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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_{Ca}) in adult transgenic mice. Cardiac-specific overexpression of CYP2J2 was achieved using the α-myosin heavy chain promoter. I_{Ca} was recorded from isolated ventricular cardiomyocytes. Compared with the wild-type cardiomyocytes (n = 60), the density of I_{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_{Ca} in both wild-type and transgenic cardiomyocytes; however, MS-PPOH inhibited I_{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_{Ca} in MS-PPOH-treated cells. Intracellular dialysis with either of two inhibitory monoclonal antibodies against CYP2J2 significantly reduced I_{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_{Ca}. In addition, the total protein level of the α1 subunit of the Ca_v1.2 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_{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 monooxygenase 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 cardiomyocyte 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

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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 adenylyl 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 overexpress 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-IF14-24), and the P450 inhibitor clotrimazole were obtained from Sigma-Aldrich (St. Louis, MO). The specific inhibitor of EET biosynthesis *N*-methylsulfonyl-6-(2-proparglyloxyphenyl)hexanamide (MS-PPOH) was synthesized as described previously (Wang et al., 1998). Working stocks of clotrimazole (50 mM) and MS-PPOH (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., 1997; Chen et al., 1999; Node et al., 2001). Isoproterenol was freshly dissolved in the bath solution at 2 μM. PKA-IF was dissolved in the internal pipette solution at 0.5 mg/ml. Two monoclonal antibodies, MAb-1 (6-2-16-1, lot A1) and MAb-2 (6-5-20-8, lot A1), against the recombinant CYP2J2 protein and a control monoclonal antibody MAb-C (Hy-Hel-9, lot 12-10-96) against egg lysozyme were generated in mouse hybridoma cells as described previously (Gelboin et al., 1998; Krausz et al., 2000). Each antibody was diluted by the internal pipet solution to a final concentration of 0.125 mg of IgG/ml for intracellular dialysis (see below). Affinity-purified anti-α_1 L-type Ca^{2+} channel (CNC1) antibody was obtained from Chemicon International (Temecula, CA). Anti-CH1923-1932P antibody, which recognizes the phosphorylated form of the α_1 subunit of the $Ca_v1.2$ L-type Ca^{2+} channel (Davare et al., 2000; Davare and Hell, 2003), was a generous gift from Dr. Johannes Hell (University of Iowa, Iowa City, IA).

Generation of CYP2J2 Transgenic Mice. The coding region of the human CYP2J2 cDNA (GenBank U37143) was cloned into the SalI-HindIII sites of the vector pBS-α-MHC-hGH, a generous gift from Dr. Jeffrey Robbins (University of Cincinnati, Cincinnati, OH). This vector contains the α-MHC promoter to drive cardiomyocyte-specific expression of the transgene and human growth hormone/polyA sequences to enhance transgene mRNA stability. The linearized transgene was microinjected into pronuclei of single-cell C57BL6/J mouse embryos, which were implanted into pseudopregnant mice. Transgenic mice were identified by a combination of polymerase chain reaction and Southern blotting of tail genomic DNAs as described previously (Seubert et al., 2004). All studies used heterozygous CYP2J2 Tr progeny of each of four overexpressing lines and age/sex-matched Wt littermate control mice. All studies were approved by Animal Care and Use Committees of the respective institutions and were in accordance with principles outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

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-^{14}C]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 MΩ 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 =

71; $P > 0.05$ versus Wt). Series resistance and membrane capacitance were electrically compensated before application of experimental protocol. Ca^{2+} currents were recorded with an Axopatch 200B amplifier and pClamp software. For the whole-cell recording the external solution contained 100 mM *N*-methyl-*d*-glucamine, 5 mM CsCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, 10 mM tetrethylammonium, pH 7.4 with tetrethylammonium-OH. The pipette solution contained 100 mM CsCl, 40 mM CsOH, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM EGTA, 5 mM Mg-ATP, 10 mM HEPES, pH 7.3 with CsOH. Experiments were carried out at 22–23°C. The extracellular solution was exchanged by a modified rapid perfusion system as described previously (Xiao et al., 1998). Ca^{2+} currents were recorded from the same myocyte before, during, and after drug treatment. Final concentrations of ethanol used in experiments had no effect on cardiac Ca^{2+} currents

Expression and Phosphorylation of the α_1 Subunit of the L-Type Ca^{2+} Channel. Hearts from CYP2J2 Tr and Wt mice were lysed for 30 min on ice in radioimmunoprecipitation buffer (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) containing the protease inhibitors pepstatin A (1 μg/ml), leupeptin (10 μg/ml), aprotinin (20 μg/ml), and phenylmethylsulfonyl fluoride (200 nM). Lysates were then centrifuged for 15 min at 10,000*g*, and supernatants were used for immunoprecipitation experiments as described previously (Davare et al., 2000; Davare and Hell, 2003). The affinity-purified anti-α_1 L-type Ca^{2+} channel (CNC1) antibody (3 μg in 300-μl sample) was used to immunoprecipitate the Ca_v1.2 L-type Ca^{2+} channel from 100 μg 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 ($y = A_0 + A_1\exp^{-t/\tau1} + A_2\exp^{-t/\tau2}$) of a double-exponential function to each current traces (Xiao et al., 1998). The results of the steady-state inactivation of I_{Ca} were fitted by a Boltzmann equation ($y = 1/\{1 + \exp[(V - V_{0.5})/K]\}$). The best-fit procedure was performed with a commercial software program (Origin 6.0; OriginLab Corp., Northampton, MA). All data are presented as mean ± 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 ± 9%, from 9.7 ± 0.6 pA/pF for Wt cardiomyocytes ($n = 60$) to 13.6 ± 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 Wt ($n = 35$) and CYP2J2 Tr ($n = 48$; $P > 0.05$) cardiomyocytes (Fig. 1E). The fast (τ1) and slow (τ2) components of inactivation were 10.36 ± 0.99 and 54.71 ± 2.39 ms for I_{Ca} of Wt cardiomyocytes, and 10.03 ± 0.80 and 49.91 ± 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 ± 0.9 and −28.4 ± 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 μM 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 ± 4% of control ($n = 4$; $P < 0.05$) and 11,12-EET partially restored the MS-PPOH-inhibited currents to 65 ± 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 μM MS-PPOH, I_{Ca} of CYP2J2 Tr cardiomyocytes was significantly inhibited by 29.0 ± 8.0% ($n = 6$; $P < 0.05$), whereas inhibition of I_{Ca} in Wt cardiomyocytes did not reach statistical significance (24.5 ± 8.1%; $n = 5$; $P > 0.05$). The degree of inhibition of I_{Ca} was greater in both Wt cardiomyocytes (50.2 ± 6.2%; $n = 10$; $P < 0.01$) and CYP2J2 Tr cardiomyocytes (64.7 ± 6.5%; $n = 10$; $P < 0.001$) when the concentration of MS-PPOH was raised to 25 μM. 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 μM clotrimazole was 52.8 ± 10.1% ($n = 6$; $P < 0.05$) and 64.3 ± 13.5% ($n = 5$; $P < 0.01$) for Wt and CYP2J2 Tr cardiomyocytes, respectively. Inhibition of I_{Ca} developed slowly and required ~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 μM MS-PPOH and 5 μM 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

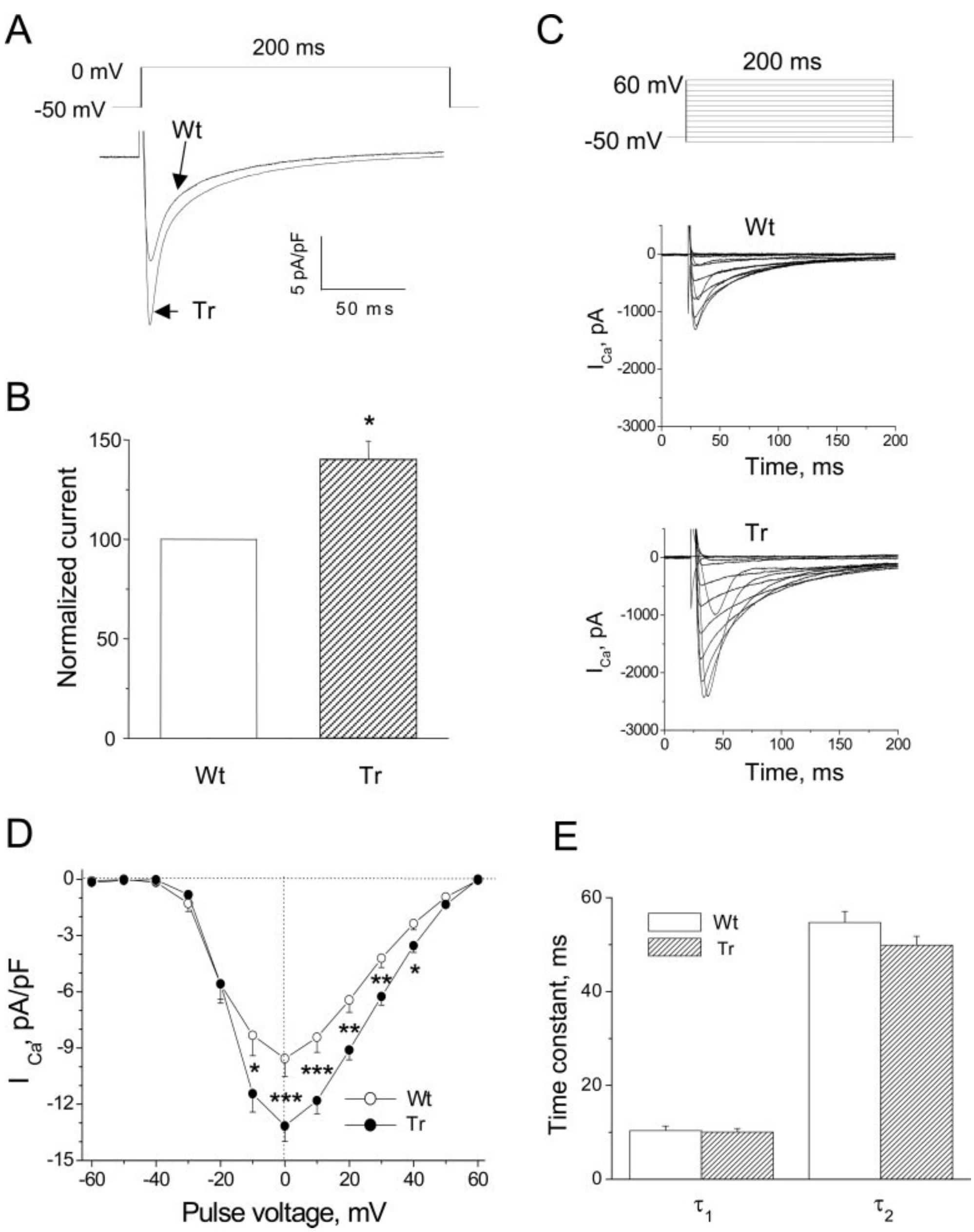

Fig. 1. Enhancement of I_{Ca} in ventricular myocytes isolated from CYP2J2 Tr mice. A, L-type Ca^{2+} currents were elicited from Wt and CYP2J2 Tr cardiomyocytes by 200-ms pulses from a holding potential of −50 to 0 mV. Shown are representative original current traces. B, normalized values of peak I_{Ca} 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 $I_{Ca,Tr}$ to $I_{Ca,Wt}$. *, $P < 0.001$ versus Wt. C, current-voltage relationship of I_{Ca} in CYP2J2 Tr and Wt cardiomyocytes. The voltage protocol was composed of a group of pulses from −60 to 60 mV with 10-mV increments every 10 s. The superimposed current traces were elicited 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. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus Wt. E, fast (τ1) and slow (τ2) 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_{Ca} recorded immediately after forming the whole-cell configuration was taken as the control value. I_{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_{Ca} was significantly suppressed in both Wt and CYP2J2 Tr cardiomyocytes (Fig. 5A). In Wt cardiomyocytes, I_{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_{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_{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_{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 ($n = 8$; $P = 0.078$) cells. Current rundown and/or nonspecific inhibition of I_{Ca} could cause this reduction of I_{Ca} by MAb-C.

The inhibition of I_{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_{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_{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_{Ca} and that cAMP can partially restore the inhibited currents.

Effects of PKA Modulation on I_{Ca}. Activation of PKA results in L-type Ca^{2+} channel phosphorylation that leads to increased I_{Ca} (Reuter, 1983; McDonald et al., 1994; Keef et al., 2001). To determine whether the effect of PKA on I_{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_{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_{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_{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_{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_{Ca}. Extracellular perfusion of 2 μM isoproterenol significantly increased the inhibited I_{Ca} recorded 15 min after dialysis with MAb-1 in both Wt and CYP2J2 Tr cardiomyocytes. Thus, compared with the control I_{Ca} recorded after dialysis with MAb-1 but before application of isoproterenol (Fig. 6C, PreIso), the normalized I_{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_{Ca} in cardiomyocytes dialyzed with MAb-2. The normalized control I_{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_{Ca} recorded after dialysis with PKA-IF, 2 μM isoproterenol had no significant effects on the inhibited I_{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_{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 cardiomyocyte (175 ± 15%

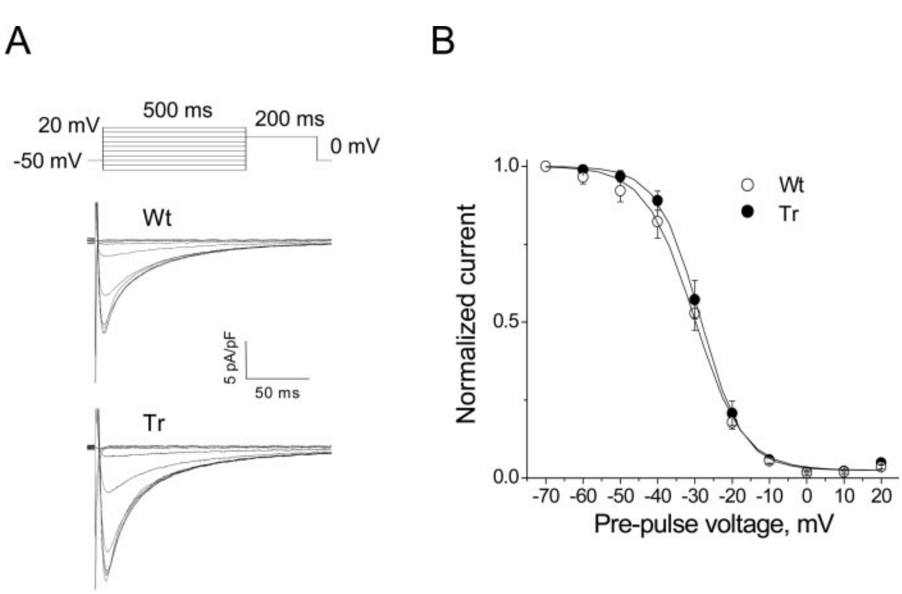

Fig. 2. Comparison of the steady-state inactivation of cardiac I_{Ca} between Wt and CYP2J2 Tr mice. The voltage protocol (A, top) had double pulses consisting of a 200-ms test pulse to 0 mV after a 500-ms conditioning prepulse varying from −70 to 20 mV in 10-mV increments at 0.1 Hz. The superimposed current traces in the lower portion of A were elicited by 200-ms test pulses from representative Wt and CYP2J2 Tr heart cells. The membrane holding potential was set at −50 mV. Normalized steady-state inactivation relationships (B) were plotted according to the densities of peak I_{Ca} of Wt (○; $n = 17$) and CYP2J2 Tr (●; $n = 15$) cardiomyocytes. Inactivation data of peak I_{Ca} were fitted to a Boltzmann equation (solid lines): $y = 1/\{1 + \exp[(V + V_{0.5})/K]\}$, where $V_{0.5}$ is the voltage at which $y = 0.5$ and K is the slope factor. The fitting parameters are $V_{0.5} = -28.4 \pm 0.3$ mV and $K = 5.6 \pm 0.2$ for Wt and $V_{0.5} = -30.2 \pm 0.9$ mV and $K = 6.0 \pm 0.5$ for CYP2J2 Tr cardiomyocytes, respectively.

of control; $n = 9$; $P < 0.01$) (Fig. 6C, Control). Likewise, I_{Ca} recorded after dialysis with MAb-C responded to stimulation with 2 μM isoproterenol in Wt cardiomyocytes (180 ± 20% of control; $n = 6$; $P < 0.05$) and CYP2J2 Tr cardiomyocytes (150 ± 15% of control; $n = 6$; $P < 0.01$) (Fig. 6C, MAb-C). Compared with the effects of isoproterenol on I_{Ca} in cardiomyocytes dialyzed with MAb-1, the increases in I_{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_{Ca} was not significantly inhibited in these two groups (Figs. 5A and 6, A and B). Together, these data demonstrate that β-adrenergic receptor stimulation increases I_{Ca} in both Wt and CYP2J2 Tr cardiomyocytes after selective inhibition of CYP2J2 with MAb-1, but not after inhibition of PKA.

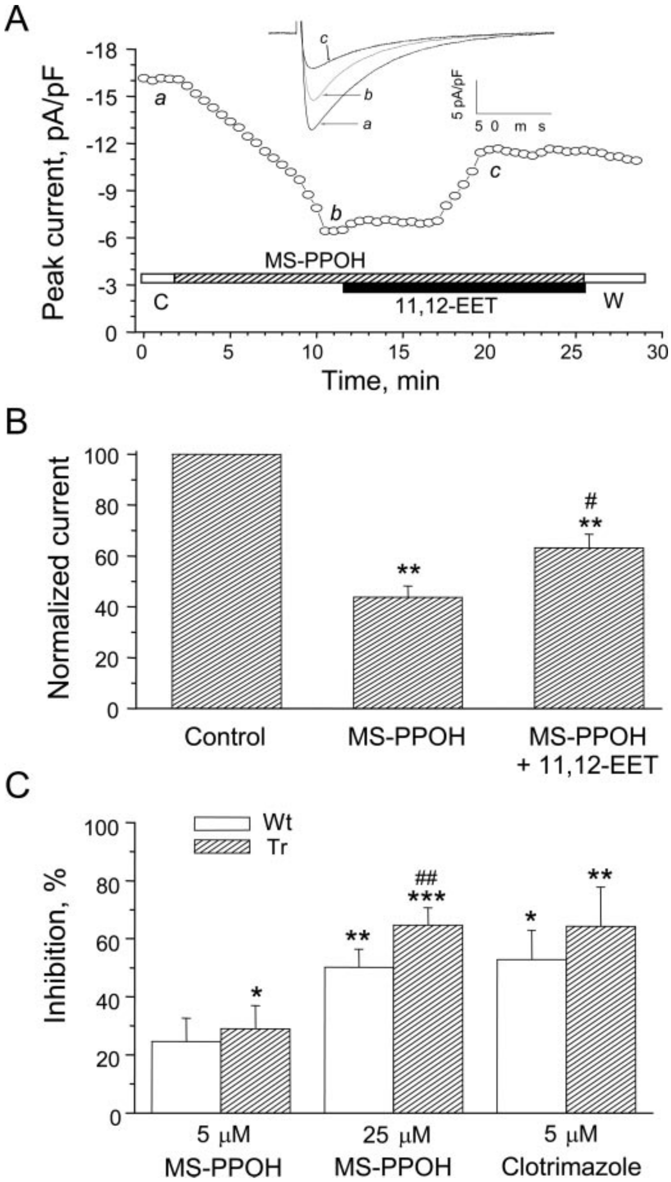

Fig. 3. Suppression of I_{Ca} by cytochrome P450 inhibitors in CYP2J2 Tr and Wt cardiomyocytes. Currents were evoked by 200-ms pulses from a holding potential of −50 to 0 mV every 30 s. A, time course of the effect of extracellular application of 25 μM MS-PPOH on I_{Ca} in a CYP2J2 transgenic cardiomyocyte. I_{Ca} was gradually inhibited and reached to a new, lower steady-state level 8 min after MS-PPOH addition. Addition of 11,12-EET at 40 nM partially restored the inhibited I_{Ca}. The inset shows the original current traces (a, b, and c) recorded at different time points corresponding to the symbols a, b, and c marked on the time course curve. C, control (open bar); MS-PPOH, perfusion with 25 μM MS-PPOH solution (striped bar); 11,12-EET, addition of 40 nM 11,12-EET (solid bar); W, washout (open bar). B, normalized values of peak I_{Ca} under control conditions, after application of 25 μM MS-PPOH, and after addition of 40 nM 11,12-EET. MS-PPOH significantly inhibited cardiac I_{Ca} in CYP2J2 transgenic hearts and 11,12-EET partially restored the MS-PPOH-inhibited I_{Ca}. **, $P < 0.01$ versus control; #, $P < 0.05$ versus MS-PPOH alone. C, MS-PPOH (5 or 25 μM) or clotrimazole (5 μM) was applied to the external perfusion solution. After reaching maximal inhibition, I_{Ca} was measured again and the percentage inhibition was calculated by comparison of the amplitudes of I_{Ca} in the absence or presence of the inhibitors. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus absence of inhibitor; ##, $P < 0.01$ versus Wt at the same dose of inhibitor.

Channel Phosphorylation in CYP2J2 Transgenic Hearts. To determine whether there were differences in expression and/or phosphorylation of the α_1 subunit of the $Ca_v1.2$ 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 α_1 subunit of the $Ca_v1.2$ 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 α_1 subunit (CH1923–1932P) to total α_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 α_1 subunit of the $Ca_v1.2$ 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; Roman, 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_{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_{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_{Ca} 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_{Ca} 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_{Ca}. The P450 inhibitor-induced suppression of cardiac I_{Ca} 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_{Ca} 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_{Ca}. Moreover, the EETs have been shown to significantly increase intracellular

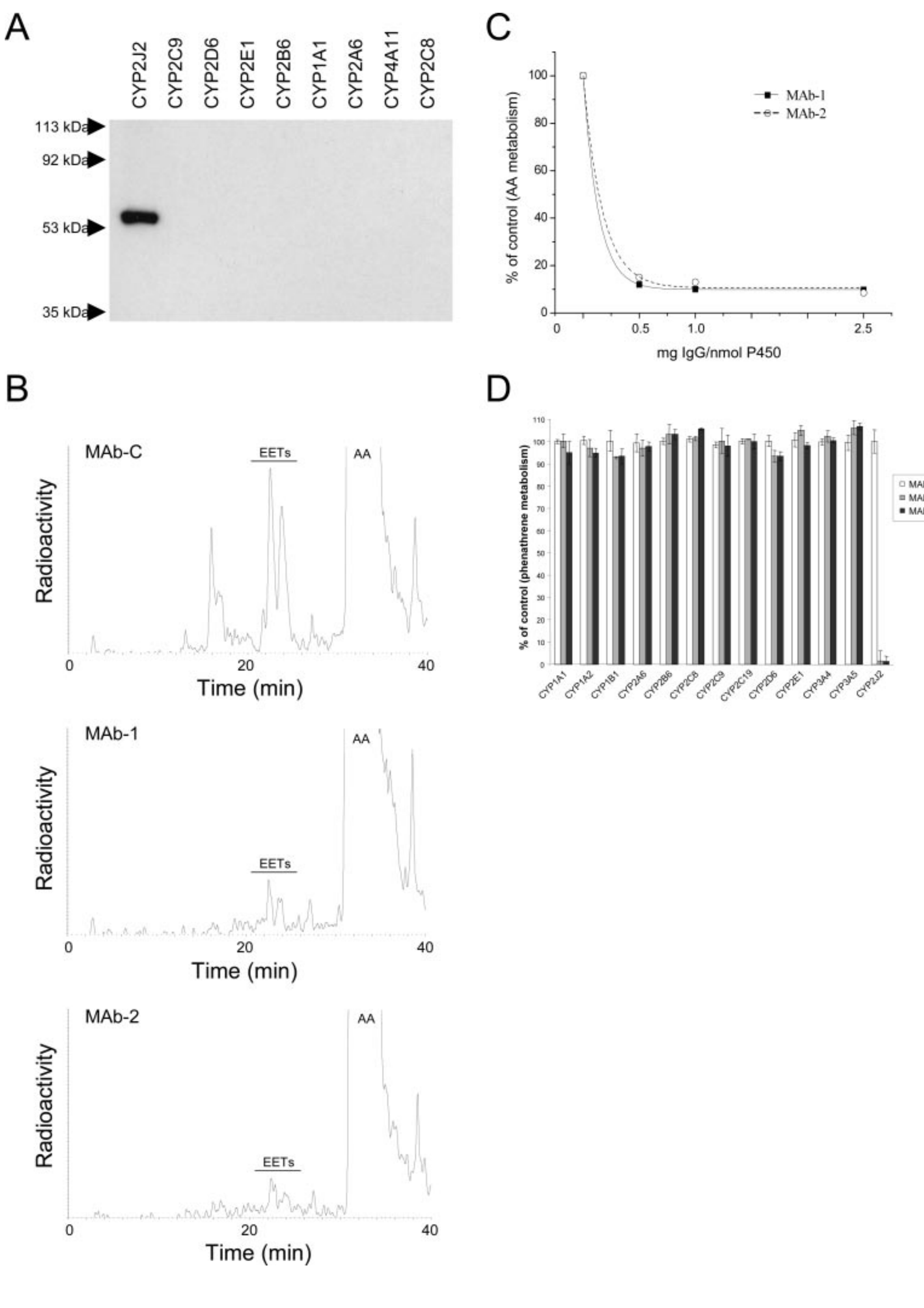

Fig. 4. Characterization of CYP2J2 monoclonal antibodies. A, immunoblot showing immunoreactivity of MAb-2 with recombinant P450s. This antibody strongly reacts with recombinant CYP2J2 but does not cross-react with non-CYP2J subfamily P450s. B, reversed-phase HPLC chromatograms showing inhibition of CYP2J2-mediated metabolism of AA by MAb-1 and MAb-2, but not by MAb-C. The MAbs were used at a final concentration of 0.5 mg of IgG/nmol of P450. Products were identified by comparing their HPLC properties with those of authentic standards as described previously (Wu et al., 1996; King et al., 2002). C, inhibition of AA metabolism by MAb-1 and MAb-2. The MAbs were used at protein to hemoprotein ratios ranging from 0 to 2.5 mg of IgG/nmol of P450. Data are expressed as a percentage of control incubations with MAb-C. D, specificity of the monoclonal antibodies with respect to inhibition of phenanthrene metabolism by a panel of recombinant P450s was examined. Data are expressed as a percentage of control incubations with buffer alone (no MAb).

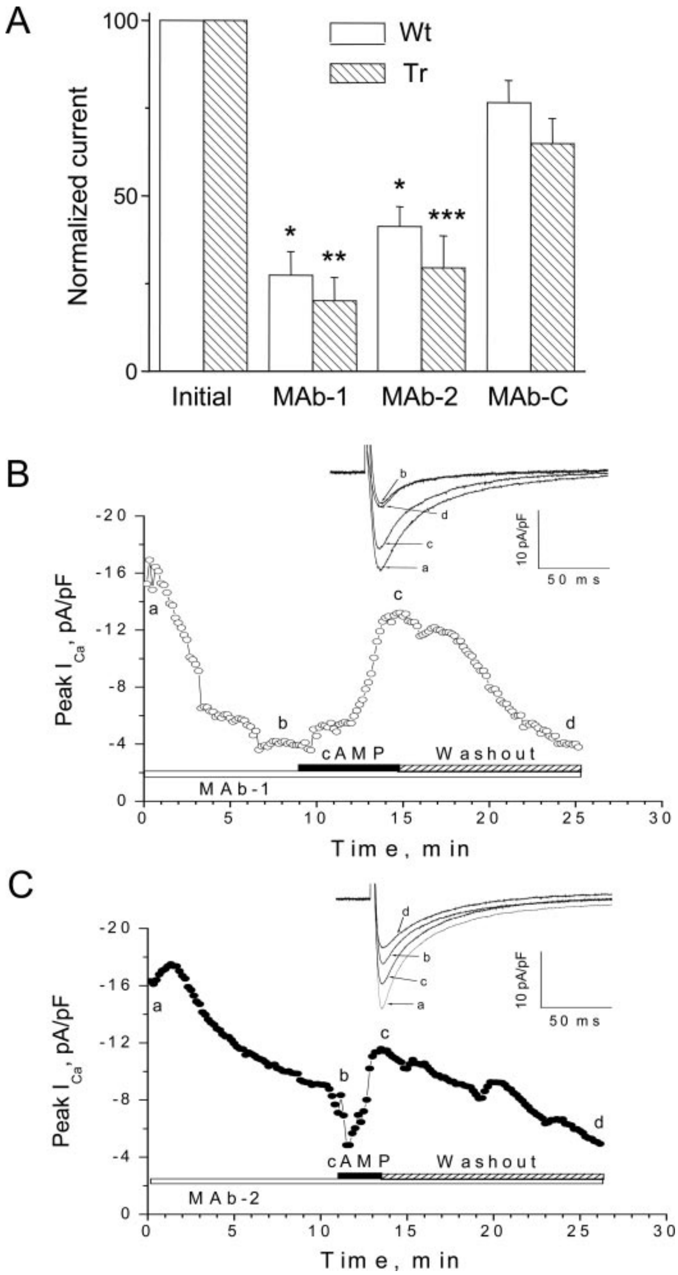

Fig. 5. Inhibitory effect of intracellular dialysis with CYP2J2 monoclonal antibodies on I_{Ca} in CYP2J2 Tr and Wt cardiomyocytes. A, currents were evoked by 200-ms pulses from a holding potential of −50 to 0 mV every 30 s. The pipette solution contained one of the monoclonal antibodies at a concentration of 0.125 mg of IgG/ml. I_{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_{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_{Ca} and response to 8-Br-cAMP in CYP2J2 Tr cardiomyocytes. I_{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_{Ca} was recorded immediately after forming the whole-cell configuration and the densities of peak I_{Ca} were plotted against the time after rupture of the cell membrane. I_{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_{Ca} was reinhibited 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.

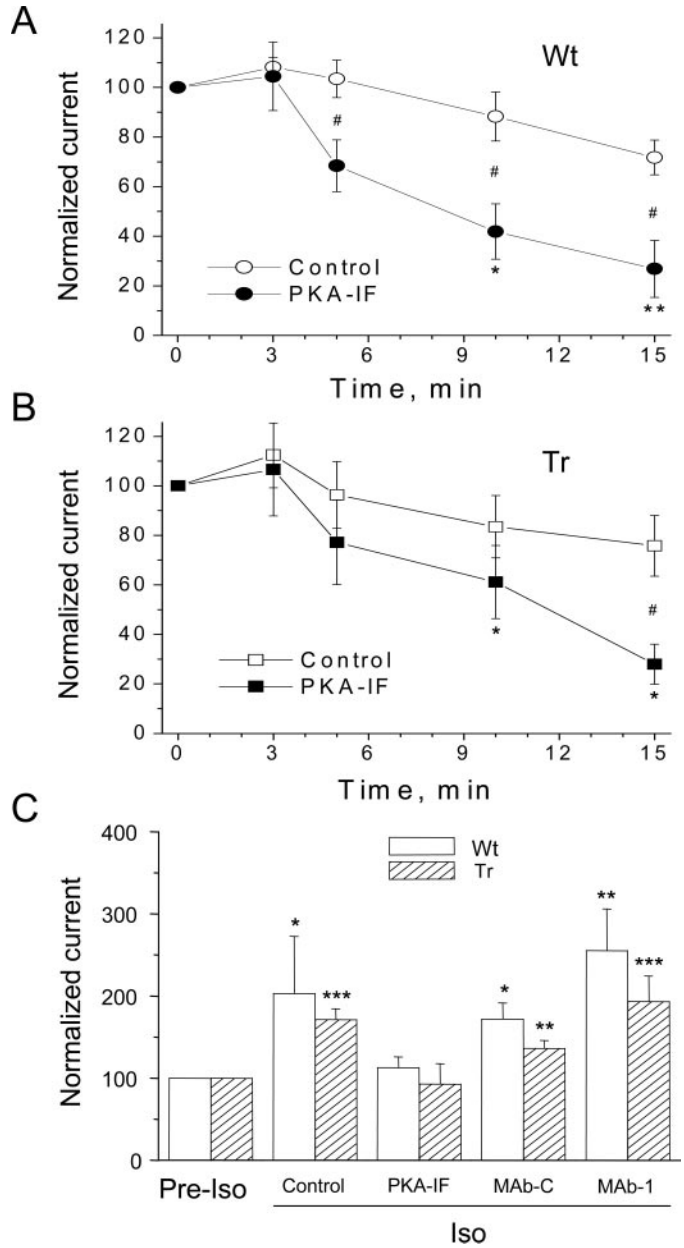

Fig. 6. Decrease of I_{Ca} in CYP2J2 Tr and Wt cardiomyocytes after intracellular dialysis with PKA-IF and effects of β-adrenergic agonist stimulation. I_{Ca} was evoked by 200-ms pulses from a holding potential of −50 to 0 mV every 30 s. Peak amplitudes of I_{Ca} were measured and normalized to their corresponding initial values recorded immediately after rupture of the cell membrane. Normalized I_{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_{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_{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_{Ca} are shown. Cardiomyocytes were dialyzed with the pipette solution alone or plus PKA-IF, MAb-C, or MAb-1. I_{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 μM significantly increased I_{Ca} in the cardiomyocytes dialyzed with the pipette solution alone (Control) or plus either of the monoclonal antibodies (MAb-C or MAb-1), but not plus PKA-IF. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus PreIso.

Ca^{2+} signals in guinea pig hearts and isolated ventricular myocytes (Moffat et al., 1993) and to enhance I_{Ca} in rat cardiomyocytes (Xiao et al., 1998). Therefore, enhancement of cardiac I_{Ca} in CYP2J2 transgenic mice most likely results from increased EET biosynthesis.

CYP2J2-derived EETs may directly affect the L-type Ca^{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_{Ca} were reversed by addition of the membrane permeable 8-Br-cAMP. Interestingly, inhibition of PKA activity significantly decreased I_{Ca} in both CYP2J2 Tr and Wt cardiomyocytes confirming that, under basal conditions, PKA-dependent phosphorylation of the L-type Ca^{2+} channel plays a crucial role in regulating I_{Ca}. Importantly, immunoblot analysis showed that, compared with Wt hearts, the level of phosphorylated α_1 subunit of the L-type Ca^{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_{Ca}. This hypothesis is consistent with our previous data that showed that 11,12-EET increased intracellular cAMP levels and enhanced L-type Ca^{2+} channel phosphorylation in rat cardiomyocytes (Xiao et al., 1998). Interestingly, although addition of the β-adrenergic agonist isoproterenol did not reverse the inhibition of I_{Ca} caused by PKA-IF, it significantly increased the inhibited I_{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 $G\alpha_s$ but not $G\alpha_{i2}$ GTP-binding activity in endothelial cells (Node et al., 2001) and are known to stimulate the ADP-ribosylation of $G\alpha_s$ 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^{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^{2+} currents. Subsequent perfusion with cAMP would enhance the Ca^{2+} currents. Inhibition of a suppressor or phosphatase activity by CYP2J2 products would also explain the increased levels of phosphorylated L-type Ca^{2+} channel subunits in the CYP2J2 transgenic hearts.

It is also possible that the enhanced I_{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_{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_{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_{Ca} in the

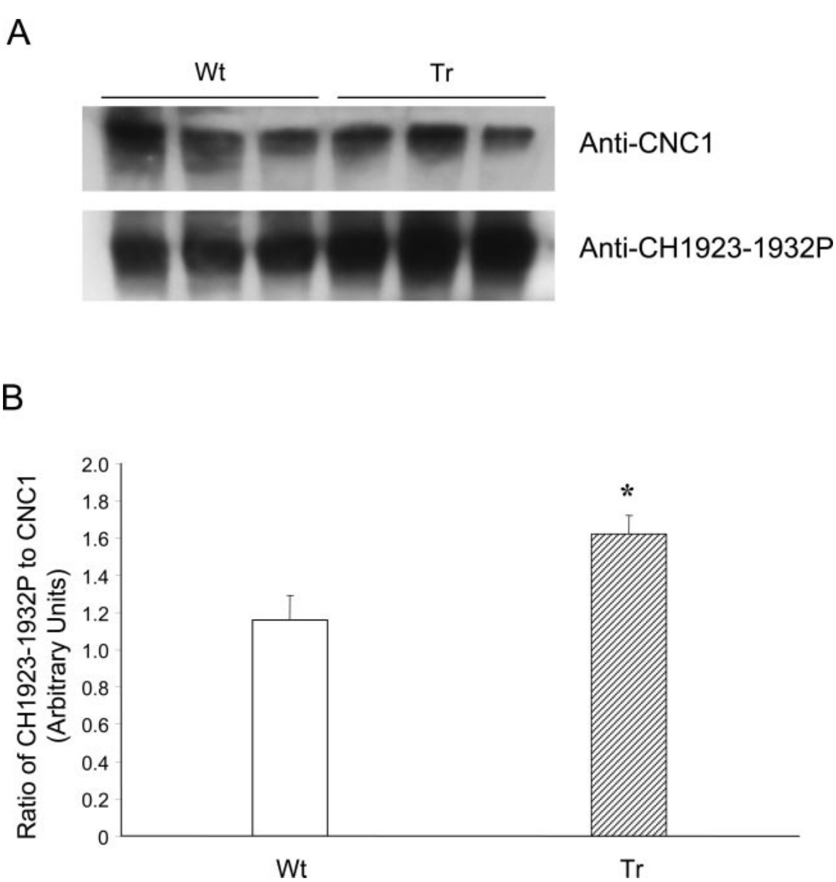

Fig. 7. Expression and phosphorylation of the $Ca_v1.2$ L-type Ca^{2+} channels in Wt and CYP2J2 Tr hearts. A, immunoblots showing expression of the $\alpha1$ subunit (anti-CNC1) and the phosphorylated form of the $\alpha1$ subunit (anti-CH1923–1932) in hearts of individual CYP2J2 Tr and Wt animals. 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 I_{Ca} 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 Ca^{2+} channel activity contribute to the enhanced postischemic functional recovery in these animals.

In conclusion, the major finding in this study is that Ca^{2+} currents are significantly increased in CYP2J2 Tr cardiomyocytes. Moreover, our data suggest that this enhancement of I_{Ca} results from an increase in L-type Ca^{2+} channel phosphorylation via a cAMP-PKA-dependent mechanism modulated by a CYP2J2-derived metabolite. Given that L-type Ca^{2+} channels play an important role in controlling excitation-contraction coupling in the heart under both normal and pathological conditions, these data suggest that CYP2J2 and its eicosanoid products may serve as an endogenous regulator of cardiac function. Moreover, because the activity of the cytochrome P450 system is exquisitely sensitive to changes in oxygen tension, CYP2J2 may be important in regulating cardiac excitability and contractile function under ischemic conditions.

Acknowledgments

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