Enhancement of Cardiac L-Type Ca\(^{2+}\) Currents in Transgenic Mice with Cardiac-Specific Overexpression of CYP2J2

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ABSTRACT

CYP2J2 is abundant in cardiomyocytes and is involved in the metabolism of arachidonic acid (AA) to epoxyeicosatrienoic acids (EETs), which affect multiple cell functions. In this study, we investigated the effect of overexpression of CYP2J2 on cardiac L-type Ca\(^{2+}\) currents (I\(_{\text{Ca}}\)) in adult transgenic mice. Cardiac-specific overexpression of CYP2J2 was achieved using the α-mycosin heavy chain promoter. I\(_{\text{Ca}}\) was recorded from isolated ventricular cardiomyocytes. Compared with the wild-type cardiomyocytes (n = 60), the density of I\(_{\text{Ca}}\) was significantly increased by 40 ± 9% in the CYP2J2 transgenic cardiomyocytes (n = 71; P < 0.001). N-Methylsulfonyl-6-(2-proparglyloxyphenyl)hexanamide (MS-PPOH), a specific inhibitor of EET biosynthesis, and clotrimazole, a cytochrome P450 inhibitor, significantly reduced I\(_{\text{Ca}}\) in both wild-type and transgenic cardiomyocytes; however, MS-PPOH inhibition of I\(_{\text{Ca}}\) to a greater extent in the CYP2J2 transgenic cells (n = 10) than in the wild-type cells (n = 10; P < 0.01). Addition of 11,12-EET significantly restored I\(_{\text{Ca}}\) in MS-PPOH-treated cells. Intracellular dialysis with either of two inhibitory monoclonal antibodies against CYP2J2 significantly reduced I\(_{\text{Ca}}\) in both wild-type and transgenic mice. Membrane-permeable 8-bromo-cAMP and the β-adrenergic agonist isoproterenol significantly reversed the monoclonal antibody-induced inhibition of I\(_{\text{Ca}}\). In addition, the total protein level of the α1 subunit of the Ca\(_{\text{1.2}}\) L-type Ca\(^{2+}\) channel was not altered in CYP2J2 transgenic hearts, but the phosphorylated portion was markedly increased. In conclusion, overexpression of CYP2J2 increases I\(_{\text{Ca}}\) in CYP2J2 transgenic cardiomyocytes via a mechanism that involves cAMP-protein kinase A-dependent phosphorylation of the L-type Ca\(^{2+}\) channel.

Cytochrome P450 (P450) and its associated monoxygenase activities have been identified in hearts from several mammalian species, including human (Comte and Gautheron, 1978; Guengerich and Mason, 1979; Abraham et al., 1987; McCallum et al., 1993; Wu et al., 1996; Wang et al., 2002). P450 epoxygenases can metabolize arachidonic acid (AA) to four regioisomeric eicosanoids, 5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acids (EETs), which have been shown to possess potent biological effects in numerous tissues (Capdevila et al., 2000; Zeldin, 2001; Kroetz and Zeldin, 2002; Roman, 2002). In the coronary circulation, the EETs are leading candidates for endothelial-derived hyperpolarizing factor, the nitric-oxide synthase, and cyclooxygenase-independent vasodilator that hyperpolarizes vascular smooth muscle cells by opening Ca\(^{2+}\)-activated K\(^+\) channels (Hecker et al., 1994; Campbell et al., 1996). EETs have also been shown to increase cardiomocyte cAMP content (Xiao et al., 1998), inhibit cardiac Na\(^{+}\) channels (Lee et al., 1999), and activate cardiac ATP-sensitive K\(^+\) channels (Lu et al., 2001, 2002). Voltage-gated L-type Ca\(^{2+}\) channels are critical for excitation-contraction coupling in the heart. The inotropic effect of

ABBREVIATIONS: P450, cytochrome P450; AA, arachidonic acid; EET, epoxyeicosatrienoic acid; α-MHC, α-myosin heavy chain; PKA, protein kinase A; Tr, transgenic; Wt, wild type; 8-Br-cAMP, 8-bromo-cAMP; MS-PPOH, N-methylsulfonyl-6-(2-proparglyloxyphenyl)hexanamide; HPLC, high-performance liquid chromatography; PKA-IF, protein kinase A inhibitory fragment; MAb, monoclonal antibody.
β-adrenergic receptor stimulation is attributed to an increase in Ca$^{2+}$ influx through the L-type Ca$^{2+}$ channel (Reuter, 1983). The binding of isoproterenol to β-adrenergic receptors is coupled to an intracellular signaling cascade by the stimulatory G protein, which activates adenyl cyclase, leading to an increase in intracellular cAMP. Activation of the cAMP-dependent protein kinase A (PKA) enhances Ca$^{2+}$ channel phosphorylation. In cardiomyocytes, the PKA-dependent phosphorylation of L-type Ca$^{2+}$ channels increases L-type Ca$^{2+}$ currents (I$_{Ca}$) (Reuter, 1983; McDonald et al., 1994; Keef et al., 2001). Several studies have shown that P450s can modulate membrane Ca$^{2+}$ influxes in cardiac and noncardiac cells. For example, P450 inhibitors can block membrane Ca$^{2+}$ channels that are activated by intracellular Ca$^{2+}$ store emptying in rat thymocytes (Alvarez et al., 1992) and in human platelets and neutrophils (Alonso et al., 1991; Sargeant et al., 1992). Similar effects of P450 inhibitors have also been found on voltage-gated Ca$^{2+}$ channels in bovine GH3 and chromaffin cells (Villalobos et al., 1992) and on L-type Ca$^{2+}$ currents in rat cardiomyocytes (Xiao et al., 1998).

Although multiple P450s are expressed in heart tissue, CYP2J2 seems to be unique in that it is primarily expressed in cardiomyocytes and active in the biosynthesis of EETs (Wu et al., 1996, 1997). Importantly, the EETs have been shown to increase I$_{Ca}$ in rat cardiomyocytes via a cAMP-dependent mechanism (Xiao et al., 1998); however, more recent data suggest that the effect of P450-derived EETs on the cardiac L-type Ca$^{2+}$ channel may be more complex (Chen et al., 1999). Recently, we used the cardiomyocyte-specific α-myosin heavy chain (α-MHC) promoter to express the human CYP2J2 cDNA in transgenic mice (Seubert et al., 2004). Hearts from CYP2J2 transgenic (Tr) mice have increased CYP2J2 protein expression and increased AA epoxygenase activity compared with wild-type (Wt) hearts (Seubert et al., 2004). Moreover, CYP2J2 Tr hearts have improved postischemic recovery of left ventricular function (Seubert et al., 2004). In the current study, we examined cardiomyocyte L-type Ca$^{2+}$ currents in this transgenic model to elucidate the effects of CYP2J2 overexpression on channel activity. Our data show that cardiac L-type Ca$^{2+}$ currents are significantly enhanced in CYP2J2 Tr mice and that this enhancement probably results from an increase in channel phosphorylation via a cAMP-PKA-dependent mechanism.

Materials and Methods

Materials. 8-Br-cAMP, (→)-isoproterenol, protein kinase A inhibitor fragment (PKA-IP14-24), and the P450 inhibitor clotrimazole were obtained from Sigma-Aldrich (St. Louis, MO). The specific inhibitor of EET biosynthesis N-methysulfonyl-6-(2-propargyloxyphenyl)hexanamide (MS-PPOH) was synthesized as described previously (Wang et al., 1998). Working stocks of clotrimazole (50 mM) were prepared in 100% ethanol and stored under argon at −20°C. 11,12-EET was prepared by total chemical synthesis and purified by reverse-phase HPLC as described previously (Wu et al., 1996; King et al., 2002). Recombinant CYP1A1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, and CYP4A11 were purchased from BD Gentest (Woburn, MA). For immunoblotting, P450s (1 pmol/lane) were electrophoresed on 12% Tris-glycine gels (Novex, San Diego, CA), and the resolved proteins were transferred to nitrocellulose membranes. Membranes were immunoblotted using MAb-2 (1:1000 dilution), goat anti-mouse IgG conjugated to horseradish peroxidase (BD Transduction Laboratories, Lexington, KY), and the SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical, Rockford, IL).

Immunoblotting. Recombinant human CYP2J2 was prepared as described previously (Wu et al., 1996; King et al., 2002). Recombinant CYP1A1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, and CYP4A11 were purchased from BD Gentest (Woburn, MA). For immunoblotting, P450s (1 pmol/lane) were electrophoresed on 12% Tris-glycine gels (Novex, San Diego, CA), and the resolved proteins were transferred to nitrocellulose membranes. Membranes were immunoblotted using MAb-2 (1:1000 dilution), goat anti-mouse IgG conjugated to horseradish peroxidase (BD Transduction Laboratories, Lexington, KY), and the SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical, Rockford, IL).

Antibody Inhibition Experiments. The CYP2J2 monoclonal antibodies MAb-1 or MAb-2, or the control monoclonal antibody MAb-C were preincubated with recombinant CYP2J2 protein (final concentration 0.1 nmol of P450/ml at protein-to-hemoprotein ratios ranging from 0 to 2.5 mg of IgG/nmol of P450 at 37°C in a buffer containing 0.05 M Tris-Cl, pH 7.5, 0.15 M KCl, and 0.01 M MgCl$_2$. After 10 min, 8 mM sodium isocitrate, 0.5 IU/ml isocitrate dehydrogenase, and [1-14C]AA (55–56 μCi/μmol; final concentration 100 μM) were added, and the reaction was initiated by adding 1 mM NADPH. After a 20-min incubation at 37°C, the reaction products were extracted and analyzed by reverse-phase HPLC as described previously (Wu et al., 1996; King et al., 2002). To determine the specificity of the monoclonal antibodies, inhibition of phenanthrene metabolism by a panel of recombinant P450s was examined as described previously (Gelboin et al., 1998; Krausz et al., 2000).

Recording of L-Type Ca$^{2+}$ Currents. Single left ventricular myocytes were isolated from hearts of adult CYP2J2 Tr and Wt mice (age 3–6 months, body weight 20–30 g) as described previously (Xiao et al., 1998). During an experiment, 20 μl of the myocyte-containing solution was pipetted into a recording chamber that was mounted on the stage of an inverted microscope (Nikon, Tokyo, Japan) and continuously superfused with the Tyrode’s solution containing 137 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 10 mM HEPES, and 10 mM glucose, pH 7.4. Recording pipettes were made from 1.5-mm outer diameter glass tubes (WPI, Sarasota, FL) with −1 Ω resistance. After forming a conventional “Gigaseal”, the capacitance of an electrode was compensated. Additional suction was used to rupture the membrane and to form a whole-cell configuration. The membrane capacitance (measured with pClamp software, version 8.2; Axon Instruments Inc., Foster City, CA) was 140 ± 5.4 pF for the Wt cardiomyocytes (n = 60) and 137 ± 4.5 pF for the Tr heart cells (n = 8).
Expression and Phosphorylation of the \( \alpha_i \) Subunit of the L-Type Ca\(^{2+} \) Channel. Hearts from CYP2J2 Tr and Wt mice were lyed for 30 min on ice in radioimmunoprecipitation buffer (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) containing the protease inhibitors pepstatin A (1 \( \mu \)g/ml), leupeptin (10 \( \mu \)g/ml), apotinin (20 \( \mu \)g/ml), and phenylmethylsulfonyl fluoride (200 nM). Lysates were then centrifuged for 15 min at 10,000g, and supernatants were used for immunoprecipitation experiments as described previously (Davare et al., 2000; Davare and Hell, 2003). The affinity-purified anti-\( \alpha_i \) type Ca\(^{2+} \) channel (CNC1) antibody (3 \( \mu \)g in 300-\( \mu \)l sample) was used to immunoprecipitate the Ca\(_{1,2,1}\) type Ca\(^{2+} \) channel from 100 \( \mu \)g of heart lysate. Immune complexes were bound to a Seize X Protein A column (Pierce Chemical), washed extensively with phosphate-buffered saline, and eluted with elution buffer (Pierce Chemical). Proteins were then separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and immunoblotted. Membranes were first incubated with anti-CH1923–1932P primary antibody (Davare et al., 1999) (1:500 dilution), goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc.) and the SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical). Blots were then stripped and reprobed with the anti-CNC1 primary antibody (1:200 dilution). Relative band intensities were quantified by densitometry using a ChemiImager 4000 Imaging System (Alpha Innotech, San Leandro, CA).

Data Analysis. The density (pA/pF) of \( I_{Ca} \) was calculated as a ratio of current amplitude to membrane capacitance of individual cardiomyocytes to avoid the possibility that differences in Ca\(^{2+} \) currents in CYP2J2 Tr and Wt cardiomyocytes resulted from differences in cell size. Inactivation time constants were determined by least-squares fitting (\( y = A_0 + A_1 \exp(-t/V_{1/2}) + A_2 + A_3 \exp(-t/V_{1/2}) \)) of a double-exponential function to each current trace (Xiao et al., 1998). The results of the steady-state inactivation of \( I_{Ca} \) were fitted by a Boltzmann equation (\( y = 1/(1 + \exp(V - V_{1/2})/K)) \). The best-fit procedure was performed with a commercial software program (Origin 6.0; OriginLab Corp., Northampton, MA). All data are presented as mean \( \pm \) S.E.M. unless otherwise stated. Paired or unpaired Student's \( t \) test or one-way analysis of variance was applied for statistical analyses as appropriate. Differences were considered significant if \( P < 0.05 \).

Results

Enhancement of Cardiac \( I_{Ca} \) in CYP2J2 Transgenic Mice. To assess the effect of CYP2J2 overexpression and enhanced EET biosynthesis on cardiac Ca\(^{2+} \) channel activity, \( I_{Ca} \) was elicited by single-step pulses from a holding potential of \(-50 \) to \( 0 \) mV in isolated left ventricular cardiomyocytes. Figure 1, A and B, shows that compared with Wt, \( I_{Ca} \) was significantly increased in CYP2J2 Tr cardiomyocytes. The density of \( I_{Ca} \) was increased by 40 \( \pm \) 9\%; from 9.7 \( \pm \) 0.6 pA/pF for Wt cardiomyocytes (\( n = 60 \)) to 13.6 \( \pm \) 0.9 pA/pF for CYP2J2 Tr cardiomyocytes (\( n = 71 \); \( P < 0.001 \)). Significant increases in the densities of \( I_{Ca} \) were also observed in CYP2J2 Tr cardiomyocytes when \( I_{Ca} \) was elicited by pulses with different voltage steps (Fig. 1C). Maximal \( I_{Ca} \) was obtained at \( 0 \) mV in both Wt and CYP2J2 Tr cells (Fig. 1D). Compared with Wt cardiomyocytes, the bell-shaped current-voltage relationship curve was not altered in CYP2J2 Tr cardiomyocytes. The inactivation time constants of \( I_{Ca} \) elicited by pulses from a holding potential of \(-50 \) to \( 0 \) mV were similar in \( n = 35 \) and CYP2J2 Tr (\( n = 48 \); \( P > 0.05 \)) cardiomyocytes (Fig. 1E). The fast (\( \tau_1 \)) and slow (\( \tau_2 \)) components of inactivation were 10.36 \( \pm \) 0.99 and 54.71 \( \pm \) 2.39 ms for \( I_{Ca} \) of Wt cardiomyocytes, and 10.03 \( \pm \) 0.80 and 49.91 \( \pm \) 1.89 ms for \( I_{Ca} \) of CYP2J2 Tr cardiomyocytes, respectively. Figure 2, A and B, shows that the steady-state inactivation curve of \( I_{Ca} \) in CYP2J2 Tr heart cells was similar to that in Wt cardiomyocytes. The \( V_{0.5} \) of the steady-state inactivation of \( I_{Ca} \) was \(-30.2 \pm 0.9 \) and \(-28.4 \pm 0.3 \) mV for Wt (\( n = 17 \)) and CYP2J2 Tr (\( n = 15 \); \( P > 0.05 \)) cardiomyocytes, respectively. Together, these data demonstrate that compared with Wt cardiomyocytes, the density of cardiac \( I_{Ca} \) was significantly increased in CYP2J2 Tr cardiomyocytes. Moreover, these differences occur without kinetic alterations in the activation or the steady-state inactivation of \( I_{Ca} \).

Suppression of \( I_{Ca} \) by Cytochrome P450 Inhibitors. To determine whether P450 activity affected cardiac \( I_{Ca} \) in CYP2J2 Tr cardiomyocytes, we added MS-PPOH to the external bath solution and then elicited \( I_{Ca} \) by single-step pulses from a holding potential of \(-50 \) to \( 0 \) mV. Extracellular application of 25 \( \mu \)M MS-PPOH gradually inhibited \( I_{Ca} \). A new, lower steady-state level of \( I_{Ca} \) was observed 8 min after MS-PPOH addition (Fig. 3A). Importantly, application of 11.12-EET (40 nM) significantly reversed the inhibition of \( I_{Ca} \) caused by MS-PPOH (Fig. 3A). Averaged data from multiple independent experiments are shown in Fig. 3B. MS-PPOH significantly reduced cardiac \( I_{Ca} \) in CYP2J2 transgenic cardiomyocytes to 45 \( \pm \) 4% of control (\( n = 4 \); \( P < 0.05 \)) and 11.12-EET partially restored the MS-PPOH-inhibited currents to 65 \( \pm \) 5% of control (\( n = 4 \); \( P < 0.05 \)).

To assess whether inhibition of P450 activity also suppressed cardiac \( I_{Ca} \) in Wt cardiomyocytes, we externally applied MS-PPOH or clotrimazole. Figure 3C shows that at 5 \( \mu \)M MS-PPOH, \( I_{Ca} \) of CYP2J2 Tr cardiomyocytes was significantly inhibited by 29.0 \( \pm \) 8.0% (\( n = 6 \); \( P < 0.05 \)), whereas inhibition of \( I_{Ca} \) in Wt cardiomyocytes did not reach statistical significance (24.5 \( \pm \) 8.1%; \( n = 5 \); \( P > 0.05 \)). The degree of inhibition of \( I_{Ca} \) was greater in both Wt cardiomyocytes (50.2 \( \pm \) 6.2%; \( n = 10 \); \( P < 0.01 \)) and CYP2J2 Tr cardiomyocytes (64.7 \( \pm \) 6.5%; \( n = 10 \); \( P < 0.001 \)) when the concentration of MS-PPOH was raised to 25 \( \mu \)M. The decrease in \( I_{Ca} \) was more profound in the CYP2J2 Tr than in the Wt cardiomyocytes (\( P < 0.01 \)) (Fig. 3C). Likewise, clotrimazole significantly suppressed cardiac \( I_{Ca} \) in both Wt and CYP2J2 Tr mice (Fig. 3C). The inhibition of the peak \( I_{Ca} \) by 5 \( \mu \)M clotrimazole was 52.8 \( \pm \) 10.1% (\( n = 6 \); \( P < 0.05 \)) and 64.3 \( \pm \) 13.5% (\( n = 5 \); \( P < 0.01 \)) for Wt and CYP2J2 Tr cardiomyocytes, respectively. Inhibition of \( I_{Ca} \) developed slowly and required \( \sim 5 \) min to reach a lower steady-state level after bath administration of clotrimazole (data not shown). Together, these results indicate that inhibition of P450 activity in mouse cardiomyocytes reduces Ca\(^{2+} \) currents and that CYP2J2 Tr cardiomyocytes are more sensitive to P450 inhibitors. It is interesting that the current densities of \( I_{Ca} \) were
significantly different between Wt \((n = 16)\) and CYP2J2 Tr \((n = 15)\) cells before treatment with 25 \(\mu\)M MS-PPOH and 5 \(\mu\)M clotrimazole \((P = 0.007)\). In contrast, there was no statistical difference in the \(I_{Ca}\) current densities between Wt and CYP2J2 Tr cells after inhibitor treatment \((P = 0.491)\).

**Inhibition of \(I_{Ca}\) by CYP2J2 Monoclonal Antibodies.**

Two monoclonal antibodies, MAb-1 and MAb-2, were developed to facilitate studies on the role of CYP2J2 metabolites in regulating cardiac L-type \(Ca^{2+}\) channel currents in mice. MAb-2 strongly reacts with recombinant CYP2J2 protein on immunoblots but does not cross-react with non-CYP2J subfamily P450s, including members of the CYP1A, CYP2A, CYP2B, CYP2C, CYP2D, CYP2E, and CYP4A subfamilies (Fig. 4A). In contrast, MAb-1 does not react with recombinant CYP2J2 or other P450s on immunoblots (data not shown). However, both MAb-1 and MAb-2 were highly selective for inhibition of CYP2J2 activity. Both antibodies inhibited >85% of CYP2J2-mediated metabolism of AA at concentrations of 0.5 mg of IgG/nmol of P450 or greater (Fig. 4, B and C). By comparison, a control antibody, MAb-C, prepared against egg lysozyme inhibited <10% of CYP2J2-mediated metabolism of AA under identical conditions (Fig. 4, B and C). None of the monoclonal antibodies significantly inhibited the metabolism of the universal P450 substrate phenanthrene by recombinant P450s of the CYP1A, CYP1B, CYP2A, CYP2B, CYP2C, CYP2D, CYP2E, and CYP3A subfamilies (Fig. 4D). In contrast, both CYP2J2 monoclonal antibodies (but not the control antibody) inhibited the metabolism of phenanthrene by recombinant CYP2J2. Based on these data, we conclude that both MAb-1 and MAb-2 are immunospecific for CYP2J2.

To assess whether the enhanced cardiac \(I_{Ca}\) in transgenic mice was related to overexpression of CYP2J2, we internally dialyzed either one of the two CYP2J2 monoclonal antibodies in cardiomyocytes to selectively inhibit CYP2J2 activity. \(I_{Ca}\) was elicited by single-step pulses from a holding potential of -50 to 0 mV. Shown are representative original current traces (Fig. 1A). Normalized values of peak \(I_{Ca}\) elicited by the voltage pulse protocol used in A are shown for Wt \((n = 60)\) and CYP2J2 Tr \((n = 71)\) cardiomyocytes. The relative currents were calculated as the ratio of \(I_{Ca,Tr}/I_{Ca,Wt}\). * \(P < 0.001\) versus Wt. ** \(P < 0.01\) versus Wt. 

C, current-voltage relationship of \(I_{Ca}\) in CYP2J2 Tr and Wt cardiomyocytes. The current-voltage curves were obtained from representative Wt and CYP2J2 Tr ventricular myocytes. The membrane holding potential was set at -50 mV. D, current-voltage relationships were plotted according to the densities of peak \(I_{Ca}\) of Wt \((n = 26)\) and CYP2J2 Tr \((n = 33)\) cardiomyocytes. * \(P < 0.001\) versus Wt. ** \(P < 0.01\) versus Wt. \(P < 0.05\) versus Wt. E, fast (\(\tau_1\)) and slow (\(\tau_2\)) components of inactivation time constants of \(I_{Ca}\) elicited by pulses from -50 to 0 mV were not significantly different between Wt \((n = 35)\) and CYP2J2 Tr \((n = 48)\) cardiomyocytes. Inactivation data of each current trace for individual cells were fit with least-squares fitting of a double exponential function as described previously (Xiao et al., 1998).
-50 to 0 mV. The amplitude of $I_{\text{ca}}$ recorded immediately after forming the whole-cell configuration was taken as the control value. $I_{\text{ca}}$ gradually decreased after intracellular dialysis with either MAB-1 or MAB-2 at antibody concentration of 0.125 mg of IgG/ml. At 15 min after initiation of dialysis with either MAB-1 or MAB-2, $I_{\text{ca}}$ was significantly suppressed in both Wt and CYP2J2 Tr cardiomyocytes (Fig. 5A). In Wt cardiomyocytes, $I_{\text{ca}}$ was reduced to 27.4 ± 6.7% (n = 6; P < 0.05) and 41.3 ± 5.6% (n = 5; P < 0.05) of control by MAB-1 and MAB-2, respectively. The reduction of $I_{\text{ca}}$ was even greater in CYP2J2 Tr cardiomyocytes after dialysis with MAB-1 (20.1 ± 6.6% of control; n = 6; P < 0.01) or MAB-2 (29.2 ± 9.2% of control; n = 7; P < 0.001). The differences in percentage of inhibition of cardiac $I_{\text{ca}}$ by MAB-1 and MAB-2 between Wt and CYP2J2 Tr cardiomyocytes did not reach statistical significance. In contrast, there was a small reduction in $I_{\text{ca}}$ in cardiomyocytes dialyzed with MAB-C (Fig. 5A), but this did not reach statistical significance in either Wt (n = 6; P = 0.212) or CYP2J2 Tr (n = 6; P = 0.078) cells. Current rundown and/or nonspecific inhibition of $I_{\text{ca}}$ could cause this reduction of $I_{\text{ca}}$ by MAB-C.

The inhibition of $I_{\text{ca}}$ after intracellular dialysis of CYP2J2 Tr cardiomyocytes with either MAB-1 or MAB-2 developed gradually and usually took 8 to 12 min to reach a new, lower steady-state level (20–30% of the control) (Fig. 5, B and C). Importantly, addition of the membrane-permeable 8-Br-cAMP at 2 mM concentration partially reversed the inhibition of $I_{\text{ca}}$ in CYP2J2 Tr cardiomyocytes dialyzed with either MAB-1 or MAB-2 (Fig. 5, B and C). The 8-Br-cAMP-induced changes in $I_{\text{ca}}$ were gradually reversed again after washout of the cyclic nucleotide (Fig. 5, B and C). Together, these results indicate that selective inhibition of CYP2J2 activity results in a significant reduction of cardiomyocyte $I_{\text{ca}}$ and that cAMP can partially restore the inhibited currents.

**Effects of PKA Modulation on $I_{\text{ca}}$** Activation of PKA results in L-type Ca$^{2+}$ channel phosphorylation that leads to increased $I_{\text{ca}}$ (Reuter, 1983; McDonald et al., 1994; Keef et al., 2001). To determine whether the effect of PKA on $I_{\text{ca}}$ was altered in CYP2J2 Tr hearts, we internally dialyzed the inhibitory fragment of PKA (PKA-IF) into cardiomyocytes. After forming the whole-cell configuration, $I_{\text{ca}}$ elicited by voltage pulses from a holding potential of −50 to 0 mV was gradually decreased after intracellular dialysis with PKA-IF in both Wt and CYP2J2 Tr cardiomyocytes (Fig. 6, A and B). At 15 min after initiation of dialysis, the density of peak $I_{\text{ca}}$ was 26 ± 11% (n = 6; P < 0.01) and 27 ± 8% of control (n = 6; P < 0.05) in Wt and CYP2J2 Tr cardiomyocytes, respectively. In contrast, dialysis with the internal solution alone for 15 min did not significantly reduce $I_{\text{ca}}$ in Wt (72 ± 7% of control; n = 8; P > 0.05) or CYP2J2 Tr (76 ± 12% of control; n = 10; P > 0.05) cardiomyocytes (Fig. 6, A and B). These results demonstrate that reduction of Ca$^{2+}$ channel phosphorylation by inhibition of PKA activity significantly decreases $I_{\text{ca}}$ to a comparable degree in both Wt and CYP2J2 Tr cardiomyocytes.

We also examined whether stimulation of β-adrenergic receptors with isoproterenol could reverse the inhibitory effect of the CYP2J2 monoclonal antibody on $I_{\text{ca}}$. Extracellular perfusion of 2 μM isoproterenol significantly increased the inhibited $I_{\text{ca}}$ recorded 15 min after dialysis with MAB-1 in both Wt and CYP2J2 Tr cardiomyocytes. Thus, compared with the control $I_{\text{ca}}$ recorded after dialysis with MAB-1 but before application of isoproterenol (Fig. 6C, Preiso), the normalized $I_{\text{ca}}$ was increased to 255 ± 69% of the control in the Wt cardiomyocytes (n = 6; P < 0.01) and to 193 ± 30% of the control in the CYP2J2 Tr cardiomyocytes (n = 12; P < 0.001) by isoproterenol application (Fig. 6C, MAB-1). We also assessed the effects of isoproterenol on $I_{\text{ca}}$ in cardiomyocytes dialyzed with MAB-2. The normalized control $I_{\text{ca}}$ recorded after dialysis with MAB-2 but before application of isoproterenol was increased to 251 ± 19% of control in Wt cardiomyocytes (n = 2) and to 200 ± 11% of control in CYP2J2 Tr cardiomyocytes (n = 2) by 2 μM isoproterenol (data not shown). In contrast, compared with the values of $I_{\text{ca}}$ recorded after dialysis with PKA-IF, 2 μM isoproterenol had no significant effects on the inhibited $I_{\text{ca}}$ in Wt cardiomyocytes (112 ± 13% of control; n = 5; P > 0.05) and CYP2J2 Tr cardiomyocytes (93 ± 24% of control; n = 5; P > 0.05) (Fig. 6C, PKA-IF). However, $I_{\text{ca}}$ recorded after dialysis with the pipette solution alone responded to stimulation with 2 μM isoproterenol in Wt cardiomyocytes (200 ± 75% of control; n = 5; P < 0.05) and CYP2J2 Tr cardiomyocytes (175 ± 15%
of control; \( n = 9; P < 0.01 \) (Fig. 6C, Control). Likewise, \( I_{\text{Ca}} \) recorded after dialysis with MAB-C responded to stimulation with 2 \( \mu M \) isoproterenol in Wt cardiomyocytes (180 \( \pm \) 20\% of control; \( n = 6; P < 0.05 \)) and CYP2J2 Tr cardiomyocytes (150 \( \pm \) 15\% of control; \( n = 6; P < 0.01 \)) (Fig. 6C, MAB-C). Compared with the effects of isoproterenol on \( I_{\text{Ca}} \) in cardiomyocytes dialyzed with MAB-1, the increases in \( I_{\text{Ca}} \) induced by isoproterenol were less, albeit not statistically so, in cardiomyocytes dialyzed with the pipette solution alone or with MAB-C in both Wt and CYP2J2 Tr mice (Fig. 6C). This is because \( I_{\text{Ca}} \) was not significantly inhibited in these two groups (Figs. 5A and 6, A and B). Together, these data demonstrate that \( \beta \)-adrenergic receptor stimulation increases \( I_{\text{Ca}} \) in both Wt and CYP2J2 Tr cardiomyocytes after selective inhibition of CYP2J2 with MAB-1, but not after inhibition of PFA.

**Channel Phosphorylation in CYP2J2 Transgenic Hearts.** To determine whether there were differences in expression and/or phosphorylation of the \( \alpha_1 \) subunit of the Ca\(_{1.2} \) L-type Ca\(^{2+} \) channel between Wt and CYP2J2 Tr hearts, the channel subunit was immunoprecipitated with anti-CNC1 and expression levels were analyzed by immunoblotting. No significant differences were observed in cardiac expression of the \( \alpha_1 \) subunit of the Ca\(_{1.2} \) L-type Ca\(^{2+} \) channel between Wt and CYP2J2 Tr mice (Fig. 7A). However, expression of phosphorylated form of the channel was significantly increased in hearts from CYP2J2 Tr mice compared with Wt mice (Fig. 7A). Hence, the ratio of phosphorylated \( \alpha_1 \) subunit (CH1923–1932P) to total \( \alpha_1 \) subunit (CNC1) expression was 30\% greater in CYP2J2 Tr hearts than in Wt hearts (\( P < 0.05 \)) (Fig. 7B). Based on these data, we conclude that overexpression of CYP2J2 is associated with increased phosphorylation of the \( \alpha_1 \) subunit of the Ca\(_{1.2} \) L-type Ca\(^{2+} \) channel.

**Discussion**

CYP2J2 is abundant in the heart and its expression is highly localized to cardiomyocytes (Wu et al., 1996, 1997). This P450 epoxygenase is a major cardiac enzyme responsible for generating biologically active eicosanoids, the EETs (Wu et al., 1996). Human and rodent hearts contain substantial quantities of EETs, which have been shown to influence cardiac function (Wu et al., 1996, 1997; Capdevila et al., 2000; Zeldin, 2001; Kroetz and Zeldin, 2002). For example, the EETs are potent coronary artery vasodilators (Hecker et al., 1994; Campbell et al., 1996) and are known to affect cardiac Na\(^+ \) and ATP-sensitive K\(^+ \) channels (Lee et al., 1999; Lu et al., 2001, 2002). The effects of EETs on cardiomyocyte L-type Ca\(^{2+} \) channels are more controversial. Xiao et al. (1998) found that EETs increase \( I_{\text{Ca}} \) in rat cardiomyocytes via a mechanism that involves changes in intracellular levels of cAMP. In contrast, Chen et al. (1999) found that EETs have a direct inhibitory effect on porcine cardiac L-type Ca\(^{2+} \) channels reconstituted into planar lipid bilayers. In light of this controversy and to further characterize the biological function of CYP2J2 in the heart, we used a recently developed transgenic mouse model (Seubert et al., 2004) to study the effects of increased CYP2J2 expression on cardiac L-type Ca\(^{2+} \) channel activity. The main finding of the current study is that cardiac \( I_{\text{Ca}} \) is significantly enhanced in CYP2J2 Tr mice relative to Wt controls. Moreover, under basal conditions, the amount of L-type Ca\(^{2+} \) current that is sensitive to P450 inhibition is substantial in both CYP2J2 Tr and Wt cardiomyocytes. In light of the fact that CYP2J2 Tr
hearts have enhanced EET biosynthesis (Seubert et al., 2004), our data suggest that these P450 epoxygenase metabolites have a net stimulatory effect on I_{C_{a}} in cardiomyocytes and play an important role in modulating basal cardiac L-type Ca^{2+} channel activity. 

Inhibition of P450 activity by MS-PPOH or clotrimazole significantly reduced cardiac I_{C_{a}} in both Wt and CYP2J2 Tr mice. This is consistent with our previous report that suppression of P450 activity reduced cardiac Ca^{2+} currents, intracellular free-Ca^{2+} signals, and cell shortening in isolated rat single ventricular myocytes (Xiao et al., 1998). It has been previously shown that MS-PPOH is a potent and selective inhibitor of P450-catalyzed AA epoxidation in vitro and in vivo (Wang et al., 1998; Brand-Schieber et al., 2000) and that clotrimazole is a powerful and selective P450 inhibitor with little effect on either cyclooxygenase or lipoxygenase pathways at concentrations similar to those used in the current studies (Capdevila et al., 1988). Interestingly, application of the CYP2J2 metabolite 11,12-EET significantly reversed the MS-PPOH-inhibited I_{C_{a}}. The P450 inhibitor-induced suppression of cardiac I_{C_{a}} in the current study is therefore probably caused by inhibition of P450 AA epoxygenase activity. Our inhibitor data also suggest that the effect on I_{C_{a}} is mediated by P450-mediated metabolites of AA rather than a direct interaction between the CYP2J2 protein and the Ca^{2+} channel. This concept is further supported by our results with the two different inhibitory monoclonal antibodies that are highly selective for inhibition of CYP2J2 activity and also caused a marked suppression of cardiac I_{C_{a}}. Moreover, the EETs have been shown to significantly increase intracellular Ca^{2+} levels in cardiomyocytes (Seubert et al., 2004).
Fig. 5. Inhibitory effect of intracellular dialysis with CYP2J2 monoclonal antibodies on \( I_{\text{Ca}} \) in CYP2J2 Tr and Wt cardiomyocytes. A, currents were evoked by 200-ms pulses from a holding potential of \(-50\) to \(0\) mV every 30 s. The pipette solution contained one of the monoclonal antibodies at a concentration of 0.125 mg of IgG/ml. \( I_{\text{Ca}} \) was recorded immediately after forming the whole-cell configuration (Initial) and again 15 min after intracellular dialysis with one of the three antibodies (MAb-1, MAb-2, and MAb-C). \( I_{\text{Ca}} \) recorded after dialysis for 15 min was normalized to the corresponding initial value for each individual cell. *, \( P < 0.05 \); **, \( P < 0.01 \); ***, \( P < 0.001 \) versus initial. B and C, time course of the CYP2J2 monoclonal antibody inhibition of \( I_{\text{Ca}} \) and response to 8-Br-cAMP in CYP2J2 Tr cardiomyocytes. \( I_{\text{Ca}} \) was evoked by 200-ms pulses from a holding potential of \(-50\) to \(0\) mV every 10 s. The pipette solution contained 0.125 mg of IgG/ml of either MAb-1 (B) or MAb-2 (C). \( I_{\text{Ca}} \) was recorded immediately after forming the whole-cell configuration and the densities of peak \( I_{\text{Ca}} \) were plotted against the time after rupture of the cell membrane. \( I_{\text{Ca}} \) was gradually inhibited after intracellular dialysis with either MAb-1 or MAb-2 and was significantly restored after extracellular perfusion of 2 mM 8-Br-cAMP. \( I_{\text{Ca}} \) was reinnhibited after washout of cAMP. The insets were the original current traces recorded at different time points corresponding to the symbols of a, b, c, and d during the time courses.

Fig. 6. Decrease of \( I_{\text{Ca}} \) in CYP2J2 Tr and Wt cardiomyocytes after intracellular dialysis with PKA-IF and effects of \( \beta \)-adrenergic agonist stimulation. \( I_{\text{Ca}} \) was evoked by 200-ms pulses from a holding potential of \(-50\) to \(0\) mV every 30 s. Peak amplitudes of \( I_{\text{Ca}} \) were measured and normalized to their corresponding initial values recorded immediately after rupture of the cell membrane. Normalized \( I_{\text{Ca}} \) was plotted as a function of time after rupture of the membrane patch for whole-cell recordings of Wt (A) and CYP2J2 Tr (B) cardiomyocytes. There was some "rundown" of \( I_{\text{Ca}} \) in cardiomyocytes after intracellular dialysis with the pipette solution alone, but there were no significant differences between the values recorded at 0 and 15 min in Wt (\( n = 8 \)) and CYP2J2 Tr (\( n = 10 \)) mice. After intracellular dialysis with 0.5 mg/ml PKA-IF for 15 min, \( I_{\text{Ca}} \) was significantly inhibited in both Wt (\( n = 6 \)) and CYP2J2 Tr (\( n = 6 \)) cardiomyocytes. *, \( P < 0.05 \); **, \( P < 0.01 \) versus 0 min; #, \( P < 0.01 \) versus control. In C, the effects of isoproterenol on \( I_{\text{Ca}} \) are shown. Cardiomyocytes were dialyzed with the pipette solution alone or plus PKA-IF, MAb-C, or MAb-I. \( I_{\text{Ca}} \) was evoked by 200-ms pulses from a holding potential of \(-50\) to \(0\) mV every 30 s and normalized to their corresponding preisoproterenol values (PreIso). Isoproterenol at 2 mM significantly increased \( I_{\text{Ca}} \) in the cardiomyocytes dialyzed with the pipette solution alone (Control) or plus either of the monoclonal antibodies (MAb-C or MAb-I), but not plus PKA-IF. *, \( P < 0.05 \); **, \( P < 0.01 \); ***, \( P < 0.001 \) versus PreIso.
Ca\textsuperscript{2+} signals in guinea pig hearts and isolated ventricular myocytes (Moffat et al., 1993) and to enhance I\textsubscript{Ca} in rat cardiomyocytes (Xiao et al., 1998). Therefore, enhancement of cardiac I\textsubscript{Ca} in CYP2J2 transgenic mice most likely results from increased EET biosynthesis.

CYP2J2-derived EETs may directly affect the L-type Ca\textsuperscript{2+} channel as proposed by Chen et al. (1999), or, alternatively, may act through an intracellular signaling pathway that leads to channel phosphorylation (Reuter, 1983; McDonald et al., 1994; Xiao et al., 1998; Keef et al., 2001). In this regard, we found that the inhibitory effects of the two CYP2J2 monoclonal antibodies on I\textsubscript{Ca} were reversed by addition of the membrane permeable 8-Br-cAMP. Interestingly, inhibition of PKA activity significantly decreased I\textsubscript{Ca} in both CYP2J2 Tr and Wt cardiomyocytes confirming that, under basal conditions, PKA-dependent phosphorylation of the L-type Ca\textsuperscript{2+} channel plays a crucial role in regulating I\textsubscript{Ca}. Importantly, immunoblot analysis showed that, compared with Wt hearts, the level of phosphorylated a\textsubscript{1} subunit of the L-type Ca\textsuperscript{2+} channel protein was significantly increased in CYP2J2 Tr hearts. Together, these data suggest that CYP2J2-derived EETs act through a cAMP-PKA-dependent mechanism, leading to increased channel phosphorylation resulting in enhanced I\textsubscript{Ca}. This hypothesis is consistent with our previous data that showed that 11,12-EET increased intracellular cAMP levels and enhanced L-type Ca\textsuperscript{2+} channel phosphorylation in rat cardiomyocytes (Xiao et al., 1998). Interestingly, although addition of the \beta-adrenergic agonist isoproterenol did not reverse the inhibition of I\textsubscript{Ca} caused by PKA-IF, it significantly increased the inhibited I\textsubscript{Ca} in cardiomyocytes dialyzed with the CYP2J2 monoclonal antibody. These results suggest that CYP2J2 metabolites modulate a step that is upstream of PKA in the signaling cascade. In this regard, EETs have been recently shown to increase G\textsubscript{\alpha} but not G\textsubscript{\alpha\textsubscript{12}} GTP-binding activity in endothelial cells (Node et al., 2001) and are known to stimulate the ADP-ribosylation of G\textsubscript{\alpha} in vascular smooth muscle cells (Li et al., 1999).

Other explanations for our findings are possible. For example, overexpression of CYP2J2 may inhibit the expression of another gene product that is involved in suppressing the phosphorylation of L-type Ca\textsuperscript{2+} channels or one that actually dephosphorylates the channels (e.g., a phosphatase). Under this scenario, inhibition of CYP2J2 by the MAb would increase the net expression of the inhibitory intermediate, lower the fraction of phosphorylated channels, and reduce the Ca\textsuperscript{2+} currents. Subsequent perfusion with cAMP would enhance the Ca\textsuperscript{2+} currents. Inhibition of a suppressor or phosphatase activity by CYP2J2 products would also explain the increased levels of phosphorylated L-type Ca\textsuperscript{2+} channel subunits in the CYP2J2 transgenic hearts.

It is also possible that the enhanced I\textsubscript{Ca} observed in the CYP2J2 transgenic hearts is due, at least in part, to reduced AA availability because extracellular application of AA has been shown to inhibit I\textsubscript{Ca} in rat cardiomyocytes (Xiao et al., 1997). Indeed, increased CYP2J2-mediated metabolism of AA would be expected to reduce intracellular levels of this free fatty acid in the CYP2J2 transgenic hearts. Likewise, inhibition of I\textsubscript{Ca} in cardiomyocytes dialyzed with the CYP2J2 monoclonal antibodies and/or treated with P450 inhibitors might result from an accumulation of AA. However, we believe that this possibility is unlikely because 8-Br-cAMP and isoproterenol significantly restored the suppressed I\textsubscript{Ca} in the CYP2J2 transgenic hearts.

![Fig. 7](https://example.com/fig7.png)

**Fig. 7.** Expression and phosphorylation of the Ca\textsubscript{1.2} L-type Ca\textsuperscript{2+} channels in Wt and CYP2J2 Tr hearts. A, immunoblots showing expression of the a\textsubscript{1} subunit (anti-CNC1) and the phosphorylated form of the a\textsubscript{1} subunit (anti-CH1923–1932) in hearts of individual CYP2J2 Tr and Wt animals. B, ratio of CH1923–1932P to CNC1 expression was determined by densitometry. *, P < 0.05 versus Wt.
presence of the CYP2J2 inhibitors or monoclonal antibodies in the CYP2J2 Tr mice in the present study, whereas isoproterenol failed to reverse the AA-induced inhibition of cardiac ICa in our previous experiments (Xiao et al., 1997).

Our group has recently described the cardiac phenotype of the CYP2J2 Tr mice (Seubert et al., 2004). In brief, there were no significant differences between the CYP2J2 Tr and Wt mice with respect to heart or individual chamber weights, echocardiographic dimensions or fractional shortening, heart rate, or hemodynamic parameters under basal conditions. Moreover, histological assessment revealed no overt pathology in the CYP2J2 Tr hearts. The major heart phenotype of these mice is that they have enhanced postischemic recovery of contractile function. Further studies will be necessary to determine whether alterations in L-type Ca2+ channel activity contribute to the enhanced postischemic functional recovery in these animals.

In conclusion, the major finding in this study is that Ca2+ currents are significantly increased in CYP2J2 Tr cardiomyocytes. Moreover, our data suggest that this enhancement of ICa results from an increase in L-type Ca2+ channel phosphorylation via a cAMP-PKA-dependent mechanism modulated by a CYP2J2-derived metabolite. Given that L-type oxygen tension, CYP2J2 may be important in regulating the body and Dr. Jeffrey Robbins for providing the vector pBS-MHC-hGH. Acknowledgments

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