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Antiarrhythmic effect and its underlying ionic mechanism of 17β-estradiol in cardiac myocytes

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1 The effects of oestrogens on action potential and membrane currents were examined in single guinea-pig atrial myocytes.

2 17β-estradiol (3–10 μM) shortened the action potential duration without significant changes in the resting membrane potential. E-4031 (1 μM) markedly prolonged the action potential duration and induced early afterdepolarization, and 17β-estradiol (10 μM) abolished it.

3 When cells were perfused in isoproterenol-containing solution, action potentials due to abnormal automaticity caused by membrane depolarization developed, and were also inhibited by 17β-estradiol.

4 Under voltage clamp conditions, the voltage-dependent Ca^{2+} currents consisted of both T-($I_{Ca.T}$) and L-type ($I_{Ca.L}$). 17β-estradiol reduced $I_{Ca.L}$ concentration-dependently, while it (10 μM) suppressed $I_{Ca.T}$ only by approximately 10%. 17β-estradiol did not affect time courses of $I_{Ca.L}$ inactivation, but it shifted the steady-state inactivation curve to more negative potentials.

5 17β-estradiol (10 μM) did not affect the time-dependent K^+ current (I_K), referred to as I_{Kr} and I_{Ks}, and inwardly rectifying K^+ current. However, 17β-estradiol (30 μM) or diethylstilbestrol (10 μM) inhibited K^+ currents.

6 DES and ethinylestradiol (EES) also suppressed $I_{Ca.L}$, but testosterone and progesterone failed to inhibit $I_{Ca.L}$. The potency of the inhibitory effect on $I_{Ca.L}$ was DES > EES > 17β-estradiol.

7 17β-estradiol and DES also inhibited the cyclic AMP-enhanced $I_{Ca.L}$, but cyclic GMP in the pipette or pretreatment of L-NAME could not block the effects of oestrogen on $I_{Ca.L}$.

8 These results suggest that oestrogen specifically has antiarrhythmic effects, possibly by acting the L-type Ca^{2+} channels. The antiarrhythmic effects of oestrogens may contribute to the cardioprotective actions of oestrogens.

Keywords: 17β-estradiol; single atrial myocyte; antiarrhythmic effect; oestrogen; voltage-dependent L-type calcium current; whole cell clamp technique; diethylstilbestrol; enthinylestradiol; testosterone

Abbreviations: BAPTA, 1,2-bis(2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid; cyclic AMP, cyclic adenosine 5′-monophosphate; cyclic GMP, cyclic guanosine 5′-monophosphate; DES, diethylstilbestrol; EES, ethinylestradiol; EGTA, ethylene glycol bis-(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid; $I_{Ca.L}$, voltage-dependent L-type Ca^{2+} current; $I_{Ca.T}$, voltage-dependent T-type Ca^{2+} current; I_K, delayed outward K^+ currents; I_{Kr}, rapid I_K; I_{Ks}, slow I_K; I_{K1}, inwardly rectifying K^+ current; L-NAME, N^G-monomethyl-L-arginine; TEA, tetraethylammonium; TTX, tetrodotoxin

Introduction

Oestrogen replacement therapy reduces the risk of cardiovascular events (Godsland *et al*., 1987; Knopp, 1988) and cardiovascular mortality (Bush *et al*., 1987; Colditz *et al*., 1987) in previously healthy postmenopausal women. Also, it decreases the mortality rate in postmenopausal women with angiographically documented coronary artery diseases (Sullivan *et al*., 1988; Barrett-Connor & Bush, 1991). A number of mechanisms for the protective effect of oestrogen on cardiovascular systems have been proposed. Oestrogen has a beneficial effect on plasma lipoproteins (Bush *et al*., 1993), and inhibits endothelial hyperplasia (Fischer *et al*., 1981) and acts as a modulator of coronary vasoreactivity (Chang *et al*., 1980; Williams *et al*., 1992; Collins *et al*., 1993; 1994; Reis *et al*., 1994).

In addition to these actions of oestrogens, oestrogen therapy has been reported to decrease heart rate as well as blood pressure in humans (Luotola, 1983). Also, *in vitro*, 17β-estradiol has been shown to decrease the contraction of perfused rabbit hearts and the cell shortening in single ventricular myocytes isolated from the guinea-pig (Raddino *et al*., 1986; Jiang *et al*., 1992). The receptors for oestrogens then exist around the nucleus and cytoplasma in cardiac myocytes, especially atrial myocytes (Stumpf *et al*., 1977). These observations suggest that oestrogen may act on heart cells, which may also contribute to the cardioprotective effects of oestrogen. It has been reported that the negative inotropic effect of 17β-estradiol may be induced by inhibiting the voltage-dependent L-type Ca^{2+} current ($I_{Ca.L}$) (de Beer & Keizer, 1982; Jiang *et al*., 1992), but the effects of oestrogen on the other membrane currents and electrical activities in hearts have not been investigated in details. Furthermore, Rosano *et al*. (1996) showed that a cyclical variation of episodes of supraventricular tachycardia exists in women and to correlate such variation with cyclical variation in plasma ovarian hormones (i.e. 17β-estradiol). Therefore, the purpose of the present study is to clarify whether oestrogens have antiarrhythmic effects, and to gain insights into the underlying ionic mechanisms. Here, we provide evidence that oestrogen specifically has antiarrhythmic actions, possibly by inhibiting

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L-type Ca^{2+} channel, which may contribute to the cardioprotective effects of oestrogen.

Methods

Preparation

Single atrial myocytes were obtained from female guinea-pig hearts by enzymatic dissociation as described previously (Isenberg & Klockner, 1982; Kurachi *et al*., 1986; Nakajima *et al*., 1992). Briefly, the animals were anaesthetized with sodium pentobarbital, their hearts rapidly removed and retrogradely Langendorff-perfused at 35–37°C with an oxygenated Tyrode solution. The hearts were then perfused with Ca^{2+}-free Tyrode solution for approximately 10 min and subsequently with the same solution containing collagenase (0.04% w v^{-1} Type 1, Sigma Chemical, St. Louis, MO, U.S.A.) for 17–20 min. The digested hearts were stored in a high K^+ low Cl^- solution at 5°C for later experimentation. The atria were then removed and cells were obtained by gentle mechanical agitation. This procedure consistently yielded an acceptable number of quiescent and relaxed atrial cells.

Solution and drugs

The control Tyrode solution contained (in mM): NaCl, 136.5; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 0.53; glucose, 5.5 and HEPES-NaOH buffer 5; pH 7.4. The Ca^{2+}-free Tyrode solution was identical to the Tyrode solution, except that $CaCl_2$ was omitted. The patch pipette was filled with a solution of the following composition (in mM): K aspartate, 130, KCl, 20; KH_2PO_4, 1; disodium adenosine 5′-triphosphate (Na_2 ATP), 3; guanosine-5′-triphosphate (GTP, sodium salt, Sigma), 0.1; $MgCl_2$ 1; ethylene glycol bis-(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA), 5 and HEPES-KOH buffer, 5; pH 7.3. To record voltage-dependent Ca^{2+} currents, K^+ currents were eliminated by the internal Cs and external Ba (5 mM). The composition of the internal solution was as follows (in mM): CsCl, 140; Na_2ATP, 3; GTP, 0.1; $MgCl_2$, 1; EGTA, 5 and HEPES-CsOH, 5; pH 7.3. In experiments where the cells were held at −80 mV to record the voltage-dependent T-type Ca^{2+} current ($I_{Ca.T}$), the bath was perfused with the following solution (in mM) to block the voltage-dependent Na^+ current: Tetraethylammonium chloride (TEA-Cl), 140; $BaCl_2$, 5; $MgCl_2$, 0.53; glucose, 5.5; tetrodotoxin (TTX), 0.01 and HEPES-CsOH buffer, 5; pH 7.4. And, the patch pipette was filled with high EGTA and 1,2-bis(2-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid (BAPTA)-containing solution to lesson intracellular Ca^{2+} sufficiently (in mM): CsCl, 140; EGTA, 10; BAPTA, 2; Na_2ATP, 3; GTP, 0.1; $MgCl_2$, 1 and HEPES-CsOH buffer, 5; pH 7.3. the high K^+/low Cl^- solution contained (in mM): K glutamate, 50; KCl, 40; KH_2PO_4, 20; taurine, 20; oxalic acid, 10; $MgCl_2$, 3; EGTA 0.5 and HEPES-KOH buffer, 10 (pH 7.4).

17β-estradiol, diethylstilbestrol (DES), ethinylestradiol (EES), testosterone, progesterone, (±)-isoproterenol, cyclic adenosine 5′-monophosphate (cyclic AMP), cyclic guanosine 5′-monophosphate (cyclic GMP) and N^G-monomethyl-L-arginine (L-NAME) were purchased from Sigma (St Louis, MO, U.S.A.). Each hormone was dissolved in ethanol to give a stock solution of 10 mM. Ethanol (1 : 2000 v v^{-1}) was usually added to the normal Tyrode solution. Various concentrations of each substance was added to the bathing solution. E-4031 was a gift from Eisai Pharmaceutical Co. Ltd (Tokyo, Japan).

Recording technique and data analysis

The action potential and membrane currents were recorded with glass pipettes in the whole-cell clamp condition (Hamill *et al*., 1981), using a patch-clamp amplifier (EPC-7, List Electrics Darmstadt, Germany). The heat-polished patch pipettes, filled with an artificial internal solution, had a tip resistance of 3–6 MΩ. Membrane potential and currents were continuously monitored with a high-gain storage oscilloscope (COS 5020-ST, Kikussi Electronics, Tokyo, Japan). At the start of each experiment, the series resistance was compensated. The data were stored on video cassettes using a PCM converter system (RP-880, NF electronic circuit design, Tokyo). Later, the data were reproduced, low-passed filtered at 2 kHz (−3 dB) with a

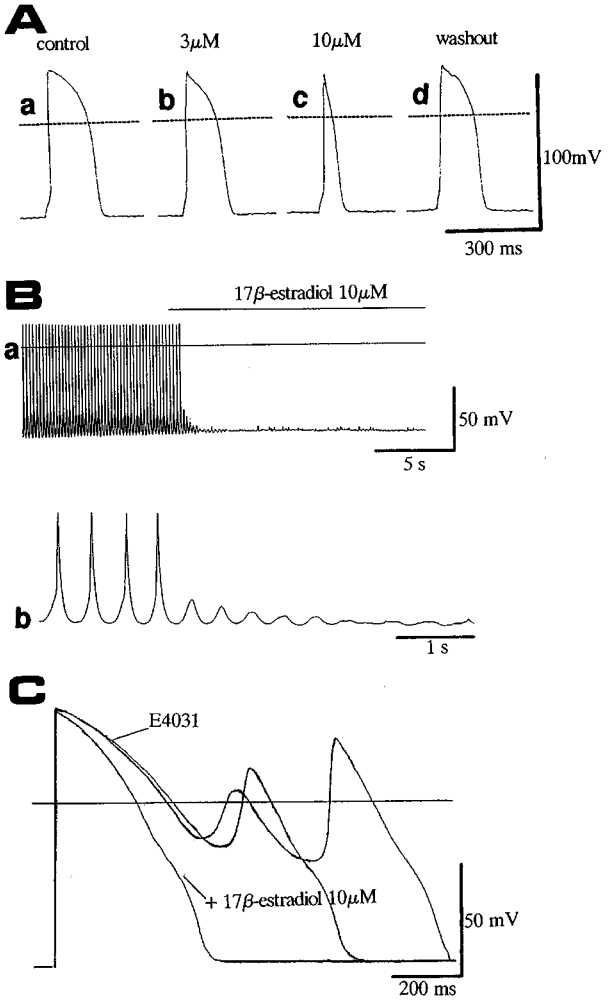

Figure 1 Effects of 17β-estradiol on action potential and its antiarrhythmic actions in single atrial myocytes. (A) Effects of 17β-estradiol on action potential. The action potentials were elicited at a stimulation frequency of 0.2 Hz. Typical action potentials recorded in control (a), after application of 3 μM (b), 10 μM 17β-estradiol (c) and after the washout (d) are indicated. The zero membrane potential level is indicated by the dashed lines. (B) Effects of 17β-estradiol on abnormal automaticity in a cell perfusing isoproterenol (1 μM) containing solution. Note that action potentials due to abnormal automaticity were abolished by 17β-estradiol (10 μM). The trace of (b) is time-expanded. (C) Effects of 17β-estradiol on E-4031-induced early afterdepolarization and triggered potentials. Action potentials were elicited at a stimulation of 0.1 Hz in a cell treated with E-4031 (1 μM). Note that 17β-estradiol (10 μM) abolished the development of early afterdepolarization from a potential of −20∼ −40 mV. The zero membrane potential is indicated by a line.

Bessel filter (FV-665, 48 dB/octave slope attenuation), sampled at 5 kHz and analysed off-line on a computer using p-Clamp software (Axon Instruments, CA, U.S.A.). In general, we used a holding potential of -40 mV to inactivate the voltage-dependent Na^+ current. In experiments to evaluate the contribution of T-type Ca^{2+} currents ($I_{Ca.T}$), a holding potential of -80 mV was used in combination with the high TEA solution containing Ba^{2+} (5 mM) in place of Ca^{2+} and the patch pipette was filled with high EGTA and BAPTA-containing solution. Statistical results are expressed as means $\pm$ s.d. Student's *t*-tests were performed, with a value of $P<0.05$ considered significant.

To measure the amplitude of the voltage-dependent Ca^{2+} currents, we subtracted from the peak amplitude of Ca^{2+} currents in the original trace to the current level in the presence of Cd^{2+} (1 mM). For obtaining the steady-state activation curve (d_∞), the current elicited by a depolarizing pulse (I_{test}) from a holding potential of -40 mV was divided by driving force and normalized. The steady-state inactivation parameters (f_∞) of the voltage-dependent L-type Ca^{2+} currents ($I_{Ca.L}$) were analysed with double-pulse protocols. Conditioning voltage pulses (3 s in duration) for various membrane potentials (V_{test} between -50 mV to $+10$ mV were followed by a 10 ms step to the holding potential (-50 mV); then, the current (I_{test}) elicited by a depolarization to the evaluation potential ($+10$ mV) for 200 ms was measured. The ratio between the amplitude of the Ca^{2+} currents with the conditioning pulse (I_{test}) and that without conditioning pulse ($I_{test\ (max)}$) was plotted for the membrane potential of each conditioning pulse. The interval between sets of double pulses was 20 s.

In a series of experiments to record time-dependent K^+ currents (I_K), Na^+ current was inactivated by holding potential at -40 mV; L-type Ca^{2+} current was inhibited by perfusing nifedipine (1 μM). In addition, to further separate I_{Kr} (rapid I_K) and I_{Ks} (slow I_K) and record I_{Ks}, extracellular Ca^{2+} was removed to shift I_{Ks} activation to more positive potentials (Sanguinetti & Jurkiewicz, 1992).

Results

Antiarrhythmic effects of 17β-estradiol

Figure 1A shows the effects of 17β-estradiol on action potential in single atrial myocytes obtained from the guinea-pig. 17β-estradiol (3, 10 μM) dose-dependently shortened the action potential duration (Figure 1Ab, c), but it failed to affect the resting membrane potential significantly, which were compatible with the previous reports in atrial myocytes (de

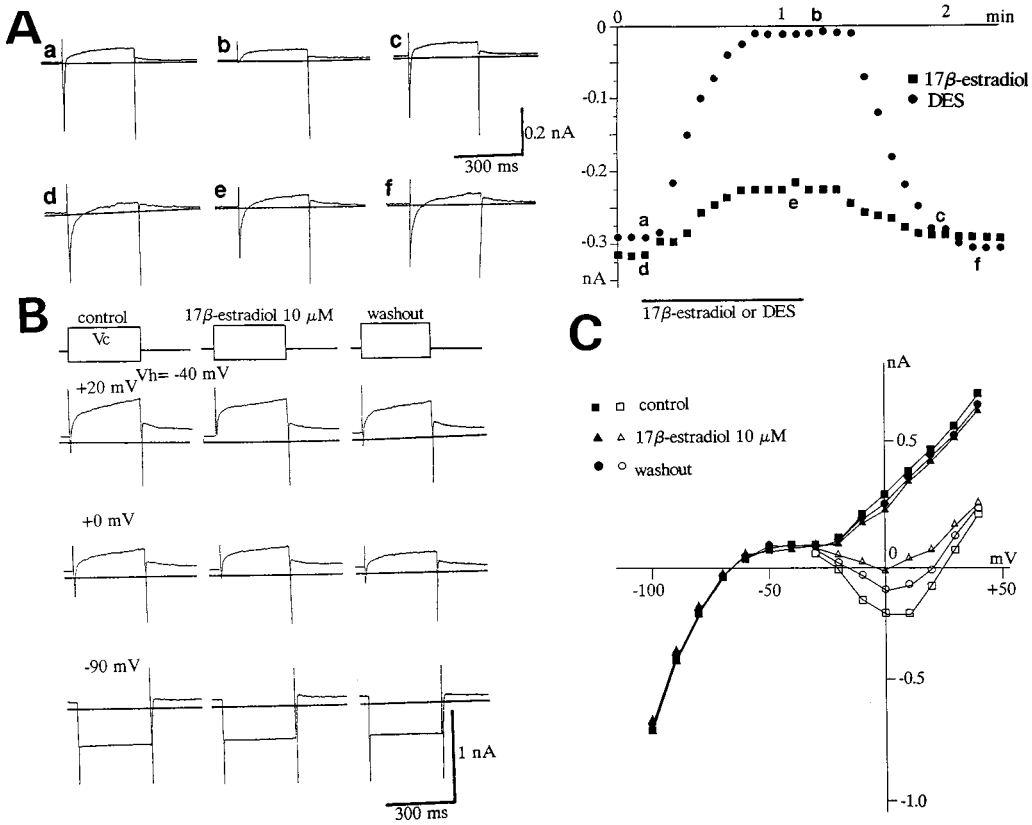

Figure 2 (A and B) Effect of 17β-estradiol and diethylstilbestrol on the L-type Ca current ($I_{Ca.L}$) in single atrial myocytes. (A) Data were obtained from two different cells. The cells were held at -40 mV and the command voltage steps to $+0$ mV were applied at 0.2 Hz. The current traces indicated in (A) were recorded in control (a, d), after application of 17β-estradiol (10 μM, e) or diethylstilbestrol (DES, 10 μM, b), and after the washout (c, f). The zero current levels are shown by dotted lines. The time course of changes in the peak $I_{Ca.L}$ measured from the zero current level is shown in the right part. The location of each current trace (a–f) in (A) is illustrated by (a–f) in the right part. The protocols for perfusing 17β-estradiol or DES are indicated by the bars. (B) Effects of 17β-estradiol on membrane currents. The voltage pulses (320 ms in duration) to various membrane potentials (V_c) were applied from a holding potential of -40 mV at 0.2 Hz. The original current traces are recorded in control, after application of 17β-estradiol (10 μM) and after the washout of 17β-estradiol. (C) The current voltage relationships measured at the peak of the inward current and at the end of the pulse are indicated in control, in the presence of 17β-estradiol (10 μM) and after the washout.

Beer & Keizer, 1982) and guinea-pig ventricular myocytes (Jiang *et al*., 1992). The effect of 17β-estradiol on action potential was reversible as indicated in Figure 1Ad. The similar results were obtained from four different cells tested. In addition, to investigate whether oestrogen has antiarrhythmic effects, we examined the effects of 17β-estradiol on isoproterenol-induced abnormal automaticity or E-4031-induced early afterdepolarization (Figure 1B and C). In the presence of isoproterenol (1 μM), action potentials were elicited spontaneously due to abnormal automaticity (Figure 1B), probably by depolarization of the membrane caused by isoproterenol (Imanishi & Surawicz, 1976; Katzung & Morgenstern, 1977; Harvey *et al*., 1990). When 17β-estradiol (10 μM) was added into the bathing solution, the action potentials induced by abnormal automaticity were abolished. Figure 1C shows the actions of 17β-estradiol (10 μM) on E4031-induced early afterdepolarization and triggered potentials. In a cell treated with E-4031 (1 μM), a class III antiarrhythmic agent (Sanguinetti, 1992), early afterdepolarization was frequently observed. In this condition, when 17β-estradiol (10 μM) was applied, the early afterdepolarization and the repetitive responses were abolished (Figure 1C). 17β-estradiol (10 μM) shortened the action potential, and then inhibited the early afterdepolarization and triggered potentials. Similar results were obtained from another three different cells. These results provide evidence that 17β-estradiol may act as an antiarrhythmic agent.

Inhibitory effect of oestrogens on the voltage-dependent L-type Ca^{2+} current ($I_{Ca.L}$)

To clarify the ionic mechanisms of oestrogen on action potentials and its antiarrhythmic effects, we examined the effects of 17β-estradiol (10 μM) or diethylstilbestrol (DES), a synthetic oestrogen (10 μM), on $I_{Ca.L}$. The cells were held at -40 mV and command voltage steps (320 ms in duration) to $+0$ mV were applied every 5 s. In control (Figure 2Aa and d), a transient inward current and a subsequent outward current were evoked during each voltage pulse. The inward current was blocked by verapamil (1 μM) and nifedipine (100 nM), indicating that it was $I_{Ca.L}$. After application of 17β-estradiol (Figure 2Ae) or DES (Figure 2Ab), $I_{Ca.L}$ decreased and reached to a steady level. DES (10 μM) decreased $I_{Ca.L}$ by $78\pm9\%$ ($n=4$), and 17β-estradiol (10 μM) decreased it by $32\pm8\%$ ($n=4$). The time-course of the peak $I_{Ca.L}$ measured from the zero current level is shown in the right part of Figure 2A. After the washout, $I_{Ca.L}$ gradually returned to the control level (Figure 2Ac and f). The effects of 17β-estradiol on membrane currents were investigated at various membrane potentials in Figure 2B. The cell was held at -40 mV, and command voltage pulses (320 ms in duration) were applied to various membrane potentials at every 5 s. $I_{Ca.L}$ and the delayed outward current (I_K) were elicited during depolarizing steps. The tail current for I_K was also observed upon returning to the holding potential. The current/voltage (I-V) relationships

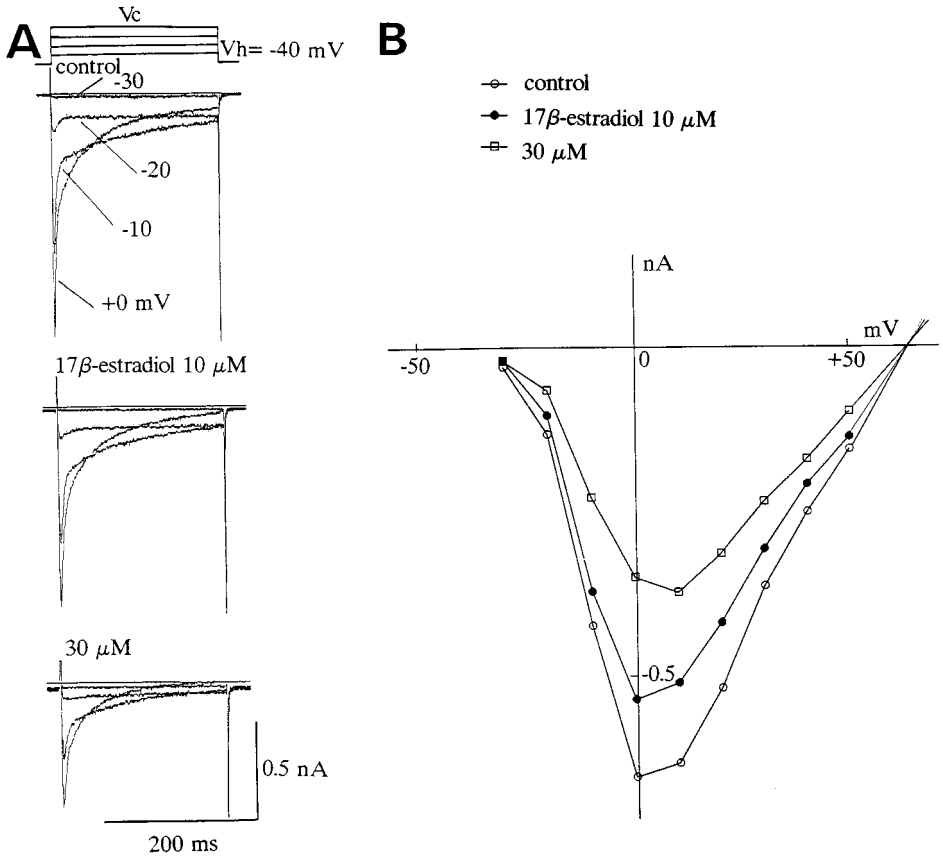

Figure 3 Effect of 17β-estradiol on $I_{Ca.L}$. The patch pipette contained CsCl-internal solution and 5 mM $BaCl_2$ was added into the normal Tyrode solution to block K^+ currents. The cell was held at -40 mV and the command voltage pulses to various membrane potentials were applied at 0.2 Hz. The original current traces shown in (A) were recorded in control and after application of 17β-estradiol (10 and 30 μM). (B) The current/voltage (I-V) relationships measured at the peak of the inward current are plotted in control and after application of 17β-estradiol (10 μM) and 30 μM.

measured at the peak of the inward current and at the end of the pulse are shown in Figure 2C. The I-V relationship between −40 mV and −100 mV did not differ significantly before and during application of 17β-estradiol, suggesting that 17β-estradiol (10 μM) did not affect the inward rectifier K^+ current (I_{K1}). Also, 17β-estradiol (10 μM) did not affect I_K significantly. However, it decreased the amplitude of $I_{Ca.L}$ at each command voltage. After the washout, $I_{Ca.L}$ returned to a control level. To clarify the effect of oestrogen on $I_{Ca.L}$, CsCl-containing pipette solution was used and 5 mM $BaCl_2$ was added to normal Tyrode solution to block K^+ currents. Figure 3 illustrates the effect of 17β-estradiol (10 and 30 μM) on $I_{Ca.L}$. The I-V relationship measured at the peak is indicated in Figure 3B. 17β-estradiol (10 and 30 μM) decreased the amplitude of $I_{Ca.L}$ without producing any significant change in the current-voltage relationships. On average, 10 μM 17β-estradiol decreased peak $I_{Ca.L}$ at +0 mV by 29±8% ($n=5$), and 30 μM 17β-estradiol decreased it by 49±5% ($n=5$). However, the extrapolated reversal potential for $I_{Ca.L}$ in each case was approximately +63 mV. Thus, the reversal potential was not altered by 17β-estradiol significantly (+59±5 mV ($n=4$) in the control vs +58±6 mV ($n=4$) and +57±4 mV ($n=4$) in the presence of 10 and 30 μM 17β-estradiol). These results suggest that 17β-estradiol lower than 10 μM selectively decreases $I_{Ca.L}$ in atrial myocytes isolated from the guinea-pig, which underlies the ionic mechanisms of the action potential shortening induced by 17β-estradiol.

Effects of 17β-estradiol on the kinetic parameters of $I_{Ca.L}$

Figure 4A shows the effects of 17β-estradiol on the inactivation time course of $I_{Ca.L}$. Under the conditions where the cell was perfused with normal Tyrode solution, the inactivation time courses of $I_{Ca.L}$ were well fitted by the sum of two exponentials as previously described (Nakajima *et al*., 1991). 17β-estradiol (30 μM, Figure 4A)) decreased the amplitude of both fast component (A1) and slow component (A2), but did not affect the time courses of inactivation of $I_{Ca.L}$ significantly. The differences between the values of Tau1 and Tau2 in the control (4.5±0.7 ms and 42±12 ms ($n=4$)) and those in the presence of 17β-estradiol (30 μM) (4.6±0.8 ms and 45±8 ms ($n=4$)) were not statistically significant.

Furthermore, the effects of 17β-estradiol on the steady-state variables (d_∞ and f_∞) were examined in Figure 4B. For obtaining the steady-state activation curve (d_∞), the current amplitude of $I_{Ca.L}$ elicited by a depolarizing pulse (I_{test}) from a holding potential of −40 mV was divided by driving force and normalized. The activation curve was fitted by Boltzmann equation. The computer fit shows the same values for the slope of the curve (k, −6.5±2.0 mV ($n=4$) in the control vs −6.9±2.3 mV ($n=4$) and −7.2±2.1 mV ($n=4$) in the presence of 10 and 30 μM 17β-estradiol) and the potential of half maximal activation (Vh, −8±4 mV ($n=4$) in the control vs −9±3 mV ($n=4$) and −7 mV±5 mV ($n=4$) in the presence of 10 and 30 μM 17β-estradiol). Figure 4B also

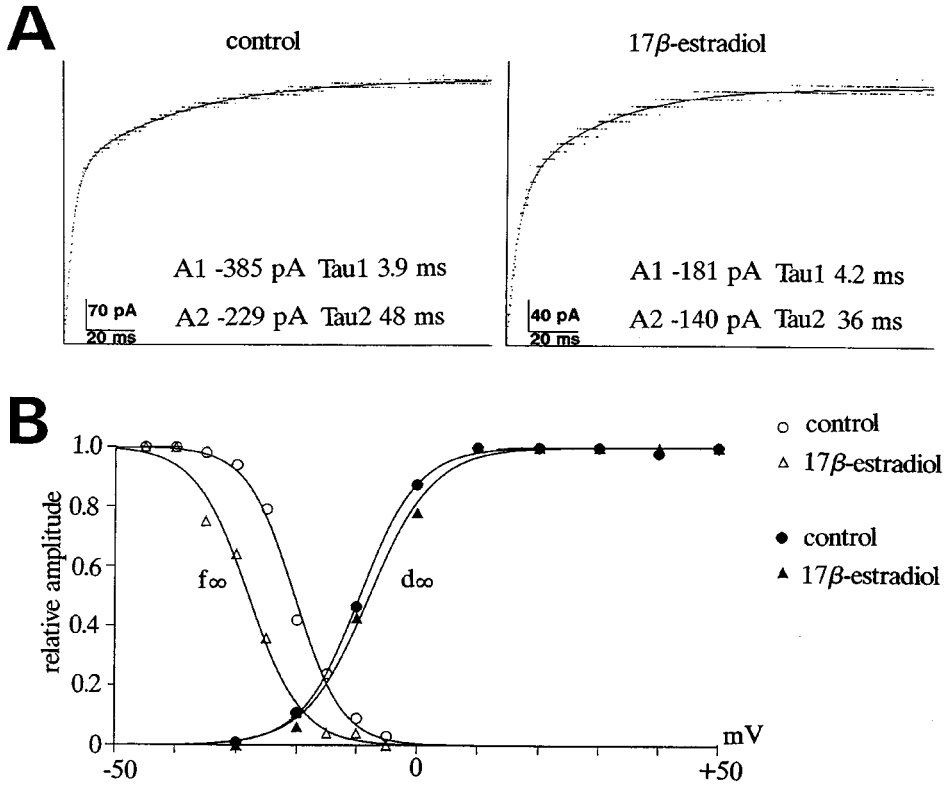

Figure 4 Effects of 17β-estradiol on the kinetic properties of $I_{Ca.L}$. (A) Effects of 17β-estradiol (30 μM) on the time courses of inactivation of $I_{Ca.L}$. The computer curve-fitting algorithm was used to determine the fast (Tau1) and slow (Tau2) time constants for inactivation of $I_{Ca.L}$ before and application of 17β-estradiol (30 μM). The cell was held at −40 mV, and the command voltage pulse to +0 mV was applied. The model used was the sum of two exponentials where A1 and A2 are the amplitude of the fast and slow components plus A3 as follows: $I(t) = -A1.\exp(-t/Tau1) - A2.\exp(-t/Tau2) + A3$; where t, the time in ms after the onset of command voltage steps; $I(t)$, the current value of $I_{Ca.L}$ at t. (B) Effects of 17β-estradiol on steady-state parameters of $I_{Ca.L}$. Effects of 17β-estradiol (30 μM) on steady-state activation variable (d_∞) and inactivation variable (f_∞) are shown. Procedures used are described in 'Methods'. Parameter values of d_∞ are control, Vh = −9.4 mV, k = 4.8 mV; 30 μM 17β-estradiol, Vh = −7.8 mV, k = 5.3 mV. Parameters values of f_∞ are control, Vh = −20.3 mV, k = 4.0 mV; 30 μM 17β-estradiol, Vh = −28.0 mV, k = 4.4 mV where Vh, the membrane potential of the half maximal activation or inactivation; k, the slope factor.

showed the effects of 17β-estradiol on the steady-state inactivation curve, by using the double-pulse protocol. To limit our measurement of Ca^{2+} current to the contribution of only $I_{Ca.L}$, the test pulse (200 ms in duration) to +10 mV from a holding potential of −50 mV was preceded by a 3 s conditioning pulse to various membrane potentials. The relationships between the membrane potential and the f_∞ value in the absence and presence of 17β-estradiol were fitted by the Boltzman equation. In the absence of the drug, the slope factor (k) and the membrane potential of the half maximal inactivation (Vh) were 5.6±1.8 mV and −21±3 mV ($n=5$), respectively. 17β-estradiol (10 and 30 μM) did not alter the slope factor (6.0±2.2 mV and 6.3±2.6 mV ($n=5$) in the presence of 10 and 30 μM 17β-estradiol), but it shifted the steady-state inactivation curve to more negative potentials. On average, 10 and 30 μM 17β-estradiol shifted the membrane potential of the half maximal inactivation to more negative values (−24±3 mV ($n=5$, $P<0.05$) and −28±2 mV ($n=5$, $P<0.05$) in the presence of 10 and 30 μM 17β-estradiol).

Effects of various oestrogens and the related sex hormones on $I_{Ca.L}$

Figure 5 indicates the effects of various oestrogens and the related sex hormones on $I_{Ca.L}$. The cells were held at −40 mV and the command voltage pulses to +0 mV were applied. Progesterone (30 μM, Figure 5Ac) and testosterone (30 μM, Figure 5B) did not affect $I_{Ca.L}$ significantly, but 17β-estradiol (30 μM, Figure 5Aa) and diethylstilbestrol (DES, 30 μM, Figure 5A, b) decreased $I_{Ca.L}$. The concentration-response relations for the inhibotory effect of these agents on $I_{Ca.L}$ are plotted in Figure 5B. 17β-estradiol, DES and ethinylestradiol (EES) inhibited $I_{Ca.L}$. The inhibitory effect of oestrogens on $I_{Ca.L}$ was reversible and the potency among these agents was DES > EES > 17β-estradiol. The inhibitory effect of these agents on $I_{Ca.L}$ was observed at concentrations more than 0.3 μM (Figure 5B). DES (30 μM) almost completely inhibited $I_{Ca.L}$ as Cd^{2+} (0.5 mM) did (Figure 5Aa). On the other hand, both progesterone and testosterone (30 μM) did not significantly affect $I_{Ca.L}$, indicating that $I_{Ca.L}$ is specifically inhibited by oestrogen in cardiac myocytes.

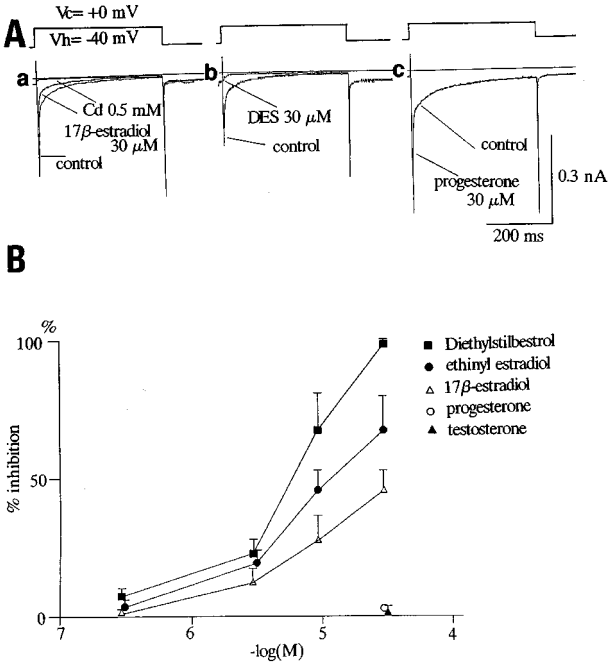

Figure 5 Inhibitory effect of oestrogens and the related sex hormones on $I_{Ca.L}$ in cardiac myocytes. (A) The original current traces obtained from two different cells are illustrated. The cell was held at −40 mV, and the command voltage steps to +0 mV were applied at 0.2 Hz. Effects of 17β-estradiol (30 μM, a), Diethylstilbestrol (DES, 30 μM, b) and progesterone (30 μM, c) are shown. (B) The concentration-response relations for the effect of oestrogens (17β-estradiol, diethylstilbestrol, and ethinylestradiol and the related sex hormones (progesterone and testosterone). The amplitude of $I_{Ca.L}$ in the presence of these agents were compared with that of I_{Ca} in control, and the per cent inhibition was plotted. Each point represents mean±s.d. obtained from four different cells.

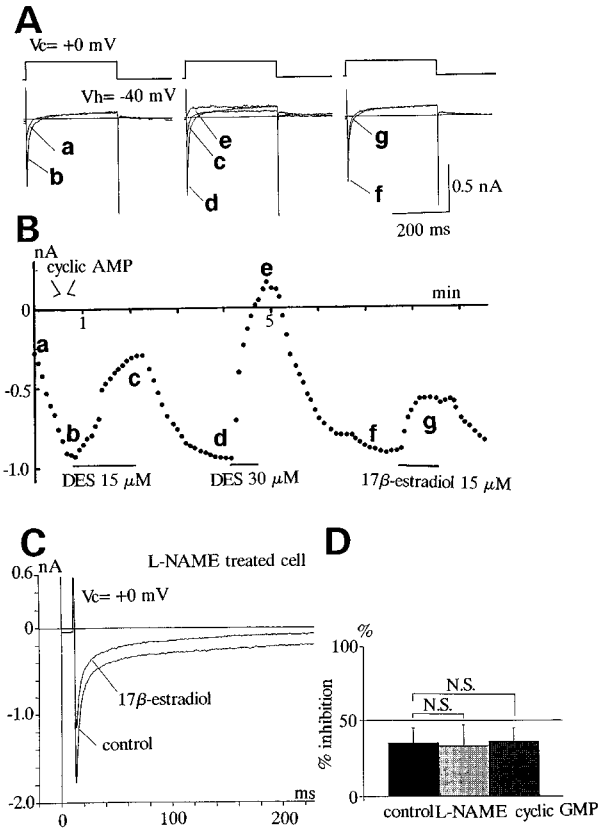

Figure 6 (A and B) Effect of 17β-estradiol and diethylstilbestrol (DES) on cyclic AMP-enhanced $I_{Ca.L}$. The patch pipette contained cyclic AMP (100 μM). The cell was held at −40 mV and the command voltage pulses to +0 mV were applied at 0.2 Hz. The current trace (a) was recorded immediately after the rupture of the membrane. Note that when cyclic AMP was inserted into a cell, $I_{Ca.L}$ gradually increased and reached to a steady-state level (b). The current traces (c–g) were recorded after 15 μM DES (c), after the washout (d), 30 μM DES (e), after the washout (f) and after 15 μM 17β-estradiol (g), respectively. (B) The time courses of the changes of the peak $I_{Ca.L}$ measured from the zero current level are indicated. The location of each current trace (a–g) in (A) is indicated by (a–g) in (B). The bars in the graphs are the protocols for perfusing DES or 17β-estradiol. (C and D) Effects of L-NAME or cyclic GMP on the inhibitory effects of 17β-estradiol on $I_{Ca.L}$. In C, the cell was pretreated with L-NAME (1 mM) for approximately 1 h. The cell was held at −40 mV, and command voltage steps to +0 mV were applied at 0.2 Hz. The current traces were shown in control and in the presence of 17β-estradiol (10 μM) in a cell treated with L-NAME. (D) The per cent inhibition by 17β-estradiol (10 μM) is shown in control cells, cells treated with L-NAME (1 mM) or cells loaded with cyclic GMP (100 μM) in the patch pipette. Cyclic GMP (100 μM) was loaded in cells through the patch pipette. The data are shown as mean±s.d. value obtained from five different cells.

Effects of oestrogens on cyclic AMP-enhanced $I_{Ca.L}$

Figure 6A and B show the effect of 17β-estradiol and DES on cyclic AMP-enhanced $I_{Ca.L}$. The patch pipette contained 100 μM cyclic AMP. After breaking the patch membrane, the amplitude of $I_{Ca.L}$ quickly increased and reached to a steady-level, due to the phosphorylation of $I_{Ca.L}$ (Figure 6Ab). DES (15 and 30 μM, Figure 6Ac and e) and 17β-estradiol (15 μM, Figure 6Ag) significantly decreased $I_{Ca.L}$. These results suggest that the inhibitory effect of oestrogens on $I_{Ca.L}$ is independent of cyclic AMP. Also, inclusion of GDPβS (2 mM) in the patch pipette to inhibit GTP-binding proteins could not block the effects of oestrogens on $I_{Ca.L}$ (data not shown). In addition, to investigate the possible involvement of nitric oxide or cyclic GMP on the inhibitory effects of oestrogen on $I_{Ca.L}$, the effects of L-NAME or cyclic GMP were examined (Figure 6C and D). Even in a cell treated with L-NAME approximately for 1 h, a nitric oxide synthesis inhibitor, 17β-estradiol (10 μM) inhibited $I_{Ca.L}$ in a similar manner as control cells (Figure 6C and D). Also, it inhibited $I_{Ca.L}$ in a cell loaded with cyclic GMP (100 μM) through the patch pipette (Figure 6D). Thus, it is unlikely that oestrogens inhibit $I_{Ca.L}$ *via* nitric oxide production or cyclic GMP cascade.

Effects of 17β-estradiol on the voltage-dependent T-type Ca^{2+} current ($I_{Ca.T}$)

The existence of two distinct voltage-dependent Ca^{2+} currents has been shown for cardiac myocytes (Bean, 1985; Mitra & Morad, 1986; Cerbai *et al*., 1988; Tytgat *et al*., 1988). $I_{Ca.T}$ activates at low voltages and inactivates quickly; $I_{Ca.L}$ activates at high voltages and inactivates slowly. In addition, $I_{Ca.T}$ is equally permeable to Ca^{2+} and Ba^{2+} ions, and has the same inactivation kinetics in Ba^{2+} as in Ca^{2+}; $I_{Ca.L}$ is more permeable to Ba^{2+}, and has dramatically slower inactivation time in Ba^{2+} than Ca^{2+}. To clarify whether both types of voltage-dependent Ca^{2+} currents can be identified, we carried out under the conditions in which extracellular Na^{+} ions were totally replaced by impermeable TEA^{+} and 5 mM Ba^{2+} was added in place of Ca^{2+}. Sodium removal induced cell contracture, but under our conditions with EGTA (10 mM) and BAPTA (2 mM) in the patch pipette, the cell attached to the patch electrode survives, probably owing to the diffusion of EGTA and BAPTA in the cytosol. The cell was held at -40 mV or -80 mV (Figure 7), and command voltage steps (320 ms in duration) were applied to various membrane potentials. The current-voltage relationships of the peak

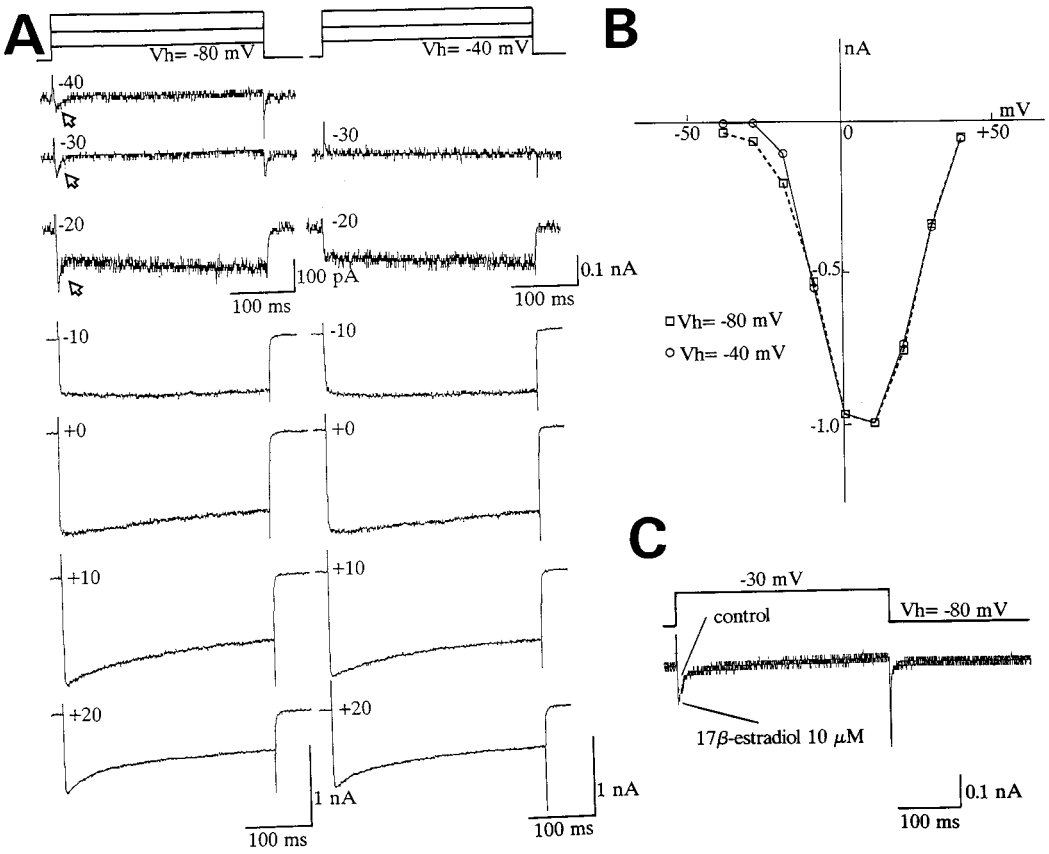

Figure 7 Effects of 17β-estradiol on T-type Ca^{2+} currents in guinea-pig atrial myocytes. (A) Voltage-dependent Ca^{2+} currents. The cell was held at -40 mV and -80 mV, respectively, and command voltage pulses (Vc, 320 ms in duration) were applied at 0.2 Hz to various membrane potentials. The bath was perfused with high TEA solution with TTX (10 μM) in place of Na^{+}. Extracellular Ca^{2+} was totally replaced by 5 mM Ba^{2+}, and the patch pipette contained CsCl$-$ internal solution with 10 mM EGTA and 2 mM BAPTA. The original current traces obtained at a holding potential of -40 mV and -80 mV are shown at various command voltage steps. Note that the transient component (as shown in arrows) was observed at command steps (-40 mV to -20 mV) from a holding potential of -80 mV. (B) The current-voltage relationships of the peak of the voltage-dependent Ca^{2+} current are shown for a holding potential of -40 mV and -80 mV. (C) Effects of 17β-estradiol (10 μM) on the T-type Ca^{2+} current. The cell was held at -80 mV, and the command voltage steps to -30 mV were applied. The current traces are shown in the control and in the presence of 17β-estradiol (10 μM).

inward current are shown in Figure 7B. At a holding potential of −40 mV, the inward current was elicited at positive potentials to −30 mV (Figure 7A, right panel). There was very small fraction of current inactivated at the command pulses to −20 mV and −10 mV. On the other hand, when the cell was held at −80 mV, the transient inward current was recorded at a command potential of −40 mV and −30 mV, and was overlapped on the non-inactivated component at a command potential of −20 mV (Figure 7A, left panel). The transient inward current rapidly inactivated within 50 ms, and could be discriminated from the sustained component. Nifedipine (1 μM, data not shown) failed to inhibit the transient component. These findings suggest that both types of voltage-dependent Ca^{2+} currents exist in guinea-pig atrial myocytes. Figure 7C shows the effects of 17β-estradiol on $I_{Ca.T}$. The T-type Ca^{2+} current was elicited at a command voltage to −30 mV from a holding potential of −80 mV. 17β-estradiol (10 μM) inhibited the amplitude of $I_{Ca.T}$ only by 9±3% ($n=4$).

High doses of 17β-estradiol and diethylstilbestrol (DES) inhibit K^+ currents

Figure 8 shows the effects of 17β-estradiol (30 μM, Figure 8A and B) and DES (10 μM, Figure 8C and D) on membrane currents. The current/voltage relationships measured at the peak and at the end of the command pulse are indicated in control and in the presence of these agents. 17β-estradiol (30 μM) or DES (10 μM) inhibited I_{K1}, and also decreased I_K. The amplitude for I_K tail was also inhibited. In addition, I_K has been known to be composed of two different types of channels, a rapidly activating component (K_{Kr}) and a slowly activating component (I_{Ks}), in several kinds of mammalian cardiac myocytes (Sanguinetti & Jurkiewcz, 1990; 1991). Therefore, to clarify whether both types of I_K exist, the effects of E-4031, which can preferentially block I_{Kr} (Sanguinetti, 1992), were examined as shown in Figure 9. The cell was held at −40 mV, and the command voltage steps to various membrane potentials were applied. The bath was perfused with nifedipine (1 μM) to block $I_{Ca.L}$. The depolarizing steps with long pulse duration (Figure 9A) or short pulse duration (Figure 9B) elicited I_K. Upon repolarization, the tail currents for I_K was observed in control conditions. The current-voltage relationships of the end of the pulse measured from the zero current level and the amplitude of the tail are shown in Figure 9C. E-4031 (5 μM) decreased the holding current into the inward direction, due to the blockade of I_{K1} at high concentration of E-4031. However, E-4031 preferentially blocked the time-dependent outward K^+ current at command potentials more negative to +0 mV with short pulses (Figure 9B, lower part). The tail currents at these command voltages were completely abolished by E-4031 (5 μM). On the other hand,

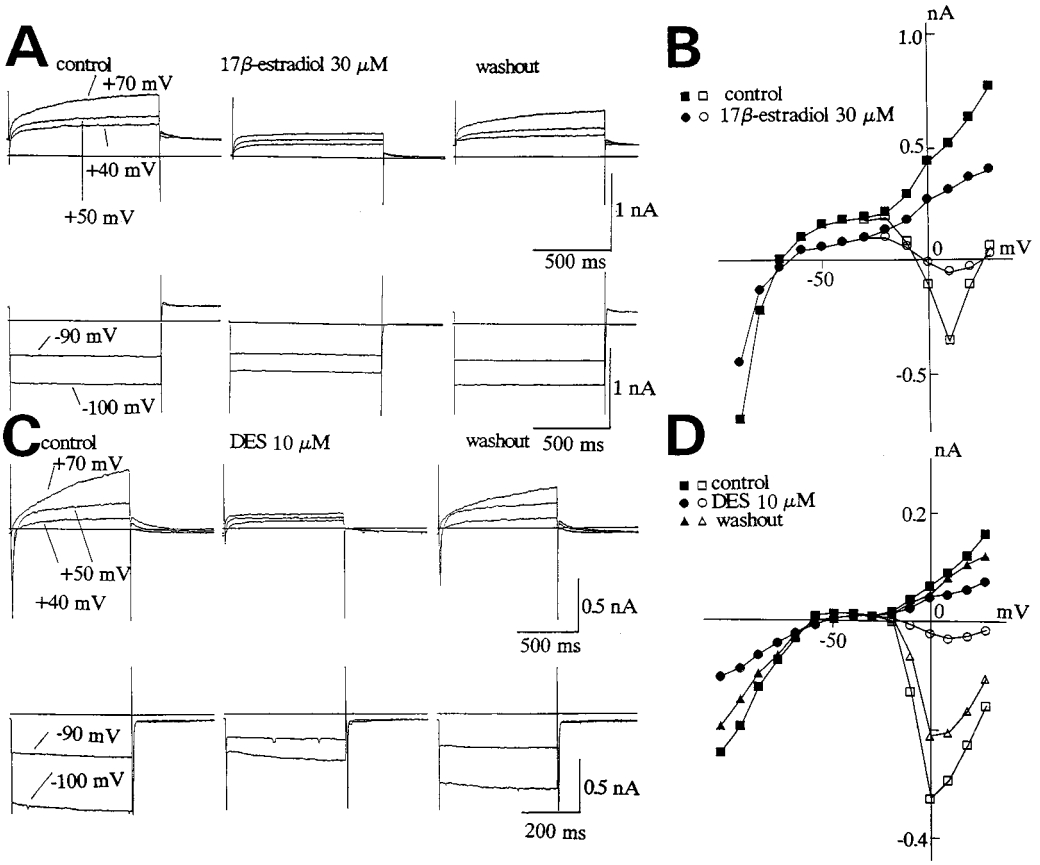

Figure 8 Effects of 17β-estradiol (30 μM) or diethylstilbestrol (10 μM) on membrane currents in single atrial myocytes. The cells were held at −40 mV and the command voltage steps were applied at 0.2 Hz. (A and B) Effect of 17β-estradiol (30 μM) on membrane currents. Note that 17β-estradiol (30 μM) inhibited I_{K1} and I_K as well as $I_{Ca.L}$. (B) The current-voltage relationships measured at the peak of the inward current and at the end of the pulse are plotted in control and after application of 17β-estradiol (30 μM), respectively. (C and D) Effect of diethylstilbestrol (DES, 10 μM) on membrane currents. (D) The current-voltage relationships measured at the peak of the inward current and at the end of the pulse are plotted in control, after application of DES (10 μM) and after the washout, respectively.

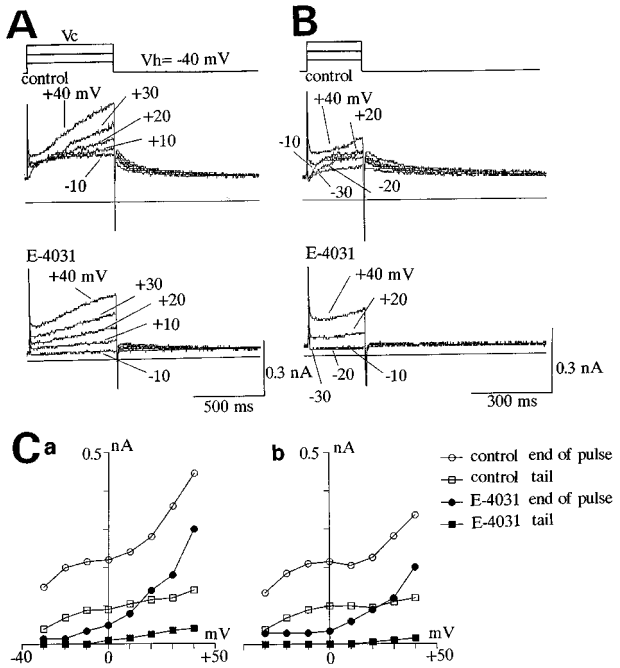

Figure 9 Two components of the delayed K^+ currents in guinea-pig atrial myocytes. The cells were held at −40 mV, and the command voltage steps to various membrane potential were applied at 0.2 Hz. Nifedipine (1 μM) was added to the bath solution. (A) Currents recorded during 620 ms pulses to −10, +10, +20, +30, and +40 mV before (upper part) and after exposure to 5 μM E-4031 (lower part). (B) Currents recorded during 220 ms pulses to −30, −20, −10, +20, and +40 mV before (upper part) and after exposure to 5 μM E-4031 (lower part). The data of (A and B) were obtained from the same cell. The current voltage-relationships of the end of the pulse measured from the zero current level (circle) and tail currents measured on return of membrane voltages to −40 mV from indicated test potential (square) are shown in (C). Ca and Cb were obtained from the data of (A, long pulse) and (B, short pulse), respectively.

even in the presence of E-4031 (5 μM), the time-dependent K^+ current was still activated by depolarizing pulses to more positive potentials, especially with long pulse duration (Figure 9A, lower part). These findings suggest that both types of I_K exist in guinea-pig atrial myocytes. I_{Ks} predominates in long pulses than in short pulses at more positive potentials, while I_{Kr} predominates in short pulses at more negative potentials. Therefore, the effects of 17β-estradiol and DES on both types of I_K were investigated. Figure 10A and B show the effects of 17β-estradiol (10 and 30 μM) and DES (10 μM) on I_{Kr}. The cells were held at −40 mV, and the command pulses to −10 mV were applied with a short pulse to evoke I_{Kr}. 17β-estradiol (10 μM) did not inhibit the time-dependent outward current during the command voltage, and the following tail current, while E-4031 (5 μM) completely inhibited it. But, 17β-estradiol (30 μM, Figure 10A, lower part) and DES (10 μM, Figure 10B) decreased I_{Kr}. The effects of 17β-estradiol and DES on I_{Ks} were also investigated in Figure 10C. The cell was held at −40 mV, and the command voltage pulses to +40 mV from a holding potential of −40 mV with a long pulse duration were applied. The bath was perfused into Ca^{2+}-free Tyrode solution in the presence of E-4031 (5 μM). In control, I_{Ks} was elicited during the depolarizing step, and upon repolarizion, the tail current for I_{Ks} was observed. 17β-estradiol (10 μM) did not affect the outward current significantly, while 17β-estradiol (30 μM) and DES (10 μM) markedly reduced the outward current for I_{Ks} during the depolarizing pulses and the following tail current.

Discussion

The major findings of the present study are as follows: (1) 17β-estradiol shortened the action potential duration, and abolished the isoproterenol-induced automaticity or E4031-induced early afterdepolarization. (2) The inhibitory effect of 17β-estradiol on $I_{Ca.L}$ may be involved in the antiarrhythmic effects of oestrogen. (3) The synthetic oestrogens, diethylstilbestrol (DES) and ethinylestradiol (EES), also inhibited $I_{Ca.L}$, and the potency among the oestrogens was DES > EES > 17β-estradiol, while progesterone and testosterone did not inhibit it significantly. (4) High doses of 17β-estradiol and DES inhibited K^+ currents (I_{K1}, I_{Kr} and I_{Ks}).

Oestrogen inhibits the voltage-dependent L-type Ca^{2+} current ($I_{Ca.L}$) in cardiac myocytes

The present study indicates that oestrogens inhibit $I_{Ca.L}$ in single atrial myocytes from the guinea-pig. The inhibitory effect of 17β-estradiol on $I_{Ca.L}$ was compatible with the previous paper showing that 17β-estradiol inhibited $I_{Ca.L}$ in single ventricular myocytes from the guinea-pig (Jiang *et al.*, 1992), but we presented the additional evidence that the synthetic oestrogens, DES and EES, also inhibited $I_{Ca.L}$ and the potency among oestrogens was DES > EES > 17β-estradiol, which was similar to that for the oestrogen receptors. Both testosterone and progesterone failed to inhibit $I_{Ca.L}$, proposing that oestrogen specifically inhibits $I_{Ca.L}$ in cardiac myocytes. Although the receptors for oestrogen have been shown to exist around the nucleus and cytoplasm in cardiac myocytes (Stumpf *et al.*, 1977; McGill *et al.*, 1980), it seems unlikely that oestrogens act on $I_{Ca.L}$ by interacting these intracellular receptors because of the very short time delay needed for the inhibitory effects of oestrogens on $I_{Ca.L}$. Therefore, a specific interaction with membrane receptors seems likely. However, alternatively, since high dose of 17β-estradiol and DES inhibited the delayed recifying K^+ current (I_{Kr} and I_{Ks}) and the inwardly rectifying K^+ current (I_{K1}), the possibility that oestrogens inhibit $I_{Ca.L}$ independently of the receptors for oestrogens can not be ruled out.

As shown in Figure 6, 17β-estradiol and DES inhibited $I_{Ca.L}$ in control and cyclic AMP-enhanced $I_{Ca.L}$, due to the phosphorylation of the channels by cyclic AMP-dependent protein kinase A (Kameyama *et al.*, 1985). These inhibitory effects of 17β-estradiol on cyclic AMP-enhanced $I_{Ca.L}$ were different from the previous study showing that it rapidly attenuated G-protein-coupled receptors by activating protein kinase A in guinea-pig hypothalamic neurons (Lagrange *et al.*, 1997). Thus, it is likely that oestrogen inhibits $I_{Ca.L}$ independently of intracellular cyclic AMP in cardiac myocytes. 17β-estradiol also suppressed $I_{Ca.L}$, even when GDPβS (2 mM) was included in the patch pipette to block GTP-binding proteins, which completely blocked the activation of muscarinic K^+ currents by acetylcholine (Kurachi *et al.*, 1986; Nakajima *et al.*, 1992) (data not shown), proposing that the GTP-binding proteins are not involved in the inhibitory effect of oestrogen on $I_{Ca.L}$. In addition, cyclic GMP or nitric oxide has been reported to inhibit $I_{Ca.L}$ in cardiac myocytes (Levi *et al.*, 1989; Han *et al.*, 1995). However, cyclic GMP in the patch pipette or pretreatment of L-NAME could not block the effects of oestrogen on $I_{Ca.L}$, suggesting that the involvement of cyclic GMP or nitric oxide is also unlikely as described in vascular

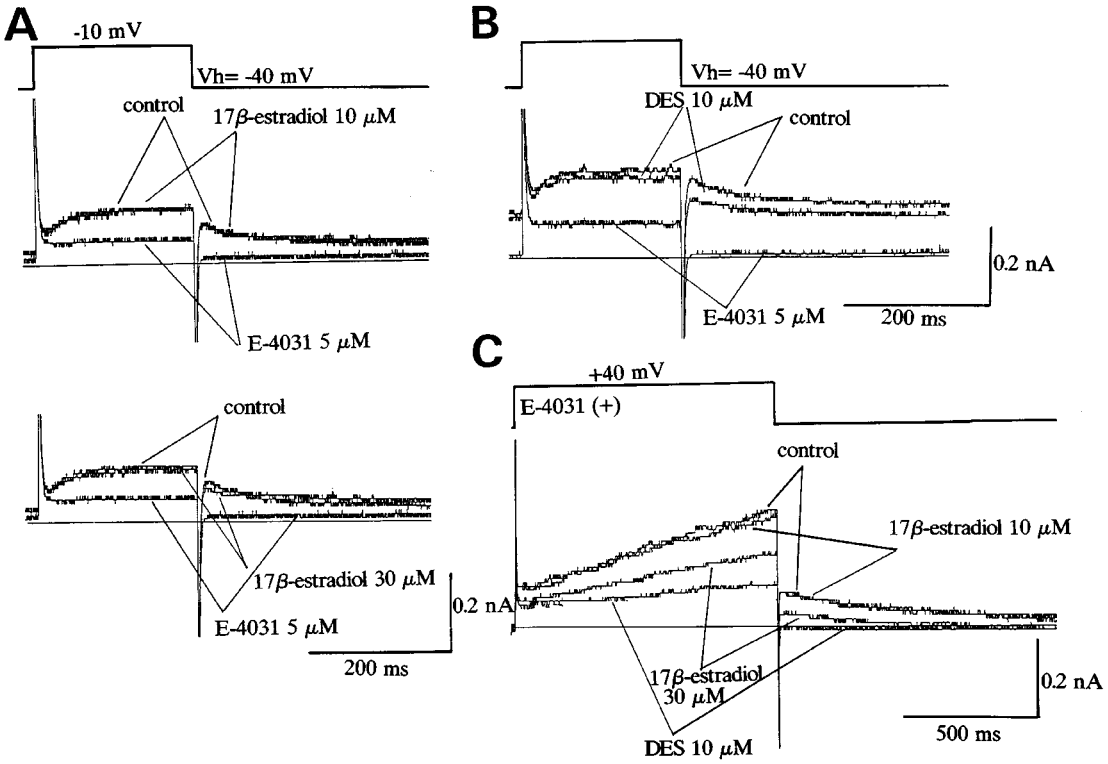

Figure 10 Effects of 17β-estradiol and diethylstilbestrol (DES) on two types of the delayed K^+ currents (I_{Kr} and I_{Ks}). (A and B) Effects of 17β-estradiol and DES on I_{Kr}. The cells were held at -40 mV, and the command pulses to -10 mV (220 ms in duration) were applied at 0.2 Hz. Nifedipine (1 μM) was added to the bath solution. (A) The effects of 10 μM 17β-estradiol (upper part), 30 μM 17β-estradiol (lower part), and E-4031 (5 μM) were shown. (B) The effects of DES (10 μM) and E-4031 (5 μM) are shown. (C) Effects of 17β-estradiol and DES on I_{Ks}. The cell was held at -40 mV, and the command pulses (620 ms in duration) to $+40$ mV were applied at 0.2 Hz. Extracellular Ca^{2+} was removed. The current traces are shown in the control, 17β-estradiol (10 and 30 μM) and DES (10 μM).

smooth muscles cells (White *et al*., 1995; Guetta *et al*., 1997). On the other hand, 17β-estradiol did not affect the time courses of inactivation of $I_{Ca.L}$, and the steady-state activation curve, while it shifted the steady-state inactivation curve to more negative potentials. In addition, the inhibitory effects of 17β-estradiol on the T-type Ca^{2+} currents were much less than on $I_{Ca.L}$. Thus, it is more likely that the inhibitory effects of oestrogens on $I_{Ca.L}$ may be voltage-dependent, and the site of action of oestrogens may be located on $I_{Ca.L}$ channel itself as classical Ca^{2+} antagonistic drugs.

Antiarrhythmic effects of oestrogen

The present studies provide evidence that 17β-estradiol may have antiarrhythmic effects in cardiac myocytes. 17β-estradiol prevented the occurrence of arrhythmia due to membrane depolarization under the perfusion of isoproterenol-containing solution or nearly afterdepolarization induced by E-4031. The first type arised from relatively high membrane potential ($-50 \sim -60$ mV) and induced sustained rhythmic activity in the form of abnormal automaticity due to membrane depolarization. The other type consists of early afterdepolarization and triggered potentials from low membrane potential ($-20 \sim -40$ mV). These two types of arrhythmia have also been reported to be induced by cesium (Cs^+), quinidine or barium (Ba^{2+}) in cardiac tissues (Levine *et al*., 1985; Roden & Hoffman, 1985; Nattel & Quantz, 1988; Tanaka & Singh, 1990).

Clinical experience indicates that torsades de pointes invariably occur in the setting of prolonged action potential duration. In the present study, E-4031, a class III antiarrhythmic agent (Sanguinetti, 1992), which inhibits I_{Kr}, prolonged the action potential duration, often resulting in the development of early afterdepolarization and triggered potentials from low membrane potentials as shown in Figure 1C. Similarly, it has been reported that quinidine and Cs^+ or Ba^{2+} can induce this type of triggered potential (Levine *et al*., 1985; Roden & Hoffman, 1985; Nattel & Quantz, 1988; Tanaka & Singh, 1990). Therefore, the overall findings suggest that prolongation of the repolarization phase of the action potential is critical to the development of this type of arrhythmia as a possible arrhythmogenic mechanism. The present study demonstrated that oestrogen abolished the development of early afterdepolarization and triggered potentials. The underlying ionic mechanism appears to be due to the inhibitory effects of oestrogen on $I_{Ca.L}$, since oestrogen at doses lower than 10 μM failed to affect K^+ currents significantly. There are several ionic mechanisms in inducing this kind of arrthythmia, i.e. $I_{Ca.L}$ and I_K. In regard to the triggered potentials from a lower membrane potential, January *et al*. (1988) showed that the L-type calcium channel agonist Bay K 8644 induced early afterdepolarization, and indicated that the Ca^{2+} current was considered as a cellular mechanism for the development of the early afterdepolarization from a low membrane potential. Thus, it is likely that 17β-estradiol exhibited pronounced suppressant effects on this type of early afterdepolarization by inhibiting $I_{Ca.L}$. 17β-estradiol eliminated the development of triggered potentials from a lower membrane potential, and the observed effects may be similar to those reported with calcium channel blockade with

verapamil, Mg^{2+} or amiodarone (Aliot *et al*., 1985; Mason, 1987; Perticone *et al*., 1986; Singh, 1988; Bailie *et al*., 1988).

In isoproterenol-perfusing solution, abnormal automaticity was frequently elicited from a potential of a higher membrane potential ($-50 \sim -60$ mV) as shown in Figure 1C. Isoproterenol is known to activate a Cl^- current (Harvey *et al*., 1990), which may produce a stable depolarization of the resting membrane potential. The depolarization of the membrane thereby can induce automatic repetitive depolarization, i.e. pace-maker like activity. Similarly, cardiac myocytes have been known to be capable of showing pace-maker like activity under certain conditions such as direct application of currents (Hauswirth *et al*., 1969; Katzung, 1975; Imanishi & Surawicz, 1976; Katzung & Morgenstern, 1977), or by exposure of Ba^{2+} ions (Tanaka & Singh, 1990). The ionic basis of the depolarization-induced automaticity may be due to the decay of time-dependent K^+ current (I_K) or the participation of inward currents ($I_{Ca.L}$ and Na^+ current) and Na^+/Ca^{2+} exchange current or both (Hauswirth *et al*., 1969; Katzung, 1975; Imanishi & Surawicz, 1976; Katzung & Morgenstern, 1977). In the present study, 17β-estradiol at concentrations lower than 10 μM failed to affect I_K significantly, proposing that the essential role of $I_{Ca.L}$ in the development of action potentials from the depolarized membrane potentials should be emphasized. 17β-estradiol inhibited this type of abnormal automaticity, which confirms this notion.

Clinical implication

The present study provides evidence that 17β-estradiol appears to act as an antiarrhythmic agent, and inhibits the development of early afterdepolarization and triggered potentials. Therefore, 17β-estradiol may prevent the occurrence of the polymorphic ventricular tachycardia (torsades de pointes) induced by antiarrhythmic drugs or in patients with long QT syndrome. In addition, since 17β-estradiol inhibits the depolarization-induced abnormal automaticity, oestrogen may inhibit the arrhythmia observed under the depolarizing conditions such as myocardial ischaemia. Clinical studies (Godsland *et al*., 1987; Bush *et al*., 1987; Colditz *et al*., 1987; Knopp, 1988) indicate that oestrogen replacement therapy reduces cardiac mortality and morbidity. It remains to be clarified whether the therapy can prevent the occurrence of fatal arrhythmia and then cardiac death. However, it is likely that the antiarrhythmic effect of oestrogen may contribute to its cardioprotective effect of oestrogen as previously reported in patients with supraventricular tachycardia (Rosano *et al*., 1996). Oestrogen therapy has been shown to decrease heart rate as well as blood pressure (Luotola, 1983). The inhibitory effect of oestrogen on $I_{Ca.L}$ may also be involved in the negative chronotropic effects of oestrogen. In addition, since the effects of oestrogens on $I_{Ca.L}$ were somewhat voltage-dependent, oestrogens may potently block $I_{Ca.L}$ in partially depolarized cells in specimens of human myocardium obtained from patients with heart disease such as myocardial infarction and dysrhythmia. However, in contrast to the acute effects of oestrogen, since voltage-dependent K^+ channel mRNA (HK2 and I_{Ks} mRNA) has been reported to be rather downregulated in cardiac ventricular tissue from chronically oestrogen-treated rabbits (Drici *et al*., 1996), further studies are needed to clarify the effects of long-term treatment of oestrogen on cardiac ionic channels.

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