Increased Expression of the Cardiac L-type Calcium Channel in Estrogen Receptor–deficient Mice

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ABSTRACT Steroid hormones control the expression of many cellular regulators, and a role for estrogen in cardiovascular function and disease has been well documented. To address whether the activity of the L-type Ca\textsuperscript{2+} channel, a critical element in cardiac excitability and contractility, is altered by estrogen and its nuclear receptor, we examined cardiac myocytes from male mice in which the estrogen receptor gene had been disrupted (ERKO mice). Binding of dihydropyridine Ca\textsuperscript{2+} channel antagonist isradipine (PN200-110) was increased 45.6% in cardiac membranes from the ERKO mice compared to controls, suggesting that a lack of estrogen receptors in the heart increased the number of Ca\textsuperscript{2+} channels. Whole-cell patch clamp of acutely dissociated adult cardiac ventricular myocytes indicated that Ca\textsuperscript{2+} channel current was increased by 49% and action potential duration was increased by 75%. Examination of electrocardiogram parameters in ERKO mice showed a 70% increase in the QT interval without significant changes in PQ or QRS intervals. These results show that the membrane density of the cardiac L-type Ca\textsuperscript{2+} channel is regulated by the estrogen receptor and suggest that decreased estrogen may lead to an increase in the number of cardiac L-type Ca\textsuperscript{2+} channels, abnormalities in cardiac excitability, and increased risk of arrhythmia and cardiovascular disease.

KEY WORDS: heart • ion channels • action potential • electrocardiogram

INTRODUCTION

Calcium channels are important regulators of cell function, but there is little information on the factors which influence their level of expression in cells. Steroid hormones are likely regulators of calcium channel expression as they control the expression of many cellular regulators and are implicated in regulation of cardiac excitability which is critically dependent on calcium channels. Premenopausal women have a lower incidence of cardiovascular disease than men and continue to have a lower risk of heart disease after menopause when receiving estrogen replacement (Collins et al., 1993). Estrogen replacement is associated with a reduction in cardiac arrhythmia in postmenopausal women (Cagnacci et al., 1992), and cyclical increases in estrogen in premenopausal women are associated with a reduction in paroxysmal supraventricular tachycardia (Rosano et al., 1996). Some of the benefits of estrogen on the cardiovascular system may derive from a reduction in cholesterol deposition on arterial walls (Chow, 1995), but estrogen may also have direct effects on arterial smooth muscle and cardiomyocytes. Estrogen in vitro reduces L-type Ca\textsuperscript{2+} channel activity and causes relaxation in both types of muscle (Jiang et al., 1992; Shan et al., 1994; Grohé et al., 1996; Ogata et al., 1996), but the effective estrogen concentrations in these experiments were in excess of those normally present in vivo. To search for effects of estrogen on L-type Ca\textsuperscript{2+} channels in the heart in vivo, we examined Ca\textsuperscript{2+} channel number and function in ventricular myocytes from mice with a disrupted estrogen receptor gene (ERKO mice) (Lubahn et al., 1993). We report here that disruption of estrogen action in vivo causes increased density of L-type Ca\textsuperscript{2+} channels in the heart as assessed from ligand binding and whole-cell voltage clamp experiments. The results demonstrate an important influence of estrogen on calcium channel expression in the heart and implicate this as one factor which may contribute to the reduced incidence of cardiovascular disease associated with estrogen action in vivo.

MATERIALS AND METHODS

Dihydropyridine Binding

The hearts from eight control (age-matched male C57B1/6j) and eight male ERKO mice were isolated quickly after anesthesia with CO\textsubscript{2}. The whole heart was minced with scissors and homogenized in 5 ml ice-cold Tris buffer (50 mM, pH 7.4) by two bursts in a Polytron (Brinkman Instruments, Inc., Westbury, NY), followed by 10 passes of a motor-driven glass-Teflon homogenizer (TRI-R Instruments Inc., Rockville Center, NY). The homogenate was filtered through four layers of cheesecloth and centri-
fuged at 45,000 g for 45 min at 4°C. The resultant pellet was suspended in 5 ml Tris buffer. The protein concentration was determined by the biuret colorimetric method using a protein assay kit (Pierce, Rockford, IL). The saturation binding assay was performed in test tubes containing 2.5 ml Tris buffer (50 mM, pH 7.4). Membrane protein (0.15–0.25 mg) was incubated with 0.01–0.6 nM 3H(+)-PN200-110 for 2 h at 37°C. Samples were then filtered over Whatman GF/B filters and washed twice with 5 ml ice-cold 50 mM Tris buffer, using a cell harvester (Brandel Instruments, Gaithersburg, MD). The radioactivity on the filters was measured by liquid scintillation counting at an efficiency of ~50%. Nonspecific binding was determined by addition of 1 μM unabeled PN200-110. The binding data were calculated by Scatchard plot using the program LIGAND (Munson and Rodbard, 1980). The results were analyzed by a standard set of pharmacological programs (Tallarida and Murray, 1981). Significance of difference was accepted at the 0.05 level. 3H(+)-PN200-110 (81.5 Ci/mmol) was purchased from DuPont NEN (Boston, MA). All other reagents were ordered from Sigma Chemical Co. (St. Louis, MO).

Cell Preparation and Electrophysiological Recording

Ventricular myocytes from six control and six ERKO adult mice (24–28 wk old) were dissociated as described (Benndorf, 1993). Briefly, after the animals were anesthetized with pentobarbital (50 mg/kg, i.p.), the hearts were excised and perfused through the aorta for 5 min (~10 ml) with 37°C, oxygenated Ca2+- and Mg2+-free Hanks’ Balanced Salt Solution (CMF HBSS, Sigma Chemical Co.) containing 10 mM HEPES (pH 7.3), then for 30 min (~10 ml, recirculated) with 37°C, oxygenated CMF HBSS to which 1 mg/ml type I collagenase (Worthington Biochemical Corp., Freehold, NJ) and 100 μM Ca2+ had been added, then for 3 min (~7 ml) with relaxing solution containing (mM units): 30 KCl, 30 KH2PO4, 50 glutamate, 20 taurine, 20 HEPES, 10 glucose, 0.5 EGTA (pH 7.3 with KOH). The ventricles were minced and triturated in relaxing solution then centrifuged and plated in fresh relaxing solution. Myocytes were stored in this solution at room temperature until use (~6 h). Four control mice were age-matched C57BL/6 mice and two were wild-type littermates (ER+/+ of the ERKO mice. Ca2+ channel current was recorded using the whole-cell configuration of the patch clamp technique. Patch pipettes were pulled from VWR micropipettes and fire-polished to produce an inner tip diameter of 4–6 μm. Patch pipettes were polished to produce an inner tip diameter of 4–6 μm. Whole-cell patch of dissociated ventricular myocytes from control and ERKO mice was first measured directly by binding of the dihydropyridine Ca2+ channel antagonist PN200-110. Dihydropyridines bind specifically and with high affinity to L-type Ca2+ channels with a 1:1 stoichiometry. The number of specific binding sites for PN200-110 (Bmax) in cardiac membranes was increased 45.6% in ERKO mice without a change in the affinity (Kd) of the channel for the dihydropyridine (Fig. 1). This increase in the number of Ca2+ channels was not due to cardiac hypertrophy in ERKO mice since circulating levels of estradiol and progesterone have been found to change dramatically in female ERKO mice but remain constant in male mice (control males, 11.8 ± 3.4 pg/ml; ERKO males 12.9 ± 3.4 pg/ml; Couse et al., 1995). Controlled circulating hormone levels were important in this study given possible direct pharmacological effects of estradiol on Ca2+ and K+ channels (Rusko et al., 1995; Ogata et al., 1996; Shan et al., 1994; Grohé et al., 1996; Jiang et al., 1992), and the recent discovery of a second estrogen receptor, named ERβ (Kuiper et al., 1996).

The number of L-type Ca2+ channels in the hearts of control and ERKO mice was first measured directly by binding of the dihydropyridine Ca2+ channel antagonist PN200-110. Dihydropyridines bind specifically and with high affinity to L-type Ca2+ channels with a 1:1 stoichiometry. The number of specific binding sites for PN200-110 (Bmax) in cardiac membranes was increased 45.6% in ERKO mice without a change in the affinity (Kd) of the channel for the dihydropyridine (Fig. 1). This increase in the number of Ca2+ channels was not due to cardiac hypertrophy in ERKO mice since neither the weight of the heart nor amount of protein in each heart was changed.

Whole-cell patch clamp of dissociated ventricular myocytes from control and ERKO mice revealed a similar increase in L-type Ca2+ channel activity. Fig. 2 A shows the mean Ca2+ channel current density (with Ba2+ as the permeant ion) in 15 myocytes of each type recorded at 0 mV. The mean current-voltage relations for control and ERKO myocytes (Fig. 2 B) showed a 17% increase at 0 mV and a 49% increase at +30 mV. The larger percentage increase in Ba2+ current at 30 mV implies a shift in the current voltage relationship to more positive membrane potentials. This shift can be seen in the superimposed data in Fig. 2 B. It is caused by a positive shift in the voltage dependence of activation (see
below). The increased density of Ca\textsuperscript{2+} channel current was independent of the ion carrying the current and was observed for Ca\textsuperscript{2+} current as well as Ba\textsuperscript{2+} current (16% increase at 0 mV, \( n = 5 \)). The kinetics of activation and inactivation of Ca\textsuperscript{2+} channel current were not changed (Fig. 2 C), indicating that the increased current in the ERKO ventricular myocytes was not due either to a reduction in Ca\textsuperscript{2+}-dependent or voltage-dependent inactivation or to acceleration of activation.

To yield a more accurate measure of Ca\textsuperscript{2+} channel activity at a broad range of membrane potentials, the current-voltage relations shown in Fig. 2 B were corrected for the effect of electrical driving force using the Goldman-Hodgkin-Katz current equation (Bargàs et al., 1994; Hille, 1992) (Fig. 2 D). Apparent reversal potentials for the two groups were not different (control, 44 ± 3 mV, \( n = 23 \); ERKO, 46 ± 5 mV, \( n = 21 \)). However, the \( V_{1/2} \) for activation was shifted from −13.3 ± 1.5 mV for controls to −8.4 ± 1.4 mV for ERKO mice (\( p < 0.05 \)). Fits to these permeability vs. voltage curves using the Boltzmann equation (Hille, 1992) show a 49 ± 5% increase in the mean membrane permeability to Ba\textsuperscript{2+} (control, 17.6 ± 2.1 × 10\textsuperscript{-6} cm/s, \( n = 23 \); ERKO, 26.2 ± 3.0 × 10\textsuperscript{-6} cm/s, \( n = 21 \); \( p < 0.05 \)). As with previous measures of cardiac hypertrophy, the mean membrane surface area of dissociated ventricular myocytes as estimated from cell capacitance was not changed in the ERKO mice (control, 17,489 ± 1,083 \( \mu \)m\textsuperscript{2}; ERKO, 16,778 ± 1,188 \( \mu \)m\textsuperscript{2}; using a conversion factor of 0.01 pF/\( \mu \)m\textsuperscript{2}). In addition, the other voltage-dependent Ca\textsuperscript{2+} channel in ventricular myocytes, the T-type Ca\textsuperscript{2+} channel, did not exhibit an increase in activity (control, 4.2 ± 1.4 \( \mu \)A/cm\textsuperscript{2}, \( n = 7 \); ERKO, 3.8 ± 0.6 \( \mu \)A/cm\textsuperscript{2}, \( n = 12 \)). The voltage-dependent Na\textsuperscript{+} channel showed a decrease in current density (control, 87.3 ± 23.9 \( \mu \)A/cm\textsuperscript{2}, \( n = 7 \); ERKO, 57.9 ± 10.9 \( \mu \)A/cm\textsuperscript{2}, \( n = 9 \)), as expected from previous evidence that block of the L-type Ca\textsuperscript{2+} channel causes an increase in cardiac Na\textsuperscript{+} channel expression (Duff et al., 1992). Thus, these data show that disruption of the estrogen receptor gene specifically increases expression of the L-type Ca\textsuperscript{2+} channel in ventricular myocytes.

An increase in Ca\textsuperscript{2+} channel current would be predicted to lengthen the ventricular action potential in the absence of other modulatory effects (Rardon and Fisch, 1994). Action potentials were recorded in control and ERKO ventricular myocytes in the whole cell recording configuration (Fig. 3, A and B). Action potential duration measured at half repolarization (APD\textsubscript{50}) was increased 75 ± 11% in ERKO myocytes compared to controls (Fig. 3 A), and action potential duration measured at 90% repolarization (APD\textsubscript{90}) was increased 53 ± 9% (Fig. 3 B). Except for action potential amplitude, which was reduced by 9% (control, 109.3 ± 3.6 mV, \( n = 6 \); ERKO, 99.6 ± 2.7 mV, \( n = 14 \); \( p < 0.05 \)), other cell membrane parameters including action potential threshold (control, −66 ± 3 mV; ERKO, −61 ± 2 mV) and membrane resistance at threshold (control, 11.9 ± 2.5 \( \Omega \); ERKO, 12.0 ± 1.3 \( \Omega \)) were not significantly changed.

Electrocardiogram (ECG) parameters were measured in anesthetized control and ERKO mice. Cardiac electrical activity recorded from electrodes placed on the skin of humans and mice consists of three waveforms, designated P, QRS, and T. The P-wave represents atrial contraction, the QRS-wave represents ventricular depolarization, and the T-wave represents ventricular repolarization (Bayes de Luna, 1993). Agents which lengthen the ventricular action potential lengthen the time interval between Q and T. A greatly prolonged ventricular action potential and QT interval leads to cardiac arrhythmia since the heart is not sufficiently repolarized between contractions (Tan et al., 1995). Examples of ECGs from control and ERKO mice are shown in Fig. 3 C. Disruption of the estrogen receptor was not found to
In contrast, disruption of the estrogen receptor profoundly altered the PQ interval (a measure of atrioventricular conduction) in the AV node, Bundle of His and Purkinje fibers (or the duration of the QRS complex—a measure of the rate of rise of the ventricular action potential). In addition, Mermelstein et al. (1996) reported that acute, membrane-receptor–mediated inhibition of L-type Ca\(^{2+}\) channel activity in neostriatal neurons. This effect was substantially greater than the levels of circulating estrogen of approximately 40–60 pM in males (Contoreggi et al., 1993; Shan et al., 1994; Farhat et al., 1996; Grohé et al., 1996). These concentrations substantially exceed the levels of circulating estrogen of approximately 1–30 \(\mu\)M in males (Contoreggi et al., 1990; Couse et al., 1995) and 90 pM to 8 nM in females (Collins et al., 1993; Couse et al., 1995; Samaan and Crawford, 1995; Volterrani et al., 1995; Rosano et al., 1996). In addition, Mermelstein et al. (1996) reported that acute, membrane-receptor–mediated inhibition of L-type Ca\(^{2+}\) currents in neostriatal neurons. This effect was observed at physiological concentrations of 17\(\beta\)-estradiol (Takimoto and Levitan, 1994) and sex steroids (Whitney et al., 1995; Drici et al., 1996) in regulation of cardiac K\(^{+}\) channels. Together, the effects of the steroid hormones on these two prominent classes of cardiovascular ion channels are likely to have important influence on the excitability of the heart.

FIGURE 2. Increased Ca\(^{2+}\) channel current in ERKO mice. Ca\(^{2+}\) channel current (carried by Ba\(^{2+}\)) was recorded in acutely dissociated ventricular myocytes from control and ERKO mice during a 100-ms depolarization from −60 mV to 0 mV. Current amplitude (normalized to membrane surface area) measured in 15 myocytes from each group was averaged to give the traces shown (A). Membrane surface area was calculated from cell capacitance as 1 \(\mu\)F/cm\(^2\). Mean capacitance was not different between cell types. (B) Mean current density-voltage relations (±SEM) for control (\(n = 23\)) and ERKO mice (\(n = 21\)) were calculated from measurements of peak current during 100-ms depolarizations from −60 mV to the potentials indicated. Solid lines represent fits of the Boltzmann and Goldman–Hodgkin-Katz current equations to the mean data with the following values: control, \(\alpha = 17 \times 10^{-6}\) cm/s, \(V_{1/2} = -13.6\) mV, \(k = 7.4\); ERKO, \(\alpha = 28 \times 10^{-6}\) cm/s, \(V_{1/2} = -9.4 \pm 0.7\) mV, \(k = 8.9\).

**Discussion**

Our results provide the first evidence to our knowledge for regulation of expression of calcium channels by a steroid hormone in vivo. Previous results have implicated glucocorticoids (Takimoto and Levitan, 1994) and sex steroids (Whitney et al., 1995; Drici et al., 1996) in regulation of cardiac K\(^{+}\) channels. Together, the effects of the steroid hormones on these two prominent classes of cardiovascular ion channels are likely to have important influence on the excitability of the heart.
but was rapidly reversible and therefore unlikely to influence our measurements on dissociated cardiac myocytes in the absence of added estrogen. Our results suggest that physiological levels of estrogen may reduce the number of cardiac Ca\textsuperscript{2+} channels through the actions of the estrogen receptor. Evidently, the estrogen receptor normally suppresses Ca\textsuperscript{2+} channel expression in the heart, and disruption of this receptor relieves this suppression. This effect may occur through a direct action of the estrogen receptor on expression of the cardiac Ca\textsuperscript{2+} channel gene or through indirect influences on other transcriptional regulators or second messenger pathways (Katzenellenbogen, 1996).

The increased Ca\textsuperscript{2+} current in cardiomyocytes of ERKO mice is accompanied by prolonged action potentials in dissociated cells and a prolonged QT interval in the heart in situ. The increased Ca\textsuperscript{2+} current would contribute to both of these effects, but other unidentified effects of estrogen receptor deficiency may also play a role. For example, reduction in the level of any of the several K\textsuperscript{+} channels involved in the repolarization phase of the cardiac action potential would also contribute to the prolonged action potentials and prolonged QT interval that we have observed (White et al., 1995; Drici et al., 1996).

This new role for estrogen in modulation of cardiac contractility may complement its role as a modulator of lipid metabolism. In both cardiac contractility and lipid metabolism, estrogen appears to maintain a favorable balance which is disrupted as estrogen levels decline with age (Farhat et al., 1996). Both men and women exhibit an inverse correlation between estrogen levels and cardiovascular disease (Contoreggi et al., 1990; Chow, 1995), and estrogen replacement in women has been found to reduce cardiac arrhythmia (Cagnacci et al., 1992) and angina pectoris (Sarrel, 1996). As treatment with blockers of L-type Ca\textsuperscript{2+} channels is an effective therapy for both atrial arrhythmias and angina pectoris (Braunwald, 1982), it is plausible that an increase in L-type Ca\textsuperscript{2+} channel activity may contribute to these conditions. In the present study, we found that disruption of the estrogen receptor in mice leads to an increase in the expression of the L-type Ca\textsuperscript{2+} channel in ventricular myocytes and a corresponding delay in cardiac repolarization. If similar changes occur in humans during a decline in estrogen and/or estrogen receptor levels, they could contribute to the increased incidence of arrhythmia and angina pectoris after menopause. Further examination of the role of the L-type Ca\textsuperscript{2+} channel plays in estrogen-induced protection against cardiovascular disease may aid in the development of more specific therapies.

**Figure 3.** Comparison of cardiac action potentials and electrocardiograms from control and ERKO mice. (A) Examples of action potentials recorded from acutely dissociated control and ERKO ventricular myocytes. The ERKO trace has been scaled to the amplitude of control for comparison of time course. (B) Mean action potential durations (±SEM) at 50% repolarization (APD\textsubscript{50}) and 90% repolarization (APD\textsubscript{90}) in control (n = 6) and ERKO mice (n = 14). Action potentials were recorded in myocytes held at −90 mV and paced at 5 Hz. Mean pulse rate (±SEM) is also shown. (C) Examples of electrocardiograms recorded from control and ERKO mice. The ERKO trace has been scaled to the amplitude of control for comparison of time course. (D) Mean electrocardiogram intervals (±SEM) measured in six control and six ERKO mice.

**Table 1.** Comparison of mean action potential durations (APD\textsubscript{50}, APD\textsubscript{90}), mean pulse rate (±SEM), and mean electrocardiogram intervals (PQ, QRS, QT, QTc) in control (n = 6) and ERKO (n = 14) mice. (A) Comparison of control (n = 6) and ERKO (n = 14) mice.

| Parameter | Control | ERKO |
|-----------|---------|------|
| APD\textsubscript{50} (ms) | 16.0 ± 1.0 | 18.0 ± 1.5 |
| APD\textsubscript{90} (ms) | 50.0 ± 2.0 | 56.0 ± 2.5 |
| Pulse rate (bpm) | 300 ± 10 | 320 ± 15 |
| PQ interval (ms) | 25 ± 3 | 30 ± 4 |
| QRS interval (ms) | 200 ± 10 | 210 ± 15 |
| QT interval (ms) | 300 ± 10 | 310 ± 15 |
| QTc interval (ms) | 40 ± 3 | 45 ± 5 |

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