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Synergic Effects of β-Estradiol and Erythromycin on hERG Currents

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Abstract The incidence rates of long QT syndrome (LQTS) and drug-induced torsades de pointes (TDP) are higher in women than men. Although gonadal steroids are assumed to play an important role in the gender-based differences in cardiac electrophysiological properties, the underlying mechanisms of the gender-based differences are not fully understood. In particular I_{Kr}, which comprises the repolarization phase of the action potential, has not been well understood in its modulation by sex hormones. To assess this, we examined the effects of the female sex hormone β-estradiol on the human *ether-a-go-go*-related gene (*hERG*)-encoded potassium current stably expressed in human embryonic kidney-293 (HEK) cells. We demonstrated that hERG currents were inhibited by β-estradiol maximally to 62% of control with an IC_{50} of 1.3 μM and a Hill coefficient of 0.87, which might account for the sex-related differences in LQTS. We also examined whether estrogen modulated drug-induced blocking effects on hERG currents or not. With simultaneous application of 10 μM erythromycin, which is known to block hERG currents but not in low doses, the blocking effects of β-estradiol on hERG currents were enhanced. Namely, hERG currents were inhibited maximally to 45.8% of control with an IC_{50} of 59 nM ($P < 0.02$) by β-estradiol with 10 μM erythromycin. We conclude here that a significant block of hERG currents by β-estradiol may account for the sex-related differences in LQTS and the synergic effects of β-estradiol and erythromycin indicate a higher risk of drug-induced TDP in women than men.

Keywords hERG current · β-Estradiol · Sex hormone · I_{kr} · Patch clamp · Erythromycin · QT prolongation

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Introduction

Gender-based differences are familiar in cardiac electrophysiology, such as the rate corrected QT (QTc) intervals in normal cardiac repolarization, the incidence rates of congenital long-QT syndrome (LQTS) and drug-induced torsades de pointes (TDP) (Pham and Rosen 2002; Makkar et al. 1993; Sanguinetti and Tristani-Firouzi 2006; James et al. 2007). Earlier studies suggested that estrogen receptor-mediated effects play a major role in the gender-based differences in the incidence of ventricular tachyarrhythmia after myocardial infarction in humans (Cupples et al. 1992). The female sex hormone estrogen (β-estradiol) is supposed to play an important role in the expression and function of ion channels in cardiac myocytes (Du et al. 2006;

Saba et al. 2002). Animal experiments have shown that early afterdepolarizations (EADs) induced by the I_{Kr} blocker E4031 are more frequently induced in 17β-estradiol-treated rabbits than in 5α-dihydrotestosterone-treated rabbits (Hara et al. 1998). On the other hand, testosterone has been reported to diminish the proarrhythmic effects of dofetilide in female rabbits (Pham et al. 2002). Recent studies also indicate that testosterone shortens action potential duration (APD) by modulating both I_{KS} and $I_{Ca,L}$ in guinea pig hearts (Bai et al. 2005), indicating that sex hormones affect ion channels and modulate the repolarization phase of action potentials. However, the precise mechanisms of the gender-based differences in cardiac electrophysiology are not fully understood. In particular I_{Kr}, which comprises the repolarization phase of the action potential, has not been well understood in its modulation by sex hormones (Trepanier-Boulay et al. 2001; Kurokawa et al. 2008).

To prove this, we investigated whether estrogen affected I_{Kr} or modulated the drug-induced blocking effects using human embryonic kidney-293 (HEK) cells stably expressing hERG. We found that hERG currents were significantly inhibited by 17β-estradiol in a dose-dependent manner and that its blocking effects were increased while coapplication of low-dose erythromycin.

Materials and Methods

Cell Preparation and Chemicals

HEK-293 cells stably expressing hERG potassium channels (gift from Dr. Craig T. January) (Zhou et al. 1998) were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum and antibiotics at 37°C in a humidified atmosphere of 95% and 5% CO_2. On the day of the experiment, cells were gently dissociated by a pipette and stored at room temperature.

Electrophysiology

Patch-clamp experiments were performed as reported previously (Kawano et al. 2003). Briefly, using a patch-clamp amplifier (Axopatch 2A and pCLAMP8; Axon Instruments, Foster City, CA), whole-cell membrane currents were recorded. Recording electrodes were made from borosilicate glass, coated with Sylgard (Dow Corning, Midland, MI) and fire-polished to a resistance of 3–7 MΩ, when filled with internal pipette solutions. Data were stored on a hard disk, digitized at 10 KHz, low-pass-filtered at 1 KHz by a filter with Bessel characteristics (octave attenuation, 48 dB) and analyzed offline on a computer (Dell VZ-6000; Epson, Tokyo, Japan).

All experiments were performed at a temperature of 35 ± 5°C, which was maintained with a TC2 temperature controller (Cell Micro Controls, Virginia Beach, VA). The input resistance and membrane capacity were always checked at the beginning and end of experiments. We omitted data where the clamp was inadequate and membrane resistance or capacity changed during experiments.

hERG currents were recorded by applying step pulses or ramp pulses. We usually started to measure the currents at 5 min after achievement of whole-cell clamp mode because of the time required for complete replacement of the internal solution. hERG currents decreased by about 22% at 5 min after starting the experiments. In pooled data, the amplitudes of the hERG currents became about 78 ± 19% of control ($n = 22$, mean ± SE). We compensated the control values by the so-called natural rundown rate (78%) in each experiment. Using this value, we estimated the true effects of drugs on hERG currents. All experiments were analyzed using this method in this study.

Solution and Drugs

For patch-clamp experiments to record membrane currents, we used HEPES buffer bath solution containing (in mM) NaCl, 137; KCl, 4; $CaCl_2$, 1.8; $MgCl_2$, 1; and HEPES, 10. pH was adjusted to 7.4 with NaOH. Internal pipette solution contained (in mM) KCl, 130; $MgCl_2$, 1; EGTA, 5; MgATP, 5; and HEPES, 10. pH was adjusted to 7.2 with KOH. Water-soluble β-estradiol (E4389), (2-hydroxypropyl)-β-cyclodextrin solution (H5784) and erythromycin were purchased from Sigma-Aldrich (St. Louis, MO). Various concentrations of β-estradiol, such as 300 nM, 3 μM, 30 μM, 300 μM and 3 mM, were used for experiments. Erythromycin was dissolved to 10 μM. E-4031 was generously donated by Eisai (Eisai Co., Ltd., Tokyo, Japan).

Statistics

Data are expressed as mean ± SD or SE, as indicated in the text. Student's paired t-test or unpaired t-test was used to assess statistical significance. $P < 0.05$ was considered significant.

Results

Effects of β-Estradiol on hERG Currents

We investigated the effects of β-estradiol on hERG current I_{Kr} using patch-clamp methods. hERG currents were elicited by repolarizing ramp pulses (0.5 V/s) from −10 to −80 mV at 0.25 Hz, as reported previously (Wu et al. 2003; Hiramatsu et al. 2004; Sasano et al. 2004). We

confirmed hERG currents by application of E-4031 (data not shown). We examined the effects of β-estradiol on hERG currents. As shown in Fig. 1a, with 300 nM β-estradiol, hERG currents were slightly inhibited. When the higher concentration of β-estradiol (30 μM) was applied to the bath solution, the amplitudes of tail currents were clearly inhibited (Fig. 1b). Therefore, we examined the β-estradiol effects on hERG currents at various membrane potentials. Steady-state currents and tail currents were recorded. A series of 4-s depolarizing pulses were applied to voltages between −60 and +50 mV with 10-mV increments from −80-mV holding potentials and then repolarized to −50 mV at 0.1 Hz. By application of 30 μM β-estradiol, hERG currents were blocked at almost all membrane potentials (Fig. 2a, b). Next, we studied the concentration–response relationships of β-estradiol among the 300 M, 3 μM, 30 μM, 300 μM and 3 mM doses. The results showed that the higher the concentration of β-estradiol, the stronger the blocking of hERG currents (Fig. 2c, d). By analyzing the tail currents from the pooled data, we concluded that hERG currents were inhibited by β-estradiol maximally to 62% of control in a dose-dependent manner (Fig. 2c). The IC_{50} value was 1.3 μM and the Hill coefficient was 0.87 (Fig. 2d). Since it has been reported that E2 is poorly soluble in aqueous buffers (Himmel 2007), we tested whether cyclodextrin-encapsulated solution affected hERG currents using (2-hydroxypropyl)-β-cyclodextrin solution. In our experiments hERG currents were not significantly affected by application of 300 μM (2-hydroxypropyl)-β-cyclodextrin (data not shown).

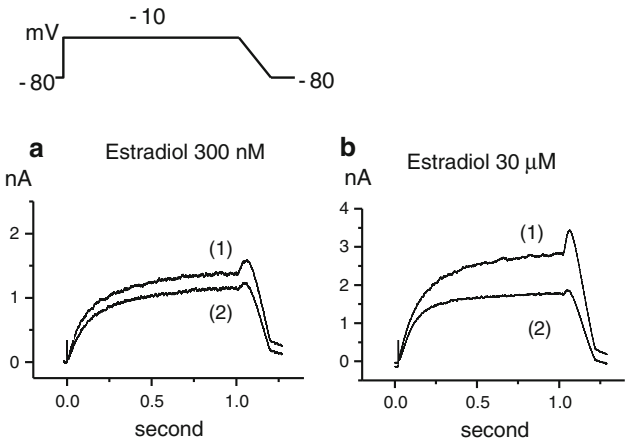

Fig. 1 Effects of β-estradiol on hERG currents. Superimposed current traces in an experiment with 300 nM (**a**) and 30 μM (**b**) β-estradiol. Whole-cell membrane currents were elicited by a 1-s depolarizing pulse to −10 mV from a holding potential of −80 mV, followed by a repolarizing ramp pulse (0/5 V/s) to −80 mV. Stimulations were applied at 0.25 Hz before and after drug. Each current shows before (*1*) and 6 min after (*2*) application of β-straddle

Blocking Properties of β-Estradiol

The blocking properties of β-estradiol were analyzed. As shown in Fig. 3a, the amplitudes of normalized tail currents in the presence of various concentrations of β-estradiol (300 nM, 3 μM, 30 μM, 300 μM and 3 mM) were blocked in a dose-dependent manner but not significantly different depending on membrane potentials between −60 and +50 mV, indicating voltage-independent block. The half-maximal activations and slope factors were analyzed. Normalized tail currents were plotted as a function of voltages (Fig. 3b), and data were fitted with a Boltzmann function: $I/I_{max} - 1/\{1 - \exp[(V_{1/2} - V_m)/S]\}$, where I represents the tail current, V_m is the test membrane potential, $V_{1/2}$ is the half-maximal activation voltage and S is the slope factor, which reflects the steepness of the voltage dependence. The voltages of half-maximal activation and the slope factors were not significantly different among these concentrations of β-estradiol.

In addition, we evaluated whether these blocking effects were use-dependent or not by applying continuous stimulations at 0.25 Hz. In the presence of 30 μM β-estradiol, as shown in Fig. 3c, hERG currents elicited by ramp pluses (0.5 V/s) from −10 to −80 mV at 0.25 Hz were gradually decreased. Thus, the amplitude of hERG current at pulse 90 was reduced to about 50% of control by application of 30 μM β-estradiol (Fig. 3d). Without continuous stimulations, the reduction of hERG currents was 50 ± 4% ($n = 7$ cells) at 6 min after application of β-estradiol, which is almost identical to that with stimulations (Fig. 3d). From these results, we concluded that the blocking effects of β-estradiol were use-independent.

Effects of β-Estradiol and Erythromycin

It is well known that several macrolides cause QT prolongation and ventricular arrhythmias (Abriel et al. 2004). Previous reports have shown that hERG currents are inhibited by various macrolides in a concentration-dependent manner (Volberg et al. 2002). It is also suggested that antibiotics induce ventricular tachycardia more frequently in females than males (Coker 2008; Pharm et al. 2008). Therefore, we hypothesized that β-estradiol might modulate the blocking effects of macrolides on hERG currents. To test this, we tested the combination effects of erythromycin and β-estradiol together on hERG currents. Since the low dose of β-estradiol or 10 μM erythromycin itself did not affect hERG currents significantly (Fig. 2), we used 10 μM erythromycin and various concentrations of β-estradiol. We found that simultaneous application of 10 μM erythromycin and 300 nM β-estradiol markedly blocked hERG currents (Fig. 4b). In Fig. 4c, the hERG current traces were superimposed in the presence of

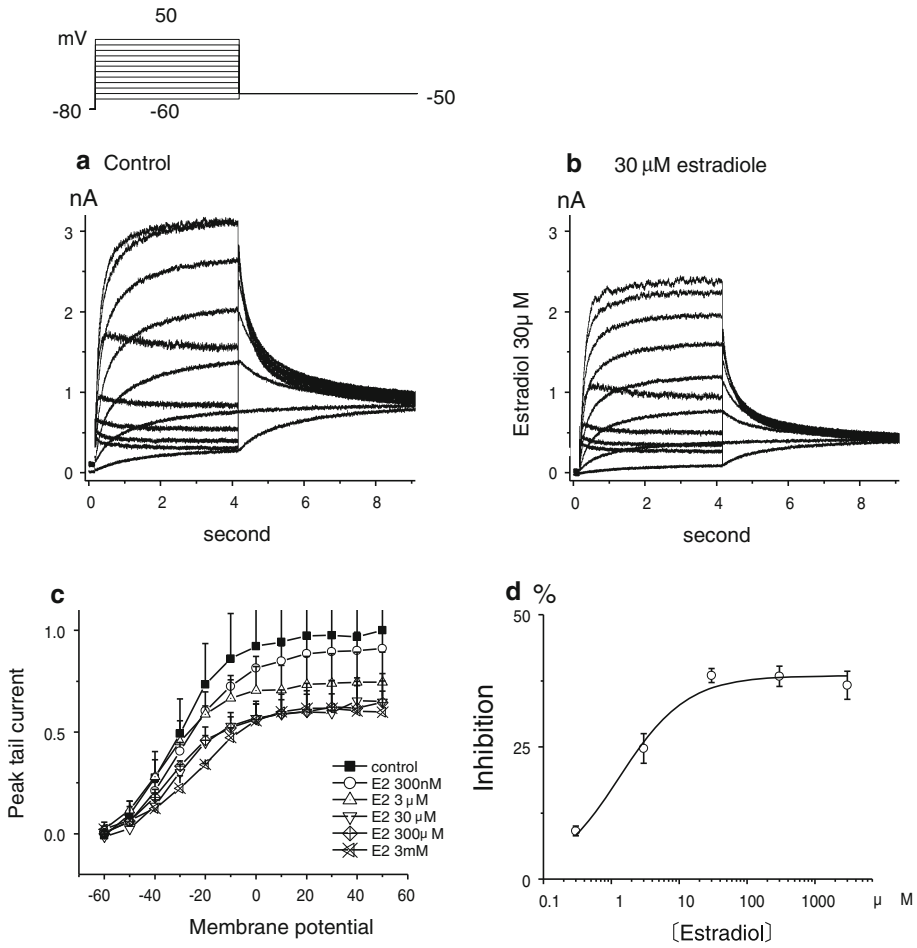

Fig. 2 β-Estradiol effects on steady-state and tail hERG currents. Currents were recorded by applying a series of 4-s depolarizing pulses to voltages between −60 and +50 mV with 10-mV increments from −80 mV holding potentials and then repolarized to −50 mV at 0.1 Hz. Currents in (**a**) and (**b**) show control and 6 min after application of 30 μM β-estradiol, respectively. In (**c**), tail currents were analyzed in the presence of various concentrations of β-estradiol. Symbols indicate control (*solid squares*), 300 nM (*open circles*), 3 μM (*open upward triangles*), 30 μM (*open downward triangles*), 300 μM (*open diamonds*) and 3 mM (*open cross-hatched triangles*), respectively. Data were normalized from five to eight experiments in each condition. In (**d**), the dose–response curve shows that the value of IC_{50} was 1.3 μM and the Hill coefficient was 0.87

300 nM β-estradiol and 10 μM erythromycin. When the two drugs were applied simultaneously, hERG currents were markedly blocked. In the presence of 10 μM erythromycin, the tail current amplitude became 65.5 ± 9.4% of control (Fig. 4a); and in the presence of 300 nM β-estradiol, it became 69.27 ± 6.7% of control, which was not much different from those in the absence of drugs (76.2 ± 16.7%). When both 10 μM erythromycin and 300 nM β-estradiol were applied together, hERG currents were markedly decreased to 45.8 ± 9.8% (Fig. 4d, e) ($n = 4$–7 cells). These results clearly indicate that simultaneous application of both β-estradiol and erythromycin markedly enhanced the blocking effects of each drug (Fig. 4e). Furthermore, examinations with various concentrations of β-estradiol in the presence of 10 μM erythromycin showed a concentration-dependent block (between 30 nM and 3 μM β-estradiol, $n = 4$–7 cells) (Fig. 4d).

The dose–response curves in the presence and absence of erythromycin were compared. In the presence of erythromycin, the half-blocking concentration was markedly shifted to the left. The IC_{50} was 1.31 μM in the absence of erythromycin and 59 nM in the presence of 10 μM erythromycin (Fig. 5). The maximal inhibition of β-estradiol was increased to 45.8 ± 9.8% ($n = 7$ cells) in the presence of erythromycin. We summarize these results in Fig. 5c. A low dose of erythromycin (10 μM) or of β-estradiol (300 nM) did not affect hERG currents; however, application of both drugs simultaneously significantly blocked hERG currents, indicating a synergic effect.

Discussion

In the present study, we clearly demonstrate that β-estradiol blocked hERG currents and that this blocking effect was enhanced by simultaneous application of erythromycin.

Blocking Effects of β-Estradiol on hERG Currents

Sex steroid hormones are known to regulate signaling pathways in the cardiovascular system (Pham et al. 2002; Du et al. 2006). Gender differences in electrophysiological properties suggest that sex hormones may directly affect

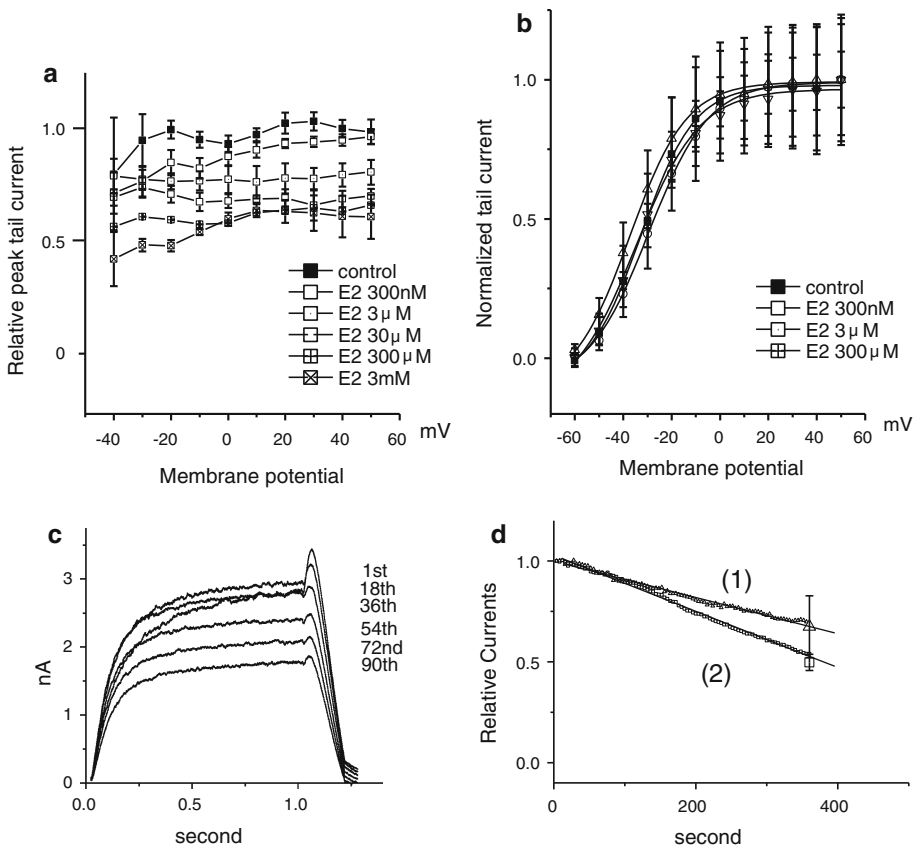

Fig. 3 Blocking properties of β-estradiol. **a** Peak late currents at various concentrations of β-estradiol were plotted. Data were obtained from five to eight experiments in each condition and normalized to the control currents at +20 mV test potential. At membrane potentials between −60 and +50 mV, the blocking by β-estradiol was not much different. **b** Voltage-dependent activations were analyzed in the presence of various concentrations of β-estradiol. Data were obtained by measuring normalized tail currents at the voltage of +20 mV and fitted with a Boltzmann function. Voltages of half-maximal activations and slope factors were not significantly different. Stimulations were applied at 0.25 Hz before and after drug. Each current shows before (*1*) and 6 min after (*2*) application of β-estradiol. **c** Superimposed current traces in the presence of 30 μM β-estradiol. The 1-s depolarizing pulse to −10 mV from a holding potential of −80 mV and a depolarizing ramp pulse (0/5 V/s) to −80 mV were applied at 0.25 Hz. Trace *1* indicates a control current and current 90 indicates one 6 min after application of β-estradiol. **d** Time courses of hERG currents were plotted and compared with and without continuous stimulations. In the presence of 300 nM β-estradiol (*1*) and 30 μM β-estradiol (*2*) amplitudes of tail currents were plotted, while applying ramp pulses at 0.25 Hz continuously. Without applying the continuous pulses, the mean amplitudes of tail currents were recorded at 6 min after application of 300 nM β-estradiol (*triangles*) and 30 μM (*squares*) (mean ± SE, $n = 7$ cells). Data in (**a**), (**b**) and (**d**) were normalized

membrane currents in the heart; however, the precise hormonal mechanism is not fully understood (Makkar et al. 1993; James et al. 2007; Coker 2008).

In electrophysiological studies, it has been reported that estrogen prolongs the QT interval and testosterone plays an important role in a shortened QT interval (Pham et al. 2002; Saba et al. 2002). Cellular examinations also demonstrate that myocytes from female mice show a prolonged action potential repolarization compared with myocytes from males (Trepanier-Boulay et al. 2001; Wu and Anderson 2002; Pham et al. 2002; Saba et al. 2002; Brouillette et al. 2005). Previously, it has been reported that testosterone regulates I_{Ks} and I_{CaL} to contribute to the QTc interval (Bai et al. 2005).

Although the hERG current is well known to involve repolarization of the cardiac action potential and to contribute to QT intervals (Sanguinetti and Mitcheson 2005; Sanguinetti and Tristani-Firouzi 2006), the modulations of hERG currents by sex hormones have not been fully evaluated. It has been reported that estradiol may modulate E4031-induced prolongation of the APD and the magnitude of EADs (Hara et al. 1998). In isolated guinea pig ventricular myocytes, 17β-estradiol prolongs the APD mainly by inhibiting the I_K components I_{Kr} and I_{Ks}, suggesting blockage of hERG currents (Tanabe et al. 1999). Recently, it was reported that physiological concentrations of E2 partially suppressed I_{Kr} (Kurokawa et al. 2008). In this study, we prove the inhibition of hERG currents by

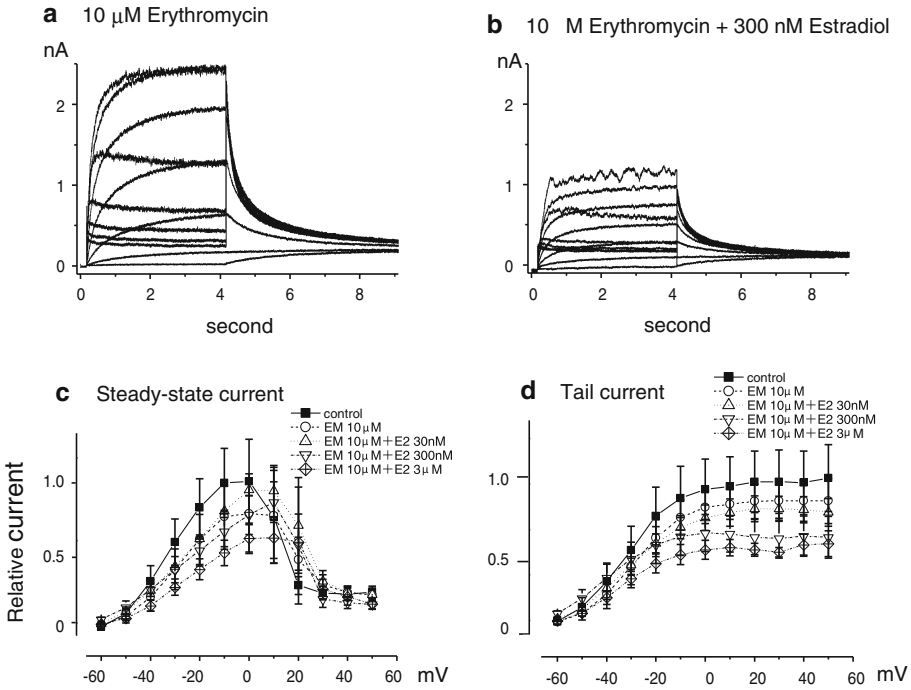

Fig. 4 Superimposed hERG currents when 10 μM erythromycin was applied (**a**) and both 10 μM erythromycin and 300 nM β-estradiol were applied (**b**). Steady-state currents (**c**) and tail currents (**d**) were plotted in the presence of 10 μM erythromycin and various concentration of β-estradiol. Control (*solid squares*), 10 μM erythromycin (*open circles*), both 10 μM erythromycin and 30 nM β-estradiol (*open upward triangles*), both 10 μM erythromycin and 300 nM β-estradiol (*open downward triangles*) and both 10 μM erythromycin and 3 μM β-estradiol (*open diamonds*) (mean ± SE, $n = 7$–8 cells). Data in (**c**) and (**d**) were normalized

β-estradiol using HEK-293 cells expressed with hERG and show a synergic effect with erythromycin, for the first time.

β-Estradiol Modulates Drug Induction

It is well recognized that many kinds of drugs, not only antiarrhythmic drugs such as class IA, IC and II but also varieties of antibiotics, neurotropic, antifungal and anti-malarial drugs, block hERG channels and prolong the repolarizing phase of the cardiac action potential to lengthen the QT interval (Volberg et al. 2002; Abriel et al. 2004; Finlayson et al. 2004; Thomas et al. 2004; Sanguinetti and Mitcheson 2005; Pharm et al. 2008; Hancox et al. 2008). A recent report demonstrates that flavonoid compounds in grapefruit juice block cardiac hERG channels and may cause prolongation of the QTc interval as a consequence (Zitron et al. 2005). These findings provide a rational basis for the potential effects of flavonoids on cardiac electrophysiology (Scholz et al. 2005). Furthermore, drug-induced LQTS and the risk of TDP are more frequent in females than males (Cupples et al. 1992; Lehmann et al. 1996; James et al. 2007; Coker 2008; Pharm et al. 2008). It is unclear whether sex-based differences in repolarization and responsiveness to I_K blockers are due entirely to gonadal steroids or are associated with other sex-related factors. In the present study, we confirmed the synergic effects of β-estradiol and erythromycin on hERG currents with simultaneous application of both drugs (Figs. 4, 5). Erythromycin is a widely used antibiotic that infrequently causes QT prolongation and TDP cardiac arrhythmias (Nattel et al. 1990). For antiarrhythmic drugs, it has been reported that women are at higher risk for these cardiac arrhythmias (Drici et al. 1998; Pharm et al. 2008). Our evidence in the present study clearly demonstrate the underlying mechanisms by which erythromycin causes a higher risk for TDP in women.

Clinical Implications

Since many I_{Kr} blocking drugs induce cardiac arrhythmias, it is very important to understand the modulation of ion channel function and how this modulation influences the response to these drugs. Our evidence in this study clarifies one of the mechanisms of the gender-based differences in cardiac electrophysiology. Therefore, the use of drugs which block I_{Kr} should be carefully considered in women. Sex-specific changes in drug transport and metabolism will result in different plasma and intracellular levels acting in a dose–response effect on I_{Kr} block. Consequently, important hormone-dependent factors such as metabolic enzymes and membrane transporters need to be investigated in new basic research studies (Hreiche et al. 2008).

We did not study the effects of progesterone or other hormones on hERG currents. Furthermore, the underlying molecular mechanisms of the β-estradiol effects on hERG currents have not been clarified yet. We need further studies.

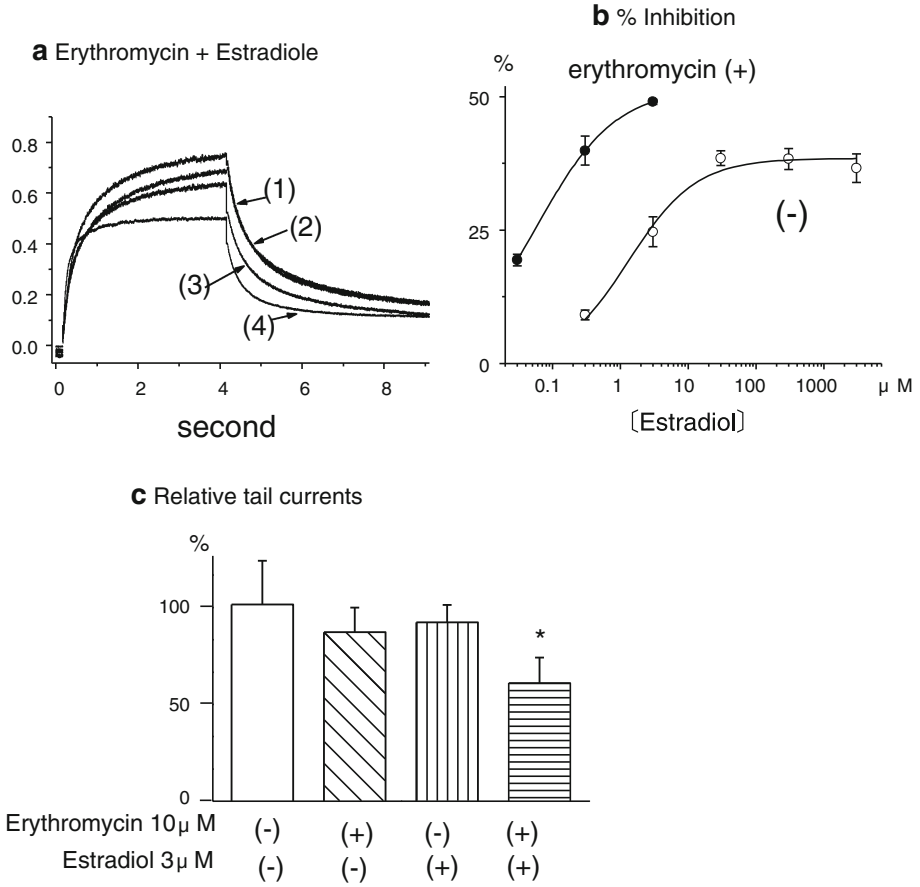

Fig. 5 **a** Superimposed currents depolarized to +10 mV test potential from −80 mV holding potentials in control (*1*), 6 min after application of 10 μM erythromycin (*2*), 3 μM β-estradiol (*3*) and both 3 μM β-estradiol and 10 μM erythromycin (*4*). Ordinate indicates relative currents normalized by control ones. **b** Dose–response curves of β-estradiol in the absence (−) and presence (+) of 10 μM erythromycin. Data were obtained by measuring normalized tail currents at a voltage of +20 mV. The apparent IC_{50} is 1.31 μM in the absence of erythromycin and 59 nM in the presence of 10 μM erythromycin. Maximal inhibition of estradiol was increased to 48% from 38% by coapplication of erythromycin. **c** Summary of blocking effects of 10 μM erythromycin or 3 μM β-estradiol on hERG currents measured by tail currents at +10 mV test potential. Data were normalized by control tail currents and indicate mean ± SE ($n = 7$ cells). Significant blocks were observed with simultaneous application of the two drugs. $^*P < 0.02$ ($n = 7$). All data were normalized

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