Facilitation and Ca\textsuperscript{2+}-dependent Inactivation Are Modified by Mutation of the Cav\textsubscript{1.2} Channel IQ Motif* \\

Montatip Poomvanicha, Jörg W. Wegener, Anne Blaich, Stefanie Fischer, Katrin Domes, Sven Moosmang, and Franz Hofmann

From Forschergruppe 923, Institut für Pharmakologie und Toxikologie, Technische Universität München, 80802 Munich, Germany

The heart muscle responds to physiological needs with a short-term modulation of cardiac contractility. This process is determined mainly by properties of the cardiac L-type Ca\textsuperscript{2+} channel (Cav\textsubscript{1.2}), including facilitation and Ca\textsuperscript{2+}-dependent inactivation (CDI). Both facilitation and CDI involve the interaction of calmodulin with the IQ motif of the Cav\textsubscript{1.2} channel, especially with Ile-1624. To verify this hypothesis, we created a mouse line in which Ile-1624 was mutated to Glu (Cav\textsubscript{1.2}I1624E mice). Homozygous Cav\textsubscript{1.2}I1624E mice were not viable. Therefore, we inactivated the floxed Cav\textsubscript{1.2} gene of heterozygous mice. The time course of Ca inactivation in I/E mice was not influenced by the use of Ba\textsuperscript{2+} as a charge carrier. Using 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid as a chelating agent for intracellular Ca\textsuperscript{2+}, inactivation of I\textsubscript{Ca} was slowed down in control but not I/E mice. The results show that the I/E mutation abolishes Ca\textsuperscript{2+}/calmodulin-dependent regulation of Cav\textsubscript{1.2}. The Cav\textsubscript{1.2}I1624E mutation transforms the channel to a phenotype mimicking CDI.

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1 Both authors contributed equally to this work.

2 To whom correspondence should be addressed: FOR923, Inst. für Pharmakologie und Toxikologie, Technische Universität München, Biedersteiner Str. 29, 80802 München, Germany. Fax: 49-89-4140-3250; E-mail: franz.hofmann@mytum.de.

3 The abbreviations used are: CDF, Ca\textsuperscript{2+}-dependent facilitation; CDI, Ca\textsuperscript{2+}-dependent inactivation; CaM, calmodulin; CaMKII, Ca\textsuperscript{2+}/CaM-dependent protein kinase II; CM, cardiomyocyte; Ctr, control; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid.
promoter (MerCreMer) and with Cav1.2 L2 mice (16) to produce inducible adult mice bearing the I1624E mutation. The intercross of the three mouse lines resulted in production of Cav1.2I1624E/L2/H11003/MerCreMer (identified as I/E) and Cav1.2L2/H11001/H11003/MerCreMer (Ctr) offspring at the expected Mendelian ratio. The experiments were performed with litter-matched mice aged 8–18 weeks on a mixed C57BL6/129/Sv background. The mice were injected with 2 mg of tamoxifen/mouse/day for 4 days. Experiments were performed 10 days after the first tamoxifen injection. All animals were maintained and bred in the animal facility of the Institut für Pharmakologie und Toxikologie, Technische Universität München, and had access to water and standard chow ad libitum. All procedures relating to animal care and treatment conformed to the institutional and governmental guidelines.

**Cell Preparation**—Ventricular myocytes were isolated as described (Alliance for Cellular Signaling (AfCS) Procedure Protocol PP00000125), maintained at 37 °C, and aerated with 5% CO2. The intercross of the three mouse lines resulted in production of Cav1.2I1624E/L2 × MerCreMer (identified as I/E) and Cav1.2L2/+/ × MerCreMer (Ctr) offspring at the expected Mendelian ratio. The experiments were performed with litter-matched mice aged 8–18 weeks on a mixed C57BL6/129/Sv background. The mice were injected with 2 mg of tamoxifen/mouse/day for 4 days. Experiments were performed 10 days after the first tamoxifen injection. All animals were maintained and bred in the animal facility of the Institut für Pharmakologie und Toxikologie, Technische Universität München, and had access to water and standard chow ad libitum. All procedures relating to animal care and treatment conformed to the institutional and governmental guidelines.

**Electrophysiological Recordings**—The whole cell L-type Ca2+ channel current (ICa) was measured at 35 °C. Stimulation and data acquisition were performed as described (17). Time constants of ICa inactivation were obtained by a fit from the peak current to the current value at the end of the voltage pulse by a two-exponential function using pCLAMP 9 (Molecular Devices). All fits showed a correlation coefficient $r=0.98$.

**Statistics**—Data plotting and statistical analysis were carried out using Prism 5 (GraphPad Software). The null hypothesis was rejected if $p$ was $<0.05$. Data are presented as means ± S.E.

**RESULTS**

Ile-1624 of the CACNA1C gene was mutated to glutamate using transgenic gene knock-in techniques (Fig. 1A). The resulting homozygous mice (genotype Cav1.211624E/I1624E on both alleles) were not viable. Therefore, we crossbred heterozygous Cav1.2I1624E mice with mice expressing the floxed Cav1.2 gene and α-myosin heavy chain-MerCreMer (16, 18), allowing tissue- and time-dependent inactivation of the Cav1.2 gene by the tamoxifen-controled α-myosin heavy chain-MerCreMer recombinase. The mutation in the resulting I/E mice (genotype Cav1.2−/11624E) was confirmed by genomic sequencing (Fig. 1B). I/E mice had a reduced life span and died within 3 weeks after treatment with tamoxifen (Fig. 1C). Western blot analysis of cardiac muscle using anti-Cav1.2 antibody detected reduced protein levels in the ventricles of I/E mice compared with litter-matched Ctr mice (genotype Cav1.2−/+) at day 10 (Fig. 1D). Reduced expression of the Cav1.211624E cDNA was confirmed in

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**Figure 1.** Generation of I/E mice. A, first row, sequence around the IQ motif of CACNA1C. Second row, genomic DNA structure of CACNA1C. Boxes represent exons 39–44 encoding part of the C terminus of Cav1.2. Third row, targeting vector. neo, neomycin resistance gene; tk, thymidine kinase gene with loxp sequence (triangles) at both sides. The I1624E substitution is shown. Fourth row, knock-in locus after homologous recombination and Cre-mediated deletion of resistance markers. N, NotI; B, BamHI; C, ClaI; X, XhoI; E, EcoRI; H, HindIII. B, sequence analysis of genomic DNA in the region coding for Ile-1624 from one WT Cav1.2 and one Cav1.211624E (Cav1.2 I/E) mouse. C, survival curve of I/E and litter-matched Ctr mice. $t=0$ represents the start of the treatment of the mice with tamoxifen (2 mg/animal on 4 consecutive days). D, immunoblot of cardiac membrane preparations. Expression of Cav1.2 protein was reduced in ventricular but not atrial preparations from I/E versus Ctr mice at day 10. GAPDH was used as a loading control. 20 μg of protein was loaded per slot and separated on a 7.5% SDS-polyacrylamide gel.
the HEK293 expression system. For further studies, I/E mice were studied at day 10 after treatment with tamoxifen.

To test the physiological significance of the mutation of Ile to Glu in the cardiac Cav1.2 channel, $I_{\text{Ca}}$ was recorded in isolated ventricular CMs from I/E and Ctr mice using the patch-clamp technique (Fig. 2A). Current traces are corrected for cell capacitance. $p_{\text{F}}$, picofarad. $B$, current-voltage relation of $I_{\text{Ca}}$. Peak current density is plotted against the voltage pulse. Data points represent means ± S.E. with $n = 60$ for Ctr and $n = 52$ for I/E mice. Data sets from Ctr and I/E mice were statistically different as revealed by two-way analysis of variance (repeated measurement design, $p < 0.05$).

$C$, recovery from inactivation of $I_{\text{Ca}}$. CMs were stimulated by the twin-pulse protocol depicted at 0.03 Hz. Fractions of current ($I_{2}/I_{1}$) are plotted against the interval duration. Data points represent means ± S.E. with $n = 13$ for Ctr, $n = 14$ for I/E mice, and $n = 3$ for I/E mice with KN-93 (1 μM). Data sets from Ctr and I/E mice were statistically different as revealed by two-way analysis of variance (repeated measurement design, $p < 0.01$).

$D$, steady-state inactivation of $I_{\text{Ca}}$. CMs were stimulated by the twin-pulse protocol depicted at 0.03 Hz. Fractions of current ($I_{2}/I_{1}$) are plotted against the prepulse voltage. Data points represent means ± S.E. $E_{50}$ was calculated to be $-16 ± 1$ mV ($n = 11$) for Ctr mice and $-27 ± 4$ mV ($n = 17$) for I/E mice after fitting the data sets with a Boltzmann equation. Some data points from I/E mice ($n = 3$) were obtained in the presence of KN-93 (1 μM). Data sets from Ctr and I/E mice were statistically different as revealed by two-way analysis of variance (repeated measurement design, $p < 0.01$).
Ca2+ inactivation was still present in CMs from I/E mice with both I/I and I/E genotypes, indicating that the I/E mutation of the Cav1.2 channel abolishes CaM/CaMKII (6, 9, 17, 19–21). Facilitation of Ica in ventricular CMs (Fig. 3A–C). As suggested previously (9, 17, 20, 21), facilitation depended on CaMKII. Inhibition of CaMKII activity by KN-93 (1 μM) abolished facilitation in CMs from Ctr mice but had no effect in I/E mice. Error bars represent means ± S.E. with n = 24 for Ctr and n = 26 for I/E mice. Data sets from Ctr and I/E mice were statistically different as revealed by two-way analysis of variance (repeated measurement design, p < 0.001). D, effect of KN-93 (1 μM) on peak facilitation of Ica in CMs from Ctr and I/E mice. Error bars represent means ± S.E. with n = 26 for Ctr and n = 24 for I/E mice. Numbers indicate the number of experiments. Each experiment was performed with and without KN-93. **, p < 0.01.

In this study, we have shown that exchange of Ile with Glu at position 1624 of the Cav1.2 channel abolishes the effects of CaMKII inhibitors on Ca1,2 channel properties (10).

Several studies have shown that facilitation of Ca1,2 currents depends on CaM/CaMKII (6, 9, 17, 19–21). Facilitation of Ica was almost abolished in CMs from I/E mice (Fig. 3A–C). As suggested previously (9, 17, 20, 21), facilitation depended on CaMKII. Inhibition of CaMKII activity by KN-93 (1 μM) abolished facilitation in CMs from Ctr mice but had no effect in CMs from I/E mice, supporting a specific effect of KN-93 in Ctr CMs (Fig. 3D). Taken together, these results support the notion that the I/E mutation of the Ca1,2 channel abolishes CaM/CaMKII-mediated effects on facilitation.

In general, CDI of Ica is attenuated by the use of Ba2+ as a charge carrier or by high concentrations of intracellular Ca2+ buffers (22). Consequently, we recorded current through L-type Ca2+ channels in the same CMs using Ca2+ and Ba2+ as the charge carrier. As expected, the fast component of inactivation observed with Ca2+ was slowed down with Ba2+ as the charge carrier, resulting in poorly inactivating currents in CMs from Ctr mice (Fig. 4A, A and C). In contrast, a fast component of inactivation was still present in CMs from I/E mice with both Ca2+ and Ba2+ as the charge carrier (Fig. 4B and C). Next, we compared the effects of buffering intracellular Ca2+ by the Ca2+ chelators EGTA and BAPTA. BAPTA has been shown to bind Ca2+ more efficiently than EGTA, thus attenuating CDI of Ica (23). Indeed, inactivation of Ica was slowed down in BAPTA- versus EGTA-dialyzed CMs from Ctr mice (Fig. 4, D and F). However, inactivation of Ica was not slowed down in BAPTA- versus EGTA-dialyzed CMs from I/E mice (Fig. 4, E and F), in which the fast component of inactivation was even faster in BAPTA- versus EGTA-dialyzed CMs. Slow components of inactivation were not different in CMs from Ctr and I/E mice. These results suggest that the mutation of Ile to Glu at position 1624 of the Ca1,2 channel abolishes the effects of Ca2+ on inactivation of Ica, most likely because the channel has already been transformed to a phenotype mimicking CDI.

DISCUSSION

In this study, we have shown that exchange of Ile with Glu in the CaM-binding motif (IQ) of the Ca1,2 channel gene is lethal.
IQ Mutation in Ca_{1.2}

to mice. Electrophysiological analysis of CMs from mice with a conditional heart-specific I/E mutation in the Ca_{1.2} gene revealed that the mutation abolishes CaM/CaMKII-mediated regulation of the Ca_{1.2} channel. In addition, the mutation transforms the Ca_{1.2} channel to a phenotype that recapitulates the properties of a Ca^{2+} -inactivated channel.

In heart muscle, Ca_{1.2} is strongly associated with a number of regulator proteins, building up a macromolecular signaling complex (24, 25). Among the association partners, CaM is permanently bound to the channel and acts as a resident Ca^{2+} sensor (26, 27). Ca^{2+}-bound CaM regulates both CDI and CDF of Ca_{1.2} (5), the latter by regulating the activity of CaMKII that is tethered to the Ca_{1.2} channel (21, 28). The major binding site for CaM is the IQ motif (amino acids 1624–1635) located in the C-terminal tail of the Ca_{1.2} channel (5, 7). Mutations in the IQ motif have been shown to inhibit CaM binding to the Ca_{1.2} channel, thus reducing facilitation and CDI (11). Especially exchange of Ile-1624 with Glu in the IQ motif of the Ca_{1.2} channel reduces CaM affinity by ∼100-fold and prohibits effective facilitation and CDI of I_{Ca} in the Xenopus oocyte expression system (11). This work clearly demonstrated that exchange of Ile-1624 with Glu in the cardiac murine Ca_{1.2} channel gene likewise altered the electrophysiological properties of I_{Ca} in CMs and reduced the life span of the mutant mice.

Experiments using peptides containing the entire IQ motif of Ca_{1.2} or the I/E mutation showed an ∼100-fold decreased affinity of the I/E mutation for CaM (11). However, no in vivo quantitative measurements of affinity changes are available for the full-length channel with this mutation. Therefore, this number must be viewed with caution. The I/A mutant, which has as strong an effect on CDI as the I/E mutant but leaves CDF intact in heterologous expression studies, showed no measurable changes in its association with CaM, as shown by both biochemical studies (11) and crystal structure (29). Therefore, one cannot rule out a possibility that the effects of the I/E mutation also result from some distortion in the structure and correspondingly in the function of the IQ domain and not only from a reduction in CaM binding.

CaMKII is a major modulator of I_{Ca} activity (2). Inhibition of CaMKII by inhibitory peptides or blockers such as KN-93 prolongs recovery from inactivation (10, 30), shifts the steady-state inactivation curve to more negative voltages (10, 31), and reduces facilitation of I_{Ca} (17, 28, 30). In addition, knock-out of CaMKIIβ slows down recovery from inactivation and reduces facilitation of I_{Ca} (9). The I/E mutation of the Ca_{1.2} channel likewise prolonged recovery from inactivation, shifted the steady-state inactivation curve to more negative voltages, and reduced facilitation of I_{Ca} (9). Thus, we conclude that the I/E mutation abolishes the effects of CaMKII on the Ca_{1.2} channel because the I/E mutation shows a reduced affinity for CaM (11), preventing activation of CaMKII. At present, we cannot rule out the possibility that the I/E mutation distorts the C terminus of Ca_{1.2} in vivo and thereby reduces the affinity for CaMKII.

The fundamental role of CaM in mediating CDI has been discussed in several excellent reviews (4, 5, 20, 32). Unfortunately, pharmacological inhibitors of CaM are not useful to characterize the role of CaM in regulating cardiac I_{Ca} (33). Instead, the role of CaM in cardiac I_{Ca} is assessed mainly by reducing intracellular [Ca^{2+}], namely by the use of Ba^{2+} as the charge carrier for currents through Ca_{1.2} channels, by the use of high concentrations of intracellular Ca^{2+} buffers, or the replacement of intracellular CaM with a CaM that does not bind Ca^{2+} (7). Each experimental condition attenuates CDI, as has been observed in part in this study using CMs from Ctr mice. In contrast, CDI was no longer observed in CMs from I/E mice. Instead, inactivation of I_{Ca} in I/E CMs was not different from that in Ctr CMs under all conditions tested. Thus, although the I/E mutation decreases significantly the affinity of the IQ motif for CaM, the mutant channel inactivates in a way that recapitulates the binding of a fully activated CaM.

In this study, we have shown that adult mice carrying the I/E mutation in the cardiac Ca_{1.2} channel gene are not viable. Preliminary experiments suggest that these mice develop dilated cardiomyopathy, in concert with a reduced contractility at an unchanged heart rate. At present, we can only speculate about the reasons for this phenotype. One reason may be that the I/E channel inactivates fast and thereby decreases the amount of Ca^{2+} entry. This lack of Ca^{2+} is not compensated and decreases cardiac contraction, which increases sympathetic tone and initiates cardiac dilation.

Another reason may be that a hindered association of CaM with the Ca_{1.2} channel reduces trafficking of the channel to the membrane during biosynthesis, as shown for cultured neurons (34), which could account for the observed reduction in channel expression and in contractility. A further reason may be the missing facilitation due to the absence of CaMKII-mediated regulation of I_{Ca} which may reduce the ability of heart muscle to adapt to exercise. Indeed, mice deficient in CaMKII show a reduced heart rate in response to work load or β-adrenergic stimulation (9). This phenotype, together with a fully inactivated I_{Ca} may limit Ca^{2+} entry and thus Ca^{2+}-induced Ca^{2+} release, leading to an insufficient contraction and finally to death of the mice. In conclusion, the mutation of Ile to Glu at position 1624 of the Ca_{1.2} channel abolishes CaM/CaMKII-dependent regulation of I_{Ca} but simultaneously transforms the channel to a phenotype mimicking CDI.

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