 0.5–0.7 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 known to be 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 β-adrenergic stimulatory effect on I_{Ks} under increased sympathetic tone. Interestingly, following β-adrenergic stimulation the peak of I_{Ks} shifted from mid plateau to the 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 β-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 β-adrenergic stimulation were experimentally recorded and quantitatively measured.

Increased adrenergic tone also increases the heart rate; hence β-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 IKs have a greater susceptibility to arrhythmias.

Effects of β-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 phase-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 (Figure-3). Previous studies of the β-adrenergic stimulation effects on I_{K1} have reported controversial results. Facilitation of I_{K1} by isoproterenol treatment was reported by Trombe et al. [26], Gadsby et al. [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 traditional V-clamp technique. Using 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 Ba^{2+}(50 μM)-sensitive current to estimate I_{K1}, whereas Zaza et al. used the I_{0K} current which was dissected out by removing K^+ from the extracellular solution. The I_{0K} obtained this way is a composite current containing all K^+ currents including I_{K1}, I_{Kr}, and I_{Ks}. The difference between the 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 Ca^{2+} buffering condition. Zaza et al. [30] used 1 mM EGTA in their pipette solution which would have buffered the intracellular Ca^{2+} and eliminate the Ca^{2+} transient (Figure 1A, 1B). Instead, the 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 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 K^+ current to the AP repolarization

The Onion-Peeling recording of all three K^+ currents from the same cell enables, for the first time, analysis of the relative contribution of each 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 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 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 role of different K^+ currents in the AP repolarization has 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 reports 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 degrees of β-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 the Class-III antiarrhythmic drugs targeting various 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 (Figure 5). Therefore, in the design of new therapeutic strategies targeting specific 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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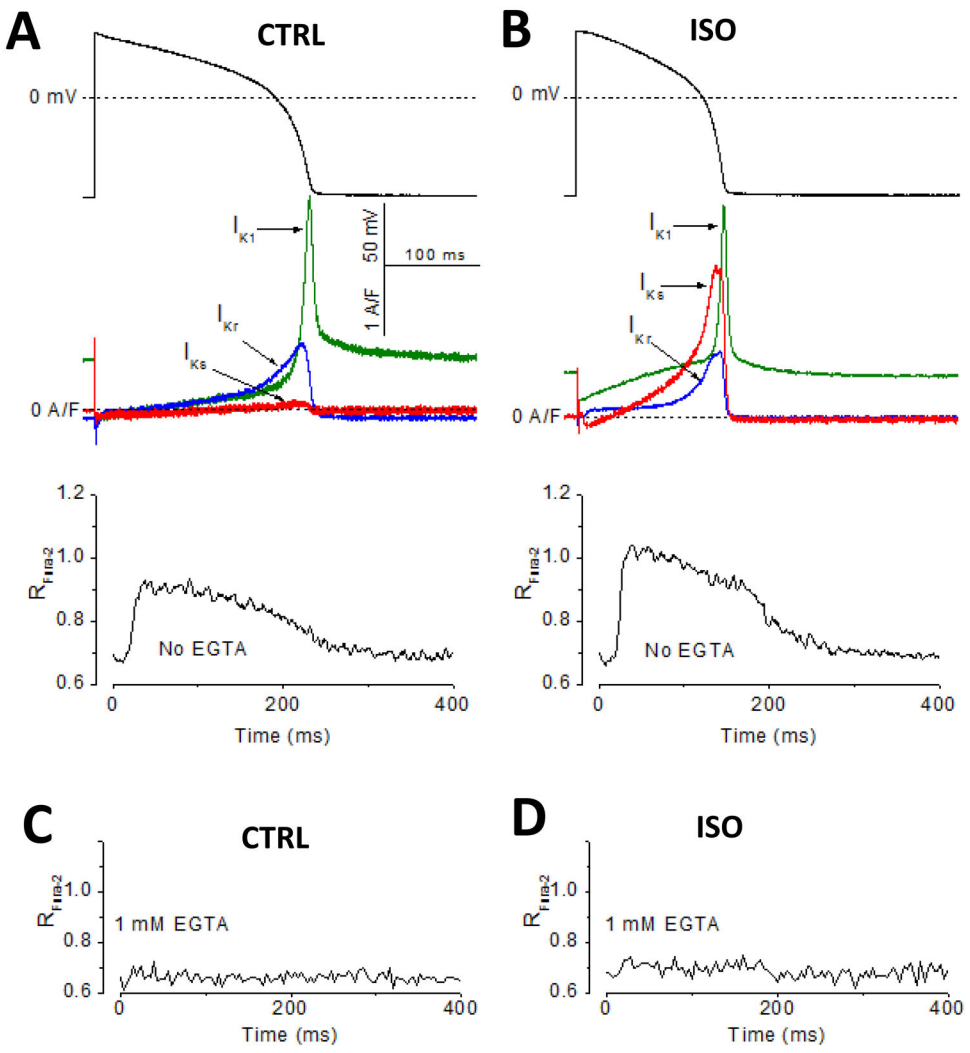

Figure 1.

β-adrenergic stimulation effects on the AP, the three K^+ currents, and the Ca^{2+} transient. Panel A and B show the AP-clamp Sequential Dissection experiments to directly record the steady-state AP (upper panel) at 1 Hz pacing rate, the three K^+ currents (mid panel) in the same cell, and the Ca^{2+} transients (lower panel) under physiological condition (CTRL) and following 30 nM isoproterenol (ISO) treatment. Note that the Ca^{2+} transient during the AP is preserved by having the endogenous Ca^{2+} buffers and without adding any exogenous Ca^{2+} buffer (No EGTA) in pipette solution. Panel C and D demonstrate that the show that the Ca^{2+} transients were largely eliminated by using 1 mM EGTA in the pipette solution under both the control condition and following ISO treatment.

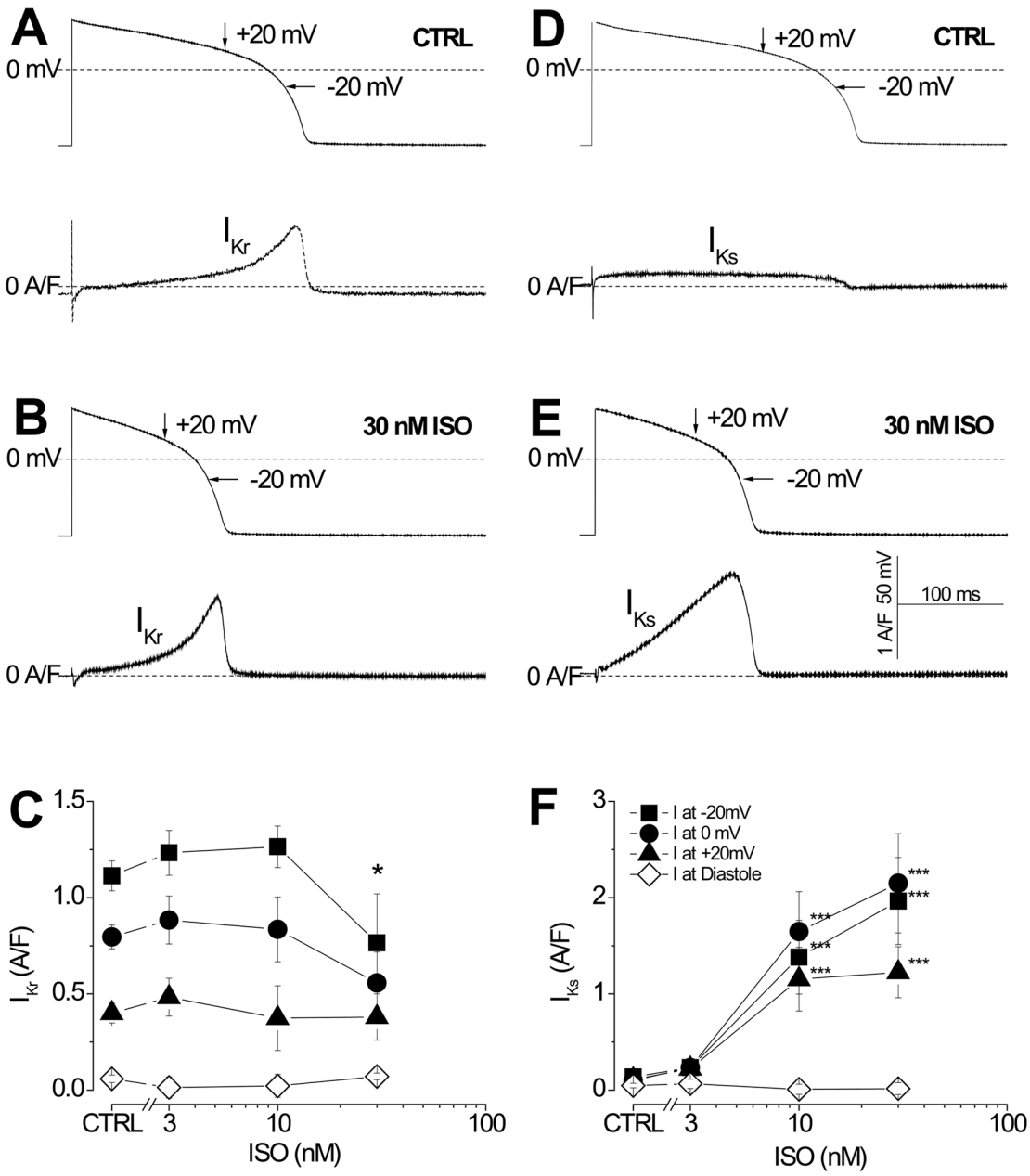

Figure 2.

Dose-dependent β-adrenergic tuning of delayed rectifier K^+ currents during the AP. Panel A and B show the I_{Kr} recorded during the cell's own AP before and after β-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 little effect on the amplitude of I_{Kr} at lower concentrations but slightly reduced I_{Kr} at 30 nM (Panel C). Panel D and E show the AP and I_{Ks} current traces before and after 30 nM ISO treatment, respectively. ISO increased I_{Ks} during AP in dose-dependent manner (Panel F). Each point on panel C and F represents averaged current values and standard error from 7–15 cells isolated from 7 hearts. Student's t-test p values: $p<0.05$*, $p<0.01$**, $p<0.001$***.

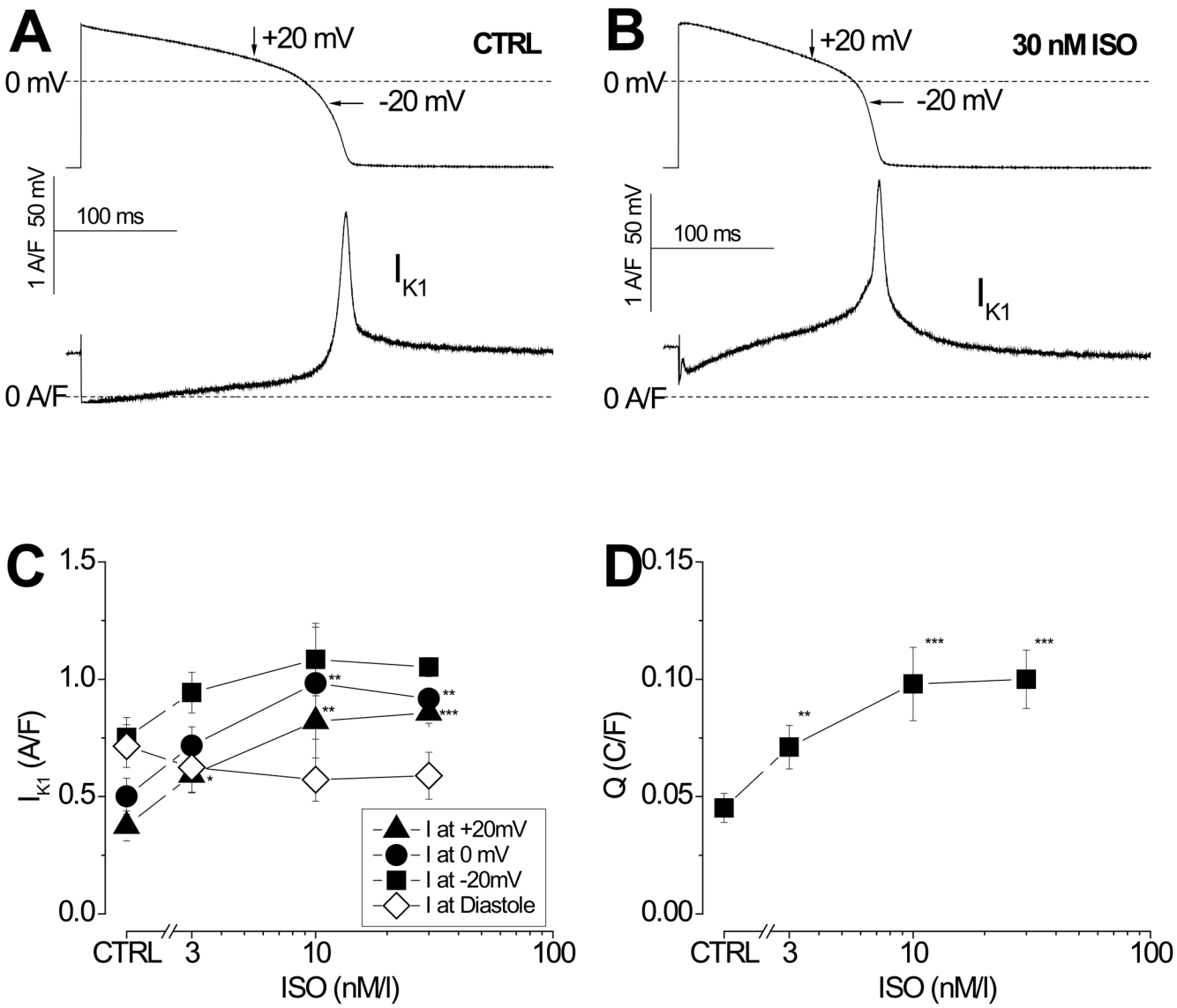

Figure 3.

Dose-dependent β-adrenergic tuning of I_{K1} current. Representative traces were recorded in the absence (panel A) and presence (panel B) of 30 nM ISO. Dose response curves for current values at different membrane potentials and total charge movement during AP are shown in panel C and D respectively. ISO increased I_{K1} during AP plateau, but left diastolic current value unaltered. n=7–15 cells from 7 animals.

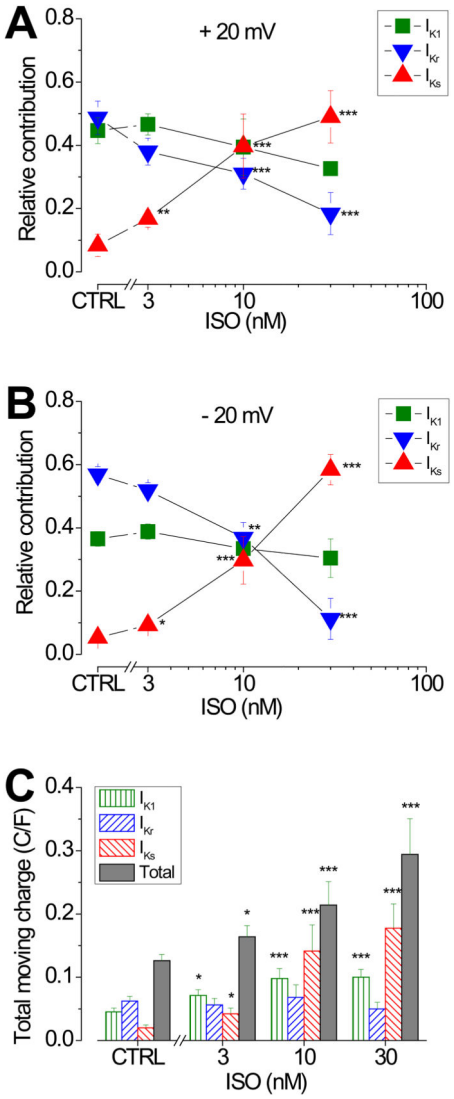

Figure 4.

β-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 mV and −20 mV are shown in panel A and B respectively. Panel C shows the 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 7 animals.

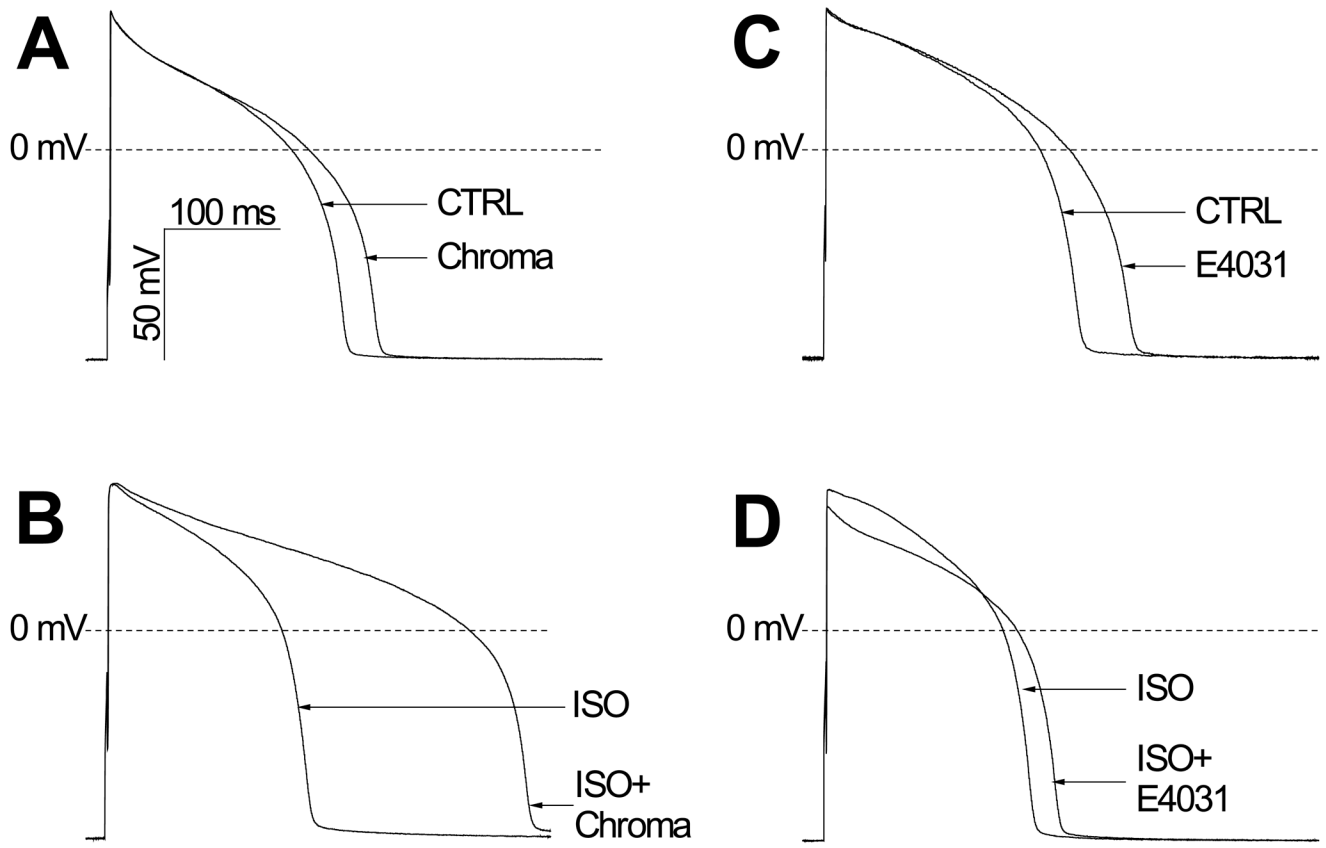

Figure 5.

β-adrenergic state alters the effects of specific K^+ channel inhibitors on modifying the AP. The AP lengthening effect of using 1 μM Chromanol-293B to block I_{Ks} is moderate under control condition (Panel A) but became prominent after 30 nM ISO treatment. The AP lengthening effect of using 1 μM E-4031 to block I_{Kr} is similar under control conditions (Panel C) and after ISO treatment (Panel D).