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Sex differences in repolarization and slow delayed rectifier potassium current and their regulation by sympathetic stimulation in rabbits

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Abstract

Slow delayed rectifier potassium current (I_{Ks}) is important in action potential (AP) repolarization and repolarization reserve. We tested the hypothesis that there are sex-specific differences in I_{Ks}, AP, and their regulation by β-adrenergic receptors (β-AR's) using whole cell patch-clamp. AP duration (APD_{90}) was significantly longer in control female (F) than in control male (M) myocytes. Isoproterenol (ISO, 500nM) shortened APD_{90} comparably in M & F, and was largely reversed by β_1-AR blocker CGP 20712A (CGP, 300 nM). Inhibition of I_{Ks} with chromanol 293B (10 μM) resulted in less APD prolongation in F at baseline (3.0% vs 8.9%, $p<0.05$ vs M) and even in the presence of ISO (5.4% vs 20.9%, $p<0.05$). This suggests that much of the ISO-induced APD abbreviation in F is independent of I_{Ks}. In F, baseline I_{Ks} was 42% less and was more weakly activated by ISO (19% vs 68% in M, $p<0.01$). ISO enhancement of I_{Ks} was comparably attenuated by CGP in M and F. After ovariectomy, I_{Ks} in F had greater enhancement by ISO (72%), now comparable to control M. After orchiectomy, I_{Ks} in M was only slightly enhanced by ISO (23%), comparable to control F. Pre-treatment with thapsigargin (to block SR Ca release) had bigger impact on ISO-induced APD shortening in F than that in M ($p<0.01$). In conclusion, we found that there are sex differences in I_{Ks}, AP, and their regulation by β-AR's that are modulated by sex hormones, suggesting the potential for sex-specific antiarrhythmic therapy.

Keywords

Potassium current; Sex differences; Repolarization; Adrenergic receptor

Introduction

The slow delayed rectifier potassium current (I_{Ks}), an important repolarizing current in heart (44, 50) that is encoded by KCNQ1 (KvLQT1) and KCNE1 (minK) genes, is an antiarrhythmic drug target (44). Sympathetic nervous system regulation of cardiac action potential duration (13, 44) is primarily mediated by β-adrenergic receptor (β-AR) activation, which increases I_{Ks} (44). In mammalian cardiomyocytes, β_1-ARs play a predominant role in regulating myocyte contractility while β_2-ARs may play a more modest role in regulating inotropic and lusitropic responses. Both receptors signal through G_s and adenylyl cyclase, although the signals are compartmentalized differently possibly due to localization in

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distinct membrane microdomains and/or dual coupling of the β_2-AR to G_s and G_i (68). I_{Ks} is modulated by levels of intracellular Ca ($[Ca]_i$) (52) such as the increase in $[Ca]_i$ during contraction due to Ca release from the sarcoplasmic reticulum (SR); so action potential duration (APD) shortening by isoproterenol (ISO) may be influenced by ISO-induced changes in SR Ca release.

Sex hormones may also play an important role in regulating repolarizing K currents, APD, and β-adrenergic responsiveness (2, 10, 14, 18, 22, 63). It has been recognized that the corrected QT interval (QT_C) is longer in women than in men (43). In addition, female sex is associated with greater drug-induced QT prolongation and accounts for more torsades des pointes in response to certain drugs that prolong ventricular repolarization (19, 37, 43, 49). Sex differences in potassium currents in females appear to underlie increased action potential (AP) duration, QT interval, and incidence of torsades des pointes. However there is little quantitative and mechanistic characterization of sex-specific differences (and the role of sex hormones) in I_{Ks} and action potential characteristics and their responsiveness to β-adrenergic stimulation.

In the present study, we investigated sex differences in repolarization, ISO-induced APD shortening, and β-adrenergic receptor subtype selectivity in left ventricular (LV) myocytes from control male and female rabbits. We used an I_{Ks} blocker to evaluate the contribution of I_{Ks} to APD and ISO-induced APD shortening. We also assessed sex differences in I_{Ks} and its responsiveness to ISO. The contribution of sex hormones to these female vs male differences was assessed with studies in ovariectomized (OVX) and orchiectomized (ORCH) rabbits. Lastly, thapsigargin (SR Ca uptake inhibitor) was used to assess sex differences in the contribution of Ca handling to APD shortening with ISO.

Materials and methods

Animals

All experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). New Zealand rabbits weighing 3.5–4.0 kg of either sex were used. In selected studies, orchiectomized male and ovariectomized female rabbits were used. The protocols were approved by the Animal Studies Committees of the University of Illinois at Chicago and the University of Alabama at Birmingham.

Ovariectomy and Orchiectomy

In brief, female and male rabbits were gonadectomized at ~4 months old and sedated with intramuscular ketamine, intubated, and mechanically ventilated. Anesthesia was maintained with 1–3% isoflurane delivered in 100% oxygen. For ovariectomy, a 3–4 cm midline skin incision was made centered over the area of the cranial pole of the bladder. The linea alba was incised to access the peritoneal cavity. The ovary was separated from the broad ligament and the suspensory ligament. The ovarian vessels were double ligated with synthetic absorbable sutures and transected between the ligatures and the ovary. The procedure was repeated on the contralateral side to remove both ovaries. For orchiectomy, a 1–3 cm incision was made on the ventral surface of the scrotum of each testicle. The testicle was exposed by incising the spermatic fascia and the parietal tunic. The spermatic cord, vas deferens, and associated blood vessels were clamped and double-ligated with synthetic absorbable sutures and the testicles were removed. The waiting period after gonadectomize is ~8 weeks before the gonadectomized rabbits were sacrificed for myocyte isolation at 6 months old.

Cardiac myocyte isolation

LV Cardiac myocytes were isolated as previously described (47, 48). Briefly, 6 months old rabbits (25.9±0.5 weeks in control females, 26.3±0.5 weeks in control males) were anesthetized (pentobarbital sodium; 50 mg/kg) after being heparinized, and the heart was quickly removed, rinsed in cold 0 mM Ca^{2+} Tyrodes, placed on a Langendorff apparatus, and perfused through the aorta with a 0 Ca^{2+} solution at 37°C. The heart was then perfused with a MEM solution containing Liberase Blendzyme 4 (Roche Applied Science). Digestion was stopped by adding a bovine serum albumin (BSA)-containing solution (1 mg/ml), and midmyocardial LV tissue was used. Furthermore we have limited the heterogeneity by cutting off both the basal 4–8 mm and the apical 5–10 mm of the rabbit hearts. Tissue was gently minced and filtered. Cells were stored in 50 μM Ca^{2+} MEM solution at room temperature.

Single cell electrophysiology

Cardiac myocytes adhering to laminin-coated glass coverslips were placed in a small transparent recording chamber mounted on the stage of an inverted microscope (TE200S; Nikon; Tokyo, Japan). The whole-cell patch-clamp technique was used to record the membrane currents in single rabbit LV myocytes. Patch-clamp micropipettes were made from glass capillaries (Harvard Apparatus Limited, Kent, United Kingdom) using a DMZ-Universal puller (Zeitz-Instruments GmbH, Munich, Germany). These electrodes were filled with an intracellular solution containing (mM) KOH 120, KCl 20, $MgCl_2$ 2, $CaCl_2$ 1.8, Mg-ATP 5, EGTA 5, HEPES 10, aspartic acid 100, adjusted with KOH to pH 7.2. The external solution contained (mM) NaCl 132, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1.2, $BaCl_2$ 0.2, HEPES 10, glucose 10, 4-aminopyridine 5, nifedipine 0.01, dofetilide 0.003, pH 7.4. A Multiclamp 700A amplifier (Axon Instruments, Union City, CA, USA) was used to record the membrane current in the whole-cell configuration of the patch-clamp technique. Capacitance was canceled and series resistance was compensated as needed. Membrane currents were recorded from a holding potential of −50 mV to test-pulse potentials ranging in 10-mV steps from −40 mV to +50 mV. Membrane currents were digitized using a Digidata 1322A data-acquisition system (Axon Instruments, Union City, CA, USA). All current measurements were normalized to total cell capacitance (i.e. pA/pF) to allow comparison between cells of various sizes. Slow delayed rectifier potassium (I_{Ks}) step current density was measure at the end of a 3s depolarizing voltage step from a holding potential of −50 mV as the dofetilide-resistant current. Slow delayed rectifier potassium tail current was measure as peak density of tail current elicited by repolarizing to −50 mV following 3s depolarizing voltage steps. Chromanol 293B (10 μM; Tocris) was used as a blocker of the slow delayed rectifier K^+ current (I_{Ks}), and I_{Ks} effect was measured as the chromanol 293B-sensitive current in action potential recordings. Action potentials were recorded in current clamp mode with whole cell patch clamp. The pipette solution contained (mM) KCl 30, K-aspartate 110, NaCl 8, Mg-ATP 5, HEPES 5 adjusted with KOH to pH 7.2. Cells were superfused with a solution containing (mM) NaCl 140, KCl 4, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 5, glucose 10, pH 7.4. Isolated rabbit LV myocytes were stimulated at a frequency of 1 Hz. The APD was measured at 90% repolarization (APD_{90}). Experiments were performed at room temperature. We recorded I_{Ks} and APD from different cells. Experiments were performed in the absence or presence (>5 min) of ISO (500 nM; Sigma), CGP 20712A (300 nM; Sigma), ICI-118,551 (100 nM; Tocris), chromanol (10 mM; Tocris) or after pretreatment with thapsigargin (2 μM; Tocris) for 15 minutes. ISO was added for β-adrenergic stimulation. CGP 20712A was used as a selective β_1-AR antagonist for ISO-mediated effects. ICI-118,551 was used as a selective β_2-AR antagonist. In selected studies, myocytes were pretreated with thapsigargin (2 μM; Tocris) to deplete SR Ca^{2+}.

ECG studies

ECG recordings were obtained from conscious control male and female rabbits in lead III at baseline and following a 90 second administration of ISO (1.0 μg/kg/min). QT intervals were measured from QRS onset to the end of the T wave by custom-built analysis software. Corrected QT (QT_C) intervals were calculated using Carlsson's formula (QT_C=QT-0.175(RR-300))(24).

Statistics

Data are reported as means ± SEM. Data analyses were performed using Clampfit 9.2 (Axon Instruments, Union City, CA), Origin 7 (Originlab, Northampton, MA), and SAS 9.2 (SAS Institute, Cary, NC). Comparisons between the changes in APD and ISO values obtained on the same group of rabbit myocytes were done using the paired t-test. Comparisons between values on two individual groups of rabbit myocytes were done using the two-group (unpaired) t-test. One-way analysis of variance (ANOVA) was used for measurements obtained from multiple groups. Analysis of covariance (ANCOVA) was used to compare APD at baseline ± ISO for myocytes from males and females treated with thapsigargin) vs untreated controls. Terms included in the ANCOVA model were sex (male or female), thapsigargin (presence or absence of), and the interaction between sex and thapsigargin. The Tukey-Kramer multiple comparisons test was then used to determine specific pairwise differences between the means. All statistical tests were two-sided and were performed using a significance level of 5% (i.e. alpha=0.05).

Results

Sex differences in action potential characteristics

Rabbit LV myocyte AP's were continuously elicited at a stimulation rate of 1 Hz. Representative AP recordings of control female and male hearts are shown in Figs. 1A & 1B. The AP duration at 90% repolarization (APD_{90}) was 11% longer in female vs male myocytes (672.7±3.7 vs 604.9±3.6 ms, n=26 (24 hearts), 29 (24 hearts), $p<0.01$ for female vs male). Resting membrane potential (RMP) was not different (−77.0±0.4 mV for female vs −77.3±0.3 mV for male; n=26 (24 hearts), 29 (24 hearts), p=NS).

Role of β_1- and β_2-adrenergic receptor stimulation in isoproterenol-induced APD shortening in control male and female rabbits

Because some cardiac channels are modulated by stimulation of β-adrenergic receptors, we examined ISO-induced APD shortening in control female and male rabbits. ISO (500 nM) shortened APD_{90} in rabbit myocytes paced at cycle length of 1000 ms comparably (p=NS) in females (by 36%; from 664.4±7.9 to 426.5±10.9 ms; n=6 (5 hearts)) and in males (by 32%; from 609.5±10.0 to 412.9±6.9 ms; n=6 (5 hearts)) comparable (p=NS). We also examined the effect of the selective β_1-adrenergic receptor blocker CGP 20712A (CGP) on APD_{90} in the presence of 500 nM ISO. As shown in Table 1, the ISO-induced APD shortening was comparably (p=NS) prevented by CGP in females (by 84.3±4.8% from 426.5±10.9 to 627.1±6.0 ms) and in males (by 87.9±0.8%, from 412.9±6.9 to 585.7±9.1 ms). Thus β_1-adrenergic receptor plays a critical role in ISO-induced APD shortening in rabbit LV myocytes.

To investigate the role of β_2-adrenergic stimulation in ISO-induced APD shortening, we examined the effect of the β_2-adrenergic receptor blocker ICI-118,551. Typical recordings are shown in Figs. 1C & 1D. ISO-induced shortening of APD was comparably (p=NS) attenuated by 9.4±0.7% in control female and 7.4±2.0% in control male (Table 1). Thus the effects of ISO-induced APD shortening in LV myocytes from control female and male

rabbits were mediated primarily by β_1-adrenergic stimulation. Data are summarized in Table 1.

Effects of chromanol 293B on ventricular action potentials in control male and female rabbits

We investigated the effects of the I_{Ks} blocker chromanol 293B on ventricular action potentials from female and male rabbit hearts. Consistent with the previous results, baseline APD_{90} was significantly longer in control females compared with control males. Representative AP's in the presence and absence of 10 μM chromanol 293B in female and male rabbit LV myocytes at a cycle length of 1000 ms are shown in Figs. 2A & 2B. Inhibition of I_{Ks} with 10 μM chromanol 293B resulted in a minimal 3.0% increase in APD_{90} in females (from 675.5±8.3 to 695.6±10.4 ms, n=6 (6 hearts)), but in a modest (8.9%) APD_{90} prolongation in males (from 604.7±7.7 to 658.4±6.6 ms, n=8 (6 hearts), $p<0.05$ vs females). The effects of chromanol 293B on APD_{90} are summarized in Table 2.

Electrophysiological effects of I_{Ks} blocker in the presence of β-adrenergic stimulation

Because I_{Ks} is modulated by sympathetic tone, we further examined the effect of chromanol 293B on ISO-induced APD shortening. Stimulation of β-adrenergic receptors by ISO shortened APD_{90} in rabbit myocytes paced at cycle length of 1000 ms comparably (p=NS) in females (by 35%; 670.1±6.2 to 435.5±5.6 ms; n=7 (6 hearts)) and in males (by 31% from 607.3±5.7 to 416.5±5.6 ms; n=8 (7 hearts)). Addition of chromanol 293B (10 μM) in the continuous presence of 500 nM ISO produced little (5.4%) APD prolongation (from 435.5±5.6 to 459.1±8.2 ms) in females, but more marked pronounced (20.9%) prolongation of APD in males (from 416.5±5.6 to 503.4±7.9 ms, $p<0.05$ vs females). Representative AP recordings at baseline, with 500 nM ISO, and with 500 nM ISO in the presence of 10μM chromanol 293B are shown in Figs. 2C & 2D. Summary data for the effects of ISO and chromanol 293B on the change in APD_{90} are in Table 2.

Effect of SR Ca^{2+} on ventricular action potentials in the presence of β-adrenergic stimulation

To assess the effects of Ca^{2+} handling on APD and its modulation by β-adrenergic stimulation, experiments were conducted after pre-incubation with 2 μM thapsigargin to deplete SR stores so that no released SR Ca^{2+} would be available to influence APD (4). Representative recordings of the effects of ISO in control female and male rabbit myocytes pretreated with thapsigargin are shown in Fig. 3. In thapsigargin-pretreated myocytes paced at cycle length of 1000 ms, ISO shortened APD_{90} by 20.7±1.8% in females (from 658.1±2.5 to 522.2±13.3 ms; n=7 (7 hearts)) and by 26.4±1.5% in males (from 592.6±3.1 to 435.8±7.8 ms; n=7 (6 hearts)). Overall, thapsigargin block of SR Ca release resulted in greater attenuation of ISO-induced APD shortening in female than in male LV myocytes ($p<0.01$ by analysis of covariance). Since chromanol 293B had minimal effects on ISO-induced APD shortening in females, these collective results suggest that Ca-sensitive channels other than I_{Ks} play a greater role in APD shortening by ISO in myocytes from females.

β-adrenergic stimulation regulates I_{Ks} in control male and female rabbits

We studied slow delayed rectifier potassium current (I_{Ks}) in control female and male rabbits. Fig. 4 shows representative I_{Ks} tracings at baseline (A, F), in the presence of ISO (B, G), and after subsequent addition of CGP (C, H). I_{Ks} in females was 42% less ($p<0.01$) than in males (at +50mV, step: 0.61±0.04 vs. 1.05±0.07 pA/pF, $p<0.01$, n=5 (5 hearts), 9 (9 hearts), $p<0.01$ for females vs males, Figs. 4A & 4F, and Fig. 5). Because sympathetic tone enhances I_{Ks}, we examined I_{Ks} in response to β-adrenergic receptor stimulation with ISO (500 nM) in female and male myocytes (Figs. 4 & 5). In females ISO increased I_{Ks} by only

19% (at +50mV, Step: 0.61±0.04 to 0.72±0.04 pA/pF) while in males ISO enhanced I_{Ks} by 68% (1.05±0.07 to 1.78±0.13 pA/pF, p<0.01 vs females; Fig. 4G & Fig. 5).

We evaluated the β-adrenergic receptor subtypes involved in this ISO-induced increase in $I_{Ks.}$ The β_1-receptor blocker CGP 20712A comparably prevented ISO enhancement of I_{Ks} by 90.2±1.3% in females and 91.8±0.8% in males (Fig. 4). We also assessed the contribution of β_2-adrenergic receptor stimulation in the I_{Ks} response to ISO. Fig. 6 shows representative traces I_{Ks} at baseline (A, F), in the presence of ISO (B, G), after subsequent addition of ICI-118,551 (ICI, 100 nM, a selective β_2-AR antagonist) (C, H), and the results of I_{Ks} current-voltage relationship in females and males. The ISO-induced increase in I_{Ks} was comparably prevented by ICI by an average of 11.2±1.2% (n=5 (5 hearts)) in females and 10.0±0.7% (n=7 (7 hearts)) in males (Fig. 6), suggesting that while β_1-adrenergic stimulation is the primary modulator of I_{Ks}, β_2-adrenergic stimulation does contribute, at least in part, to I_{Ks} responsiveness to ISO.

Sex differences in I_{Ks} in response to isoproterenol

Our results above showed that I_{Ks} was enhanced in the presence of the β-adrenergic receptor agonist ISO, most notably in control male rabbits, suggesting a role for sex hormones. We did hormonal manipulation by ovariectomy (OVX) of female rabbits and orchiectomy (ORCH) of male rabbits. Ovariectomy decreased estrogen levels from 27.3±2.2 to 10.9±1.4 pg/ml (n=4,4; p<0.01). Representative current traces and current-voltage relations in OVX females and ORCH males are shown in Fig. 7. OVX females demonstrated a 21% increase in I_{Ks} (vs control females) and a much greater enhancement with ISO (at +50mV, Step: 0.74±0.05 to 1.26±0.08 pA/pF, n=7 (5 hearts), p<0.05 vs control females; Figs. 5 & 7). Fig. 5 shows the ratio of I_{Ks} in the presence of ISO to the control for a test pulse to +50 mV; OVX increased the ratio from 1.19±0.02 in control non-OVX females to 1.72±0.02 in OVX females (p<0.05). ORCH males demonstrated a 14% decrease in I_{Ks} (vs control males) and showed only slight change with ISO (at +50mV, Step: 0.90±0.06 to 1.11±0.09 pA/pF, n=7 (5 hearts), p<0.05; Figs. 5 & 7); ORCH decreased the ISO-enhanced ratio from 1.68±0.03 in control non-ORCH males to 1.23±0.04 in ORCH males, p<0.05. Thus, we found that a 72% increase in I_{Ks} step current in OVX females was comparable to the 68% increase that we observed in control males (p=NS). Likewise, the slight 23% increase in I_{Ks} step current in ORCH male was comparable to the 19% increase seen in control females (p=NS).

Sex differences on QT_C interval in response to isoproterenol

To investigate sex differences in repolarization and the response to β-AR stimulation in vivo, we performed additional *in vivo* ECG studies in the absence and presence of ISO infusion. Figure 8A shows representative ECG recordings from control female and male rabbits at baseline and during ISO infusion. As shown in Figure 8B, QT_C at baseline were significantly longer in female vs male (167.0±5.3 vs 150.4±5.1 ms, n=5, 7, p<0.05 female vs male). ISO shortened QT_C interval to a comparable degree in females and males. QT_C intervals with ISO infusion (1.0 μg/kg/min) were comparably shortened by 17% to 137.7±6.3 ms (n=5) in females and by 16% to 126.9±4.4 ms (n=7) in males. These findings were similar to sex differences in action potential and ISO-induced APD shortening in control female and male rabbit LV myocytes.

Discussion

The present studies provide novel information about sex differences of I_{Ks} in response to β-AR stimulation, ISO-induced APD shortening, chromanol's effect on APD in the absence or presence of ISO, and responsiveness of I_{Ks} to ISO in gonadectomized rabbits.

I_{Ks} and its role in repolarization

I_{Ks} modulates the repolarization phase of the AP (31, 50), especially in the setting of β-adrenergic stimulation (65). It counterbalances the depolarizing effects of increased L-type Ca current (I_{Ca}) associated with β-adrenergic stimulation, and plays an important role in cardiac repolarization reserve, especially in the setting of increased sympathetic tone (65). The important role of I_{Ks} is evident from finding that congenital LQTS is associated with mutations in genes encoding KCNQ1 and KCNE1 (32). I_{Ks} is composed of KCNQ1 and KCNE1 as part of a macromolecular complex that includes PKA, PP1 and the targeting AKAP protein, yotiao (44). Sex differences in I_{Ks} expression and activity may be due to sex differences in I_{Ks} channel subunits (KCNQ1 and KCNE1). We find decreased KCNE1 but unchanged KCNQ1 expression in female rabbit mid myocardium LV (unpublished data). Since KCNE1 coassembles with KCNQ1 (3, 35, 51, 54, 57), this could explain the reduced I_{Ks} amplitude in females. In our electrophysiological studies, we used mid-myocardium to limit the contribution of endocardium and epicardium. We also limited heterogeneity by excluding base and apex.

We performed our studies in rabbit LV myocytes in which I_{Ks} resembles that of humans (38, 50, 62). Chromanol 293B (10 μM) blocks >90% of I_{Ks} in rabbit myocytes (46). We found that blockade of I_{Ks} with 10 μM chromanol 293B caused a mild increase in APD in males and caused little increase in females. Published results of I_{Ks} blocker effects on ventricular AP's remain controversial. In a number of whole heart and tissue studies, I_{Ks} blockers (chromanol 293B, HMR 1556 and L-735,821) did not prolong ventricular repolarization in rabbits (38, 55). On the other hand, in cellular studies chromanol 293B (10 μM) prolonged AP (42), and L-768,673 modestly increased APD of epicardial and endocardial myocytes in rabbits myocytes (69). Likely explanations for these different findings could be different experimental conditions or the low level of baseline I_{Ks}.

After β-AR stimulation (which enhances I_{Ks}), effects of I_{Ks} blockers on APD are more consistent (65). Indeed, in males we found that the I_{Ks} blocker chromanol 293B counteracted ~50% of the ISO-induced APD shortening. However, in females chromanol 293B had minimal impact on the ISO-induced APD shortening. This is totally consistent with the weaker ability of ISO to increase I_{Ks} in females (as either a percentage change (19 vs. 68%) or an absolute I_{Ks} density change (0.11 vs. 0.73 pA/pF)). Our results in males are consistent with studies by So et al, in which I_{Ks} block by HMR 1556 reversed the repolarization shortening by ISO without rate-dependence in perfused control male rabbit hearts (55). Reduced response to chromanol 293B in females may relate to lower I_{Ks} current and sex differences in β-AR responsiveness (discussed below).

β-adrenergic regulation of I_{Ks} and action potential duration

Consistent with previous results (29), we found that APD_{90} was significantly longer in control females compared to control males. While APD prolongation may enhance SR Ca load and to some degree balance out sex differences in (i.e. reduced) Ca handling and β-AR responsiveness (12, 31, 63), the end result may be detrimental, with an increased propensity in females for EADs and torsades des pointes when challenged with further block of K channels (e.g. drugs that inhibit I_{Kr} (including antiarrhythmics) or LQTS).

I_{Ks} is enhanced by β-AR stimulation (53). β-adrenergic regulation of I_{Ks} is mediated by a macromolecular signaling complex consisting of KCNQ1, KCNE1 and Yotiao (an AKAP) that regulates channel activity by PKA-dependent phosphorylation of Ser^{27} in the N-terminal KCNQ1 (44), and of Ser^{43} in the N-terminal of Yotiao (11, 36). KCNE1 is required for this transduction of PKA effects on KCNQ1 (35). ISO's effects on I_{Ks} were primarily mediated by β_1-AR's, but with a small but definite contribution of β_2-AR stimulation to

ISO's effect. KCNQ1 and β_2-AR are localized to the intercalated discs, surface sarcolemma, and transverse tubules of isolated ventricular myocytes (17). β_2-AR stimulation can modulate I_{Ks} under conditions of increased β_2-AR expression (17), and β_2-AR regulation of I_{Ks} might be greater in the setting of HF where there is increased β_2-AR arrhythmogenicity mediated in part by β_2-AR upregulation (15). β_3-AR stimulation has been shown to have effects on I_{Ks} and APD, although the results have been variable (7, 23, 33). ISO-induced β_3-AR stimulation of I_{Ks} in guinea pig ventricular myocytes (in the presence of combined β_1-AR and β_2-AR blockade) was shown to inhibit I_{Ks} (7), although in other studies β_3-AR stimulation was shown to shorten repolarization (23). Little role for β_3-AR in control rabbit ventricular myocytes is likely due to the low level of β_3-AR's in control rabbit ventricular myocytes, but this might be otherwise in states such as HF where there is β_3-AR upregulation (45).

Sex differences in repolarization and the role of sex hormones

Women are known to have longer QT_C intervals than men. Moreover, women are at greater risk for arrhythmias associated with long QT_C intervals. Repolarization-prolonging drugs induce torsades des pointes (TdP) more frequently in women than men (37, 43), and female sex is the major predictor of d-sotalol-induced mortality (49), and an independent risk factor for the incidence of syncope and sudden death in the inherited long QT syndrome. Similar to humans, female rabbits show a greater cycle length-dependent increase in QT interval than males (18, 40, 41, 60).

With regards to the role of sex hormones on repolarization, ovariectomy shortened the QT interval of isolated hearts (18), and consistent with this, 17-β-estradiol treatment in ovariectomized rabbits caused ventricular APD prolongation. QT intervals and QT prolongation with quinidine was greater in hearts from ovariectomized female rabbits treated with estradiol than those treated with testosterone (18). Protection from excess QT prolongation in males may be attributable to testosterone (46), and testosterone may be responsible for shorter QT intervals in men (46). Overall, these results suggest gonadal steroids can determine sex differences in electrophysiology at the level of control of ion channels.

We found that I_{Ks} current was substantially smaller in control female myocytes (vs their male counterparts). A number of studies have examined sex differences in repolarization and its modulation by β-AR, but there are little data on sex differences in I_{Ks} other than a study in canine Purkinje fibers that showed limited chromanol-sensitive APD shortening with ISO in females (1). Estrogen inhibits I_{Ks} in Xenopus oocytes (9) and testosterone enhances I_{Ks} in ventricular myocytes isolated from female guinea pigs (2). Testosterone-induced APD shortening is mainly caused by enhancement of I_{Ks}, although suppression of L-type Ca2+ currents ($I_{Ca,L}$) (2) may contribute. These data suggest that estrogen and testosterone may directly modulate I_{Ks} and ventricular repolarization.

In our experiments, sex differences in I_{Ks} and importantly also its responsiveness to ISO are contributors to sex-based cardiac repolarization differences. I_{Ks} has a significant impact on repolarization in the presence of ISO in males, but minimally so in females. Our results suggest that sex hormones (including estrogen and testosterone) play an important role in the regulations of I_{Ks} responsiveness to ISO. OVX and its associated estrogen withdrawal enhanced the ISO responsiveness of I_{Ks} in females, while ORCH and its associated testosterone withdrawal had the opposite effects in males.

Sex differences in β-AR responsiveness

We found that I_{Ks} and APD in females exhibited less β-AR responsiveness than their male counterparts. Two effects may explain the differential β-AR effects in male *vs* female. First, KCNE1 is required for the β-AR effects on KCNQ1 current (35). Thus, our findings of reduced I_{Ks} responsiveness to β-AR stimulation in females may be due to the lower KCNE1 expression in females. That is, KCNQ1 channels without KCNE1 fail to be activated by β-AR stimulation (35) and mutant KCNE1 reduces functional regulation of channel by PKA-dependent phosphorylation (35). Second, reduced I_{Ks} responsiveness could also be due to lower β-AR density in female cardiac myocytes, along with reduced levels of cAMP. Early observations suggested β-AR downregulation in estrogen-treated rats (22). Vizgirda et al also characterized sex differences in rat cardiac myocyte response to β-AR stimulation (63), and found lower β-AR density and reduced levels of cAMP in cardiac myocytes from female rats (63). In addition, hearts from ovariectomized rats exhibit upregulation of β_1-AR compared with hearts from control female rats (12, 58).

Repolarization reserve and the role of $[Ca^{2+}]_i$

Our findings suggest that decreased I_{Ks} in LV myocytes of females may confer less repolarization reserve (vs males) (67). This, along with the prolonged baseline APD in females, may account for the greater propensity for females to develop torsades des pointes in response to drugs that block I_{Kr} or in the setting of long QT syndrome (37, 43). However, myocytes from females still demonstrated shortening of APD in response to ISO that was comparable to that in males. The smaller I_{Ks}, the reduced β-AR responsiveness of I_{Ks} to ISO stimulation, and the lack of chromanol-induced APD prolongation in females (but not males) suggest that factors other than I_{Ks} are likely to cause ISO-induced shortening of APD. We pre-incubated myocytes in thapsigargin for 15 min to deplete SR Ca stores and reduce the rise in $[Ca^{2+}]_i$ during systole, as has been demonstrated by Bassani et al (4), and we found a greater attenuation of ISO-induced APD shortening vs. males. Our thapsigargin and chromanol studies suggest that, in females, ISO-induced APD shortening is due in large part to currents other than I_{Ks}, possibly secondary to ISO-induced changes in Ca or Na handling. The nature of the responsible current other than I_{Ks} in females remains to be determined. The smaller ISO-dependent enhancement of inward I_{Ca} and I_{NCX} (due to less Ca transient enhancement) in females than males (~30% vs ~90%) (14, 56, 63) would limit the APD-prolonging influence of these changes in females and partly explain the difference. However, there should also be a net increase in outward current as well for net APD shortening. I_{Kr} has been reported to be smaller in females (40), making it unlikely to contribute to ISO-induced APD shortening (unless I_{Kr} in females is more activated by ISO). I_{K1} is suppressed directly by ISO (34) as well as the rise in $[Ca]_i$ (14) induced by ISO, making that an unlikely explanation. ISO also stimulates outward Na/K-ATPase current in myocytes (16) and increased outward pump current is involved in reduced APD in response to increasing heart rate (26), making this a plausible contributor. Other possible explanations include Ca-activated channels such as $I_{Cl(Ca)}$ (70) and $I_{K(Ca)}$ (59), but sex-differences in these channels have not been explored.

Limitations

There is heterogeneity of I_{Ks} current, not only transmurally (known differences between endocardial, epicardial and M cells) (8), but also between different parts of the heart (RV vs LV, apex vs base) (64) that could be related to differences in sympathetic innervation. We studied myocytes from the midmyocardium of the LV and have excluded the base and apex. We cannot rule out that some of the myocytes that we studied were M cells (that have been reported to reside in midmyocardium (39), but whose presence in all mammalian species is in dispute). Even if M cells exist and have intrinsically long APD's, their contribution could easily be masked in tissue where these cells are "clamped" by adjacent cells that have

shorter APD; Recent studies showing lack of delayed repolarization in the midmyocardium of intact LV (30) supports this.

While we conducted our cellular experiments at room temperature (much like other studies in the literature (21, 25, 27, 61, 66)), similar results of sex differences in APD and I_{Ks} were found at physiological temperature. Valverde et al reported that AP duration (APD_{90}) was significantly longer in control female than in control male LV myocytes from adult rabbits at 37°C at cycle length of 5000, 1000 and 500ms (60). Moreover, the 13% difference between females and males they found are very similar to our result at room temperature where we found APD was 11.2% longer in female vs male. Our *in vivo* ECG studies also show that control females have significant longer QT_C interval than control males and that QT_C intervals were comparably shortened after ISO infusion, further supporting and validating our results.

We would assert that the mechanistic effects that we analyze at 1 Hz would be mechanistically the same at physiological pacing rate (and at 37°C), even though quantitative differences may occur. Moreover, 1 Hz may be a "physiological frequency" for room temperature in rabbit (e.g. rabbit heart rate drops from around 240 beats/min at 38°C to 64 beats/min (close to 1 Hz) at room temperature (5) and that is similar to the ~70% drop in heart rate in humans going from 37°C to room temperature (20, 28)). Since all processes are temperature-sensitive with Q_{10} in the 2–3 range this also makes sense. Indeed, for Ca transporters all are slower at room temperature *vs.* 35°C, but their relative contributions are unaltered (6).

Implications

Our findings of sex differences in I_{Ks} activity and response to β-AR stimulation, and the different mechanisms for repolarization reserve, suggest that I_{Ks} is more functionally prominent in male vs. female (both at baseline and upon β-AR stimulation). Enhancement of I_{Ks} (by direct channel effects or effects on interacting proteins that are part of the I_{Ks} macromolecular complex) may be a preferentially effective approach to prevent drug-induced QT prolongation in females. With regard to prevention of ventricular arrhythmias due to reentry, I_{Ks} blockade and possibly β-AR blockade may be more effective antiarrhythmic strategies in males than females. Indeed, our findings support the potential and feasibility of sex-specific antiarrhythmic therapy.

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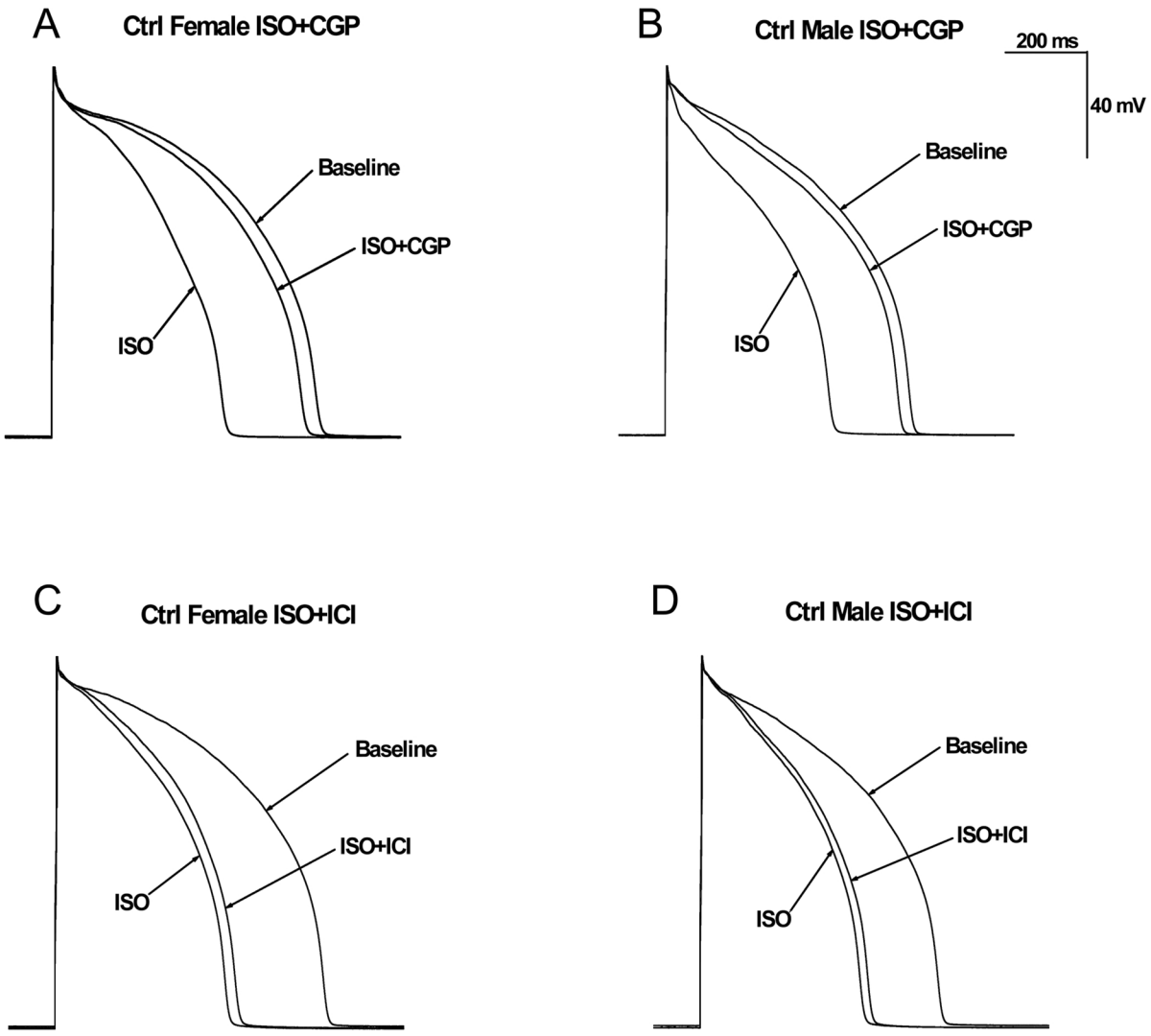

Figure 1. Effect of β_1-adrenergic and β_2-adrenergic receptor blocker on isoproterenol-induced action potential duration shortening in control male and female rabbits

A–B, Representative action potential recordings from female (A) and male (B) rabbit LV myocytes at a stimulation rate of 1 Hz at baseline, after the administration of 500nM isoproterenol (ISO) alone, or 500nM ISO plus 300nM β_1-adrenergic receptor blocker CGP 20712A (ISO+CGP). C–D, Representative AP recordings from LV myocytes from female (C) and male (D) rabbits at baseline, after 500nM ISO, or 500nM ISO + 100nM ICI-118,551 (β_2-adrenergic receptor blocker).

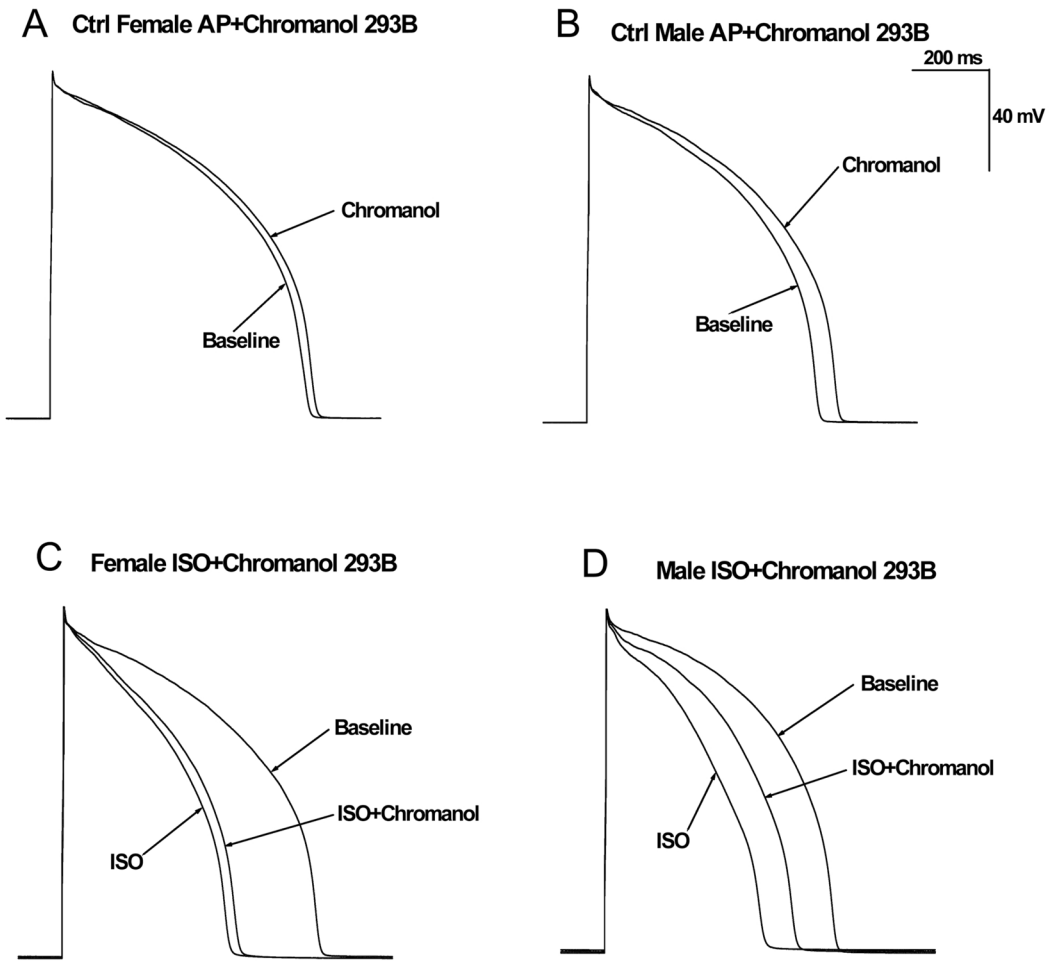

Figure 2. Effects of chromanol 293B on ventricular action potentials in the absence and presence of β-adrenergic stimulation in control male and female rabbits

A–B, Shown are representative AP's recorded in LV myocytes from a control female (A) and a control male rabbit (B) with and without 10 μM chromanol 293B (I_{Ks} blocker) at a cycle length of 1000 ms. C–D, Representative AP's recorded in myocytes from a control female (C) and a control male (D) rabbit at baseline, in the presence of 500nM ISO, and after 500nM ISO + 10 μM chromanol 293B (all at a cycle length of 1000 ms).

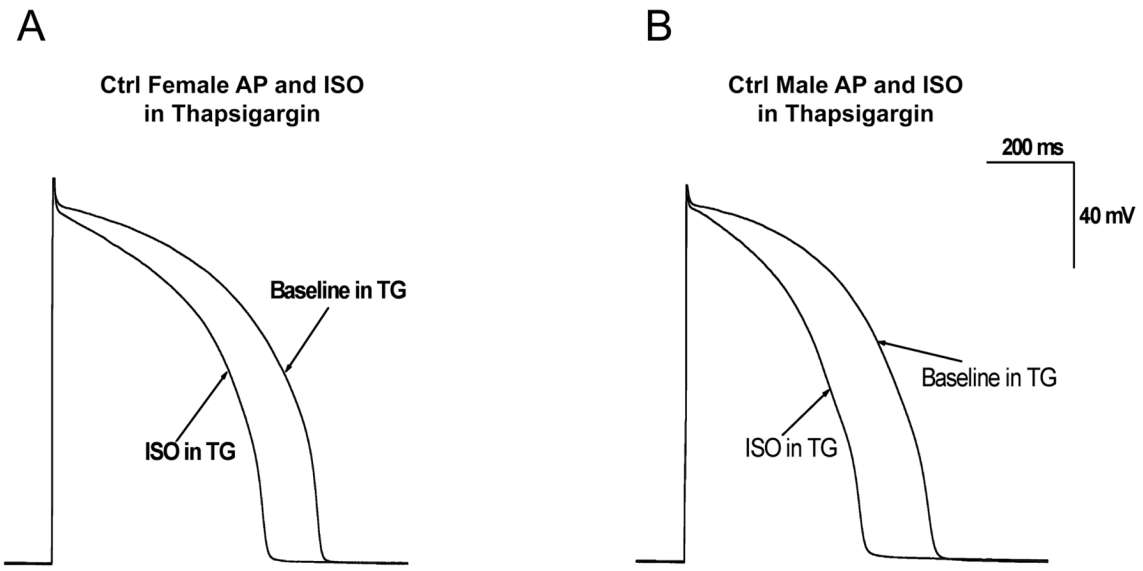

Figure 3. Effects of isoproterenol in control male and female rabbit myocytes pretreated with thapsigargin

A–B, Representative AP recordings in absence and presence of 500nM ISO from LV myocytes from control females (A) and males (B) that were pretreated with SR Ca-ATPase inhibitor thapsigargin (2 μM) for 15 min.

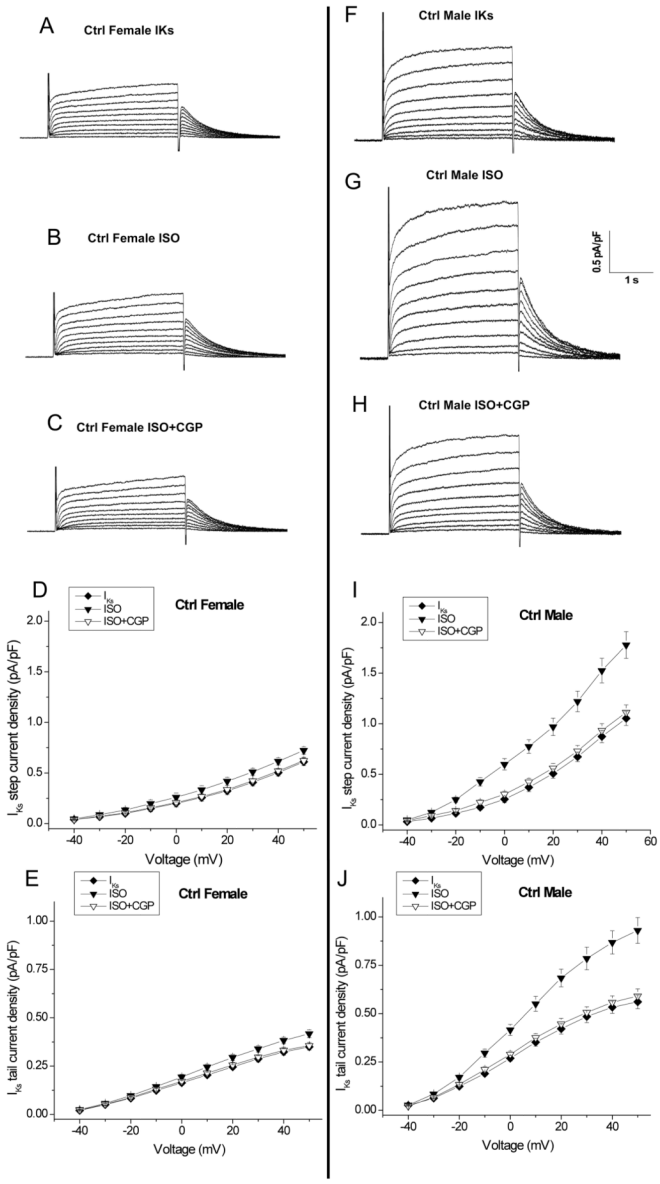

Figure 4. β_1-adrenergic stimulation regulates slow delayed-rectifier potassium current in control male and female rabbits

A–C, Representative I_{Ks} traces from control female rabbit myocytes are shown at baseline (A), in the presence of 500nM ISO (B), and after subsequent addition of 300nM CGP 20712A (β_1-adrenergic receptor blocker) (C) at test potentials ranging from −40 and +50 mV. D & E, Summarized data for step and tail current-voltage (I-V) relationship for I_{Ks} in control females (n=5 (5 hearts)). Currents are normalized to cell capacitance. Density of peak currents (mean ± SEM) were plotted vs test potentials. F–H, Representative control male I_{Ks} traces are shown at baseline, in the presence of 500nM ISO, and after subsequent addition of 300nM CGP 20712A. I–J, Summarized step and tail current-voltage (I-V) relationship for I_{Ks} at baseline and in the presence of 500nM ISO ± 300nM CGP 20712A in control male myocytes (n=9 (9 hearts)).

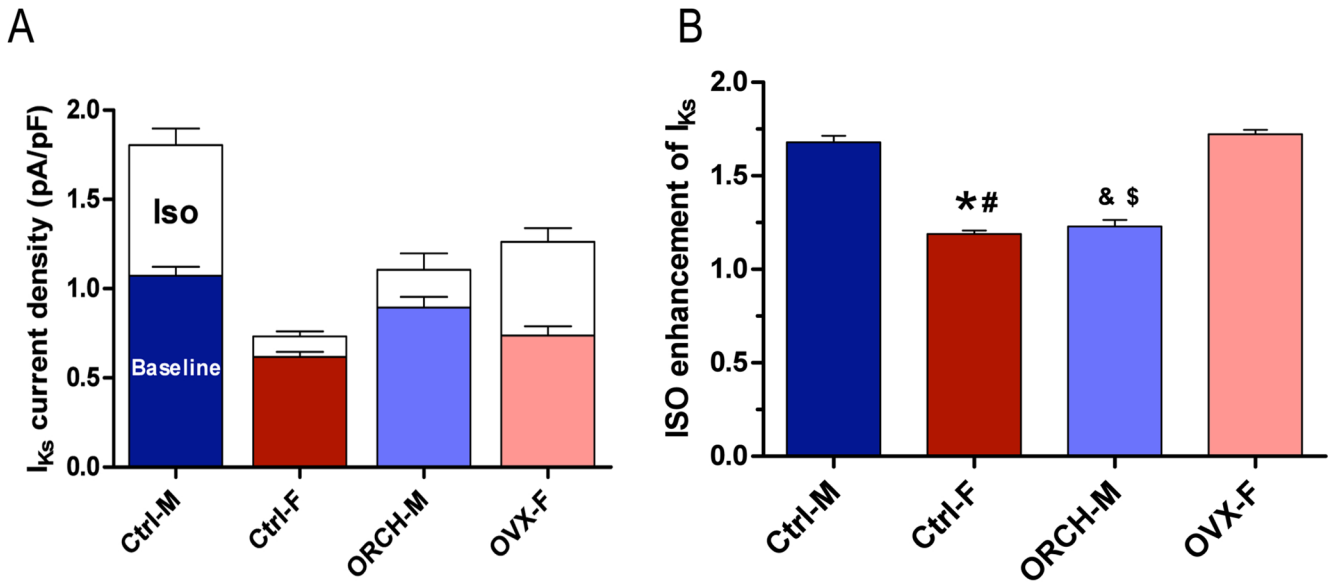

Figure 5. I_{Ks} enhancement with ISO

A, Summarized data for I_{Ks} step current at +50 mV and its response to 500nM ISO in myocytes from control male and female, orchiectomized male (ORCH-M) and ovariectomized female (OVX-F) rabbits. B, Ratio of I_{Ks} current (+50 mV test pulse) post- vs. pre- 500nM ISO application in control males, control females, ORCH males and OVX females (, $p<0.05$ vs Ctrl-M; #, $p<0.05$ vs OVX-F;&, $p<0.05$ vs Ctrl-M; $, $p<0.05$ vs OVX-F).

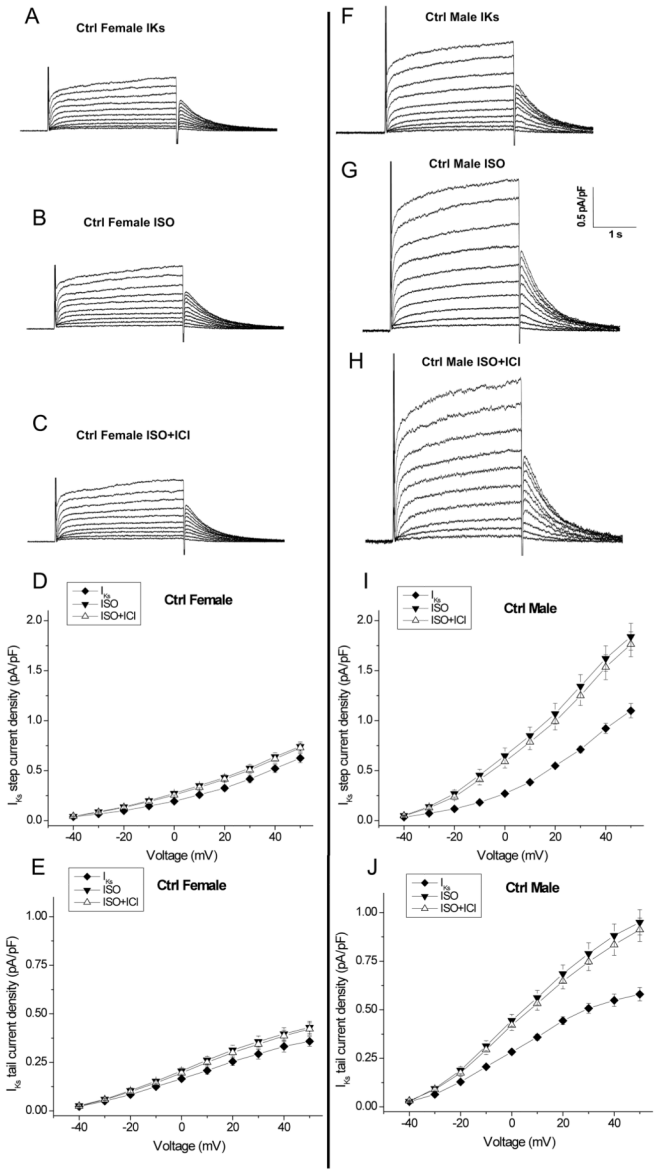

Figure 6. β_2-adrenergic stimulation enhances slow delayed-rectifier potassium current

A–C, Representative I_{Ks} traces from control females are shown at baseline and in presence of 500nM ISO ± 100nM ICI-118,551 (β_2-adrenergic receptor blocker). D–E, Summarized step and tail current-voltage (I-V) relationship for I_{Ks} in control females. F–H, Representative I_{Ks} traces from control males are shown at baseline and in presence of 500nM ISO ± 100nM ICI-118,551. I–J, Summarized step and tail current-voltage (I-V) relationship for I_{Ks} are shown at baseline and in presence of 500nM ISO ± 100nM ICI-118,551 for control males (n=7 (7 hearts)).

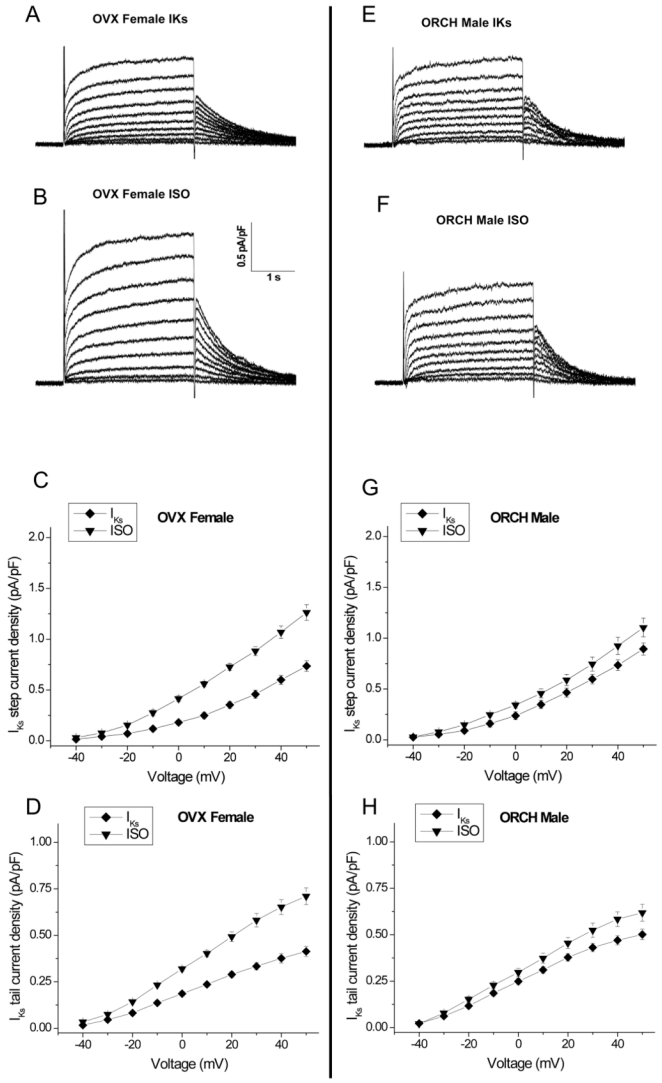

Figure 7. Effects of isoproterenol on slow delayed-rectifier potassium current in orchiectomized (ORCH) male and ovariectomized (OVX) female rabbits

A–B, Representative I_{Ks} current traces in the absence (A) or presence (B) of 500nM ISO in an OVX female rabbit. C–D, Summarized step and tail current-voltage relationships from OVX females (n=7 (5 hearts)). E–F, Representative I_{Ks} current traces in absence (E) and presence (F) of 500nM ISO in LV myocytes from an ORCH male rabbit. G–H, Summarized step and tail current-voltage relationships in ORCH males.

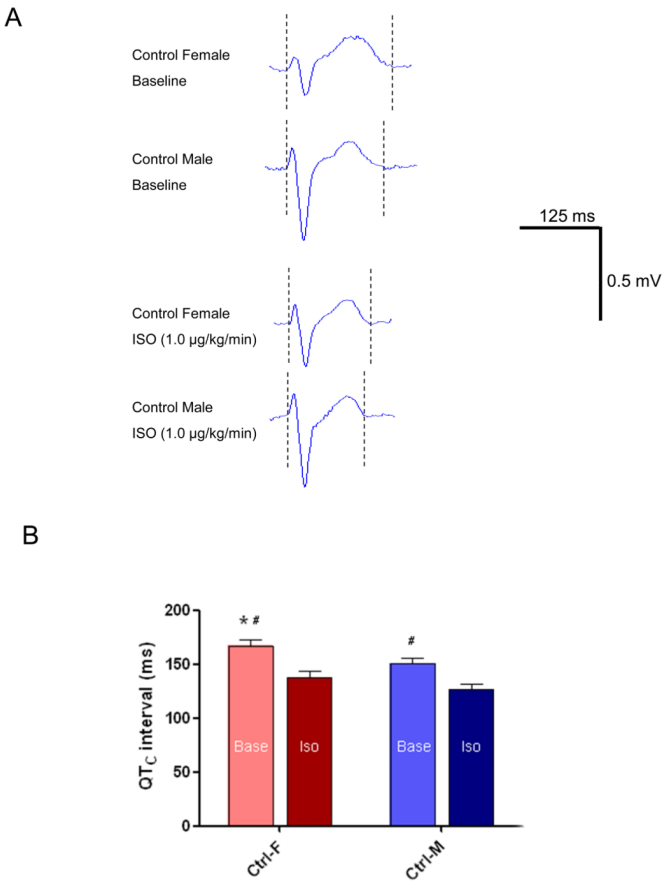

Figure 8. Sex differences in QT_C interval in response to isoproterenol

A, Representative surface ECG recordings at baseline and in response to isoproterenol (ISO, 1 μg/kg/min) in control female and male rabbits. B, Summarized data for QT_C intervals at baseline and in response to ISO from control females and males (, $p<0.05$ vs Ctrl-M; #, $p<0.05$ vs ISO).

Table 1

Comparison of APD_{90}, isoproterenol-induced APD_{90} shortening, and attenuation by CGP 20712A and ICI-118,551 in control male and female rabbit LV myocytes

	Baseline	ISO	ISO+CGP	ISO+ICI	Recovery (%)	n
Male	609.5±10.0$^{\#}$	412.9±6.9‡	585.7±9.1		87.9±0.8	6 (5 hearts)
Female	664.4±7.9$^{\#\dagger}$	426.5±10.9‡	627.1±6.0		84.3±4.8	6 (5 hearts)
Male	598.5±6.4$^{\#\dagger}$	408.3±7.6		422.4±5.5	7.4±2.0	7 (6 hearts)
Female	679.9±7.3$^{\#\dagger}$	431.7±6.5‡		455.3±5.4	9.4±0.7	7 (7 hearts)

$^{\#}$P<0.05 for Baseline vs isoproterenol (ISO).

†P<0.05 for Baseline vs ISO+CGP

‡P<0.05 for ISO vs ISO+CGP/ICI

Table 2

Effects of Chromanol 293B on APD_{90} in the absence or presence of ISO in control male and female rabbit LV myocytes

	Male				Female			
	Baseline	**Chromanol**		**n**	**Baseline**	**Chromanol**		**n**
−ISO	604.7±7.7*	658.4±6.6		8 (6 hearts)	675.5±8.3*	695.6±10.4		6 (6 hearts)
	Baseline	ISO	ISO+Chromanol	n	Baseline	ISO	ISO+Chromanol	n
+ISO	607.3±5.7#†	416.5±5.6‡	503.4±7.9	8 (7 hearts)	670.1±6.2#†	435.5±5.6‡	459.1±8.2	7 (6 hearts)

*P<0.05 for Baseline vs Chromanol

#P<0.05 for Baseline vs ISO

†P<0.05 for Baseline vs ISO+ Chromanol

‡P<0.05 for ISO vs ISO+ Chromanol