Functional Embryonic Cardiomyocytes after Disruption of the L-type α₁C (Ca₁v.1.2) Calcium Channel Gene in the Mouse*

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The L-type α₁C (Ca₁v.1.2) calcium channel is the major calcium entry pathway in cardiac and smooth muscle. We inactivated the Ca₁v.1.2 gene in two independent mouse lines that had indistinguishable phenotypes. Homozygous knockout embryos (Ca₁v.1.2−/−) died before day 14.5 postcoitum (p.c.). At day 12.5 p.c., the embryonic heart contracted with identical frequency in wild type (+/+), heterozygous (+/−), and homozygous (−/−) Ca₁v.1.2 embryos. Beating of isolated embryonic cardiomyocytes depended on extracellular calcium and was blocked by 1 μM nisoldipine. In (+/+), (+/−), and (−/−) cardiomyocytes, an L-type Ba²⁺ inward current (I_Ba) was present that was stimulated by Bay K 8644 in all genotypes. At a holding potential of −80 mV, nisoldipine blocked I_Ba of day 12.5 p.c. (+/+), and (+/−) cells with two IC₅₀ values of ~0.1 and ~1 μM. Inhibition of I_Ba of (−/−) cardiomyocytes was monophasic with an IC₅₀ of ~1 μM. The low affinity I_Ba was also present in cardiomyocytes of homozygous α₁β (Ca₁v.1.3) knockout embryos at day 12.5 p.c. These results indicate that, up to day 14 p.c., contraction of murine embryonic hearts requires an unidentified, low affinity L-type like calcium channel.

Calcium channels play an important role in the function of different tissues. The calcium entry via high voltage-activated (HVA)³ calcium channels leads to excitation-contraction coupling in the heart, tension development in smooth muscle, neurotransmitter release in brain, and endocrine secretion in gland tissues. In the cardiovascular system, voltage-activated calcium channels are essential for the generation of normal cardiac rhythm, for induction of rhythm propagation through the atrioventricular node, and for the contraction of the atrial and ventricular muscle (1). In diseased myocardium, calcium channels can contribute to abnormal impulse generation and cardiac arrhythmias (2).

Calcium channels are hetero-oligomeric complexes of up to four subunits as follows: α₁, β, α₂δ, and γ subunit. The α₁ subunit contains the voltage sensor, the selectivity filter, the ion-conducting pore, and the binding sites for all known calcium channel blockers. The other subunits are auxiliary subunits, which modulate the channel function. Calcium channels can be further modulated by a variety of hormones, protein kinases, and protein phosphatases (3–5).

High voltage-activated calcium channels have been classified as L-type and non-L-type channels. L-type channels are encoded by four distinct genes, namely Ca₁a.1 to Ca₁a.4 (6), that give rise to numerous splice variants. Mammalian L-type channels have a similar ion selectivity and inactivation kinetics and are affected by dihydropyridines at similar concentrations. The expression pattern and the electrophysiology of L-type calcium channels have been studied extensively in pre- and postnatal heart cells of the mouse (7–10). The major L-type channel expressed in the cardiac and smooth muscle is the Ca₁,2 (11, 12). In addition, the expression of the Ca₁,3 gene was reported (13–15). However, the exact function of these channels often remained unclear. To analyze the functional relevance of the L-type Ca₁,2 calcium channel for various tissues, two mouse lines were generated in which the Ca₁,2 gene was disrupted at different exons. Both mouse lines had an identical phenotype. The homozygous (−/−) embryos died before day 14.5 p.c. Surprisingly, cardiomyocytes of 12.5-day-old p.c. embryos beat spontaneously using an unidentified L-type like calcium current.

EXPERIMENTAL PROCEDURES

Vector Construction (Mouse Line A)—Murine calcium channel Ca₁v.1.2 genomic DNA was obtained from a 129SV-P1 library (Genome Systems, St. Louis, MO) by screening with two primers amplifying 204 bp of the second exon of the Ca₁v.1.2 gene. Two exons were identified in the P1 clone coding for the second and third exon of mouse Ca₁v.1.2 gene (16, 17). The third exon encoding part of the domain I of Ca₁v.1.2 was used for the construction of the targeting vector since alternative splicing has not been described for this exon. The key features of the targeting vector are shown in Fig. 1A. A neomycin resistance cassette (Neo) was placed into the MunI site of the third exon in the reverse direction of transcription. Additionally, a herpes simplex virus type I-thymidine kinase cassette (HSV-TK) was inserted 5′-terminal of the homologous region. Analysis of the genotype of the offspring and proof for the correct insertion of the Neo were performed with primer pair NeoPa (5′-GCC TGC TCT TTA CTG AAG GCT CT-3′) and VS3 (5′-ACC ATT TGA AAT CAT TAT TT ACT-3′) that amplify a 400-bp fragment of the mutated RNA and primer pair CSI (5′-AGC CCC AGC TCA TGC CAA CAT-3′) and mun3 (5′-TAA GGC CAC ACA ATT GGC AAA-3′) that amplify a 354-bp fragment of the Ca₁v.1.2 exons 2 and 3 (Fig. 1C). Vector Construction (Mouse Line B)—A second P1 plasmid was obtained from Genome Systems containing a different part of the murine Ca₁v.1.2 gene. Restriction map analysis and sequencing of this P1 plasmid revealed that exons 13–16 encoding part of repeat II of Ca₁v.1.2 were

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§The abbreviations used are: HVA, high voltage-activated; TTX, tetrodotoxin citrate; RT-PCR, reverse transcriptase-polymerase chain reaction; kb, kilobase pair; p.c., postcoitum; HP, holding potential; DHP, dihydropyridine; bp, base pair; HSV-TK, herpes simplex virus type I-thymidine kinase cassette; pIF, picofarad; ES, embryonic stem.
Disruption of L-type α₁C Calcium Channel Gene

![Diagram](image)

**Fig. 1.** Targeted disruption of the calcium channel Ca₁,L.2 gene (mouse line A). A, a partial restriction map of the Ca₁,L.2 (α₁C) wild type locus (Aa), targeting vector (Ab), and targeted locus (Ac). Exons 2 and 3 are indicated as solid boxes and introns as solid lines. The Neo cassette was inserted into the MunI site of the third exon in opposite orientation. 5' to the Neo cassette, three stop codons were inserted in the three different reading frames. Double arrowhead lines in Aa and Ac represent the expected DNA fragments after SphI digest and hybridization with the EV500 probe (striped box in Ac). S, SphI; M, MunI; B, BamHI; A, AspI; C, ClalI. B, identification of Ca₁,L.2 (+/+), wild type, (+/-, heterozygous), and (-/-, homozygous) embryos by Southern blot analysis of SphI-digested DNA. Genomic DNA was derived from a single litter of 12.5 p.c. embryos from mating of heterozygous Ca₁,L.2 embryos. PCR strategy to identify (Ca₁,L.2) wild type (+/-) mice. Hybridization with the EV500 probe yielded signals of 6.5 kb (+/+) and 4.5 kb (+/-). C, RT-PCR of RNA isolated from 12.5 p.c. embryos. PCR strategy to identify (Ca₁,L.2) wild type (+/-) and (Ca₁,L.2) mutant (-/-) RNA. Cc, the primer pair Csi/mun3 amplified a 354-bp fragment from the wild type locus but did not amplify RNA from the knockout locus. Lanes 1 and 2, the RNA was reverse-transcribed; lane 3 (K), control plasmid containing the Ca₁,L.2 cDNA; lanes 4 and 5, the RNA was not reverse-transcribed. The negative result of lane 5 shows that the amplicon of lane 2 was not derived from genomic DNA. Cd, the primer pair VS3/NeoPA yielded a 400-bp fragment only when the mutated locus is present. Lanes 1 and 2, the RNA was reverse-transcribed; lanes 3 and 4, the RNA was not reverse-transcribed. The negative result of lanes 3 and 4 shows that the amplicons of lanes 1 and 2 were not derived from genomic DNA.

**Fig. 2.** Targeted disruption of the calcium channel Ca₁,L.2 gene using the Cre/loxP system (mouse line B). Aa, a partial restriction map of the Ca₁,L.2 wild type allele. Exons 2 and 3 are indicated as solid boxes and introns as solid lines. The location of the DNA fragment used as the 5’-hybridization probe in B is shown. Ab, the targeting vector contains a Neo/HSV-TK cassette and three loxP sequences (gray triangles numbered 1-III). Two loxP sites flank the Neo/HSV-TK cassette, which is located in the intron upstream of exon 14. The third loxP site is located in the intron between exons 15 and 16. Ac, the targeted locus after homologous recombination. Ad, knockout locus after Cre-mediated excision of exons 14 and 15 and the Neo/HSV-TK cassette. The double arrowhead line in Aa and Ad shows the DNA fragment obtained after digestion with BamHI. A, Acc65I; B, BamHI; C, ClalI; El, EcoRI; H, HindIII; S, ScaI; B, Southern blot analysis. DNA isolated from embryos at day 12.5 p.c. was digested with BamHI and then hybridized with the 5’-probe yielding a 9-bp (+/-) and 17-bp (-/-) fragment. C, RT-PCR of RNA isolated from 12.5 p.c. embryos. Ca, primer pair VS11/VS16 amplifies a 329-bp fragment (arrow) of exons 14 and 15 in the wild type (+/-) and heterozygous (+/-) but not in knockout (-/-) embryos. Cb, primer pair VS9/VS18 amplifies a 341-bp fragment (arrow) of exons 15-16 in RNA from homozygous knockout embryos (-/-). Left part, schematic drawing of spliced RNA. Right part, gels of PCR products; M, marker, D, the amplicon obtained by primer pair VS9/VS18 was sequenced and aligned with the murine cDNA sequence of Ca₁,L.2 (16). Only part of the relevant sequence is shown. 1st line, exons and exon borders (); 2nd line (+/-), sequence of murine cDNA; only part of the sequence from exons 14 and 15 is shown (//, interruption of sequence). 3rd line (-/-), sequence of cDNA amplified from the RNA of a knockout embryo. Lowercase italic letters, sequence from the intron upstream of exon 15. *Stop codon by the frameshift.

*ATG-*3') to amplify exons 14 and 15 (329 bp) in (+/+, +/−), and (−/−) cells and the primer pair VS9 (5’-ACA CAG CCA ATA AAG CCC TTC TG-3’) and VS18 (5’-GCG CAG CTT CTT CTT CCT CTT-3’) to amplify the sequence between exons 13 and 16 in the (−/−) mouse (341 bp).

*Generation of Gene-targeted Mouse Lines—Sixty μg of each targeting vector were linearized with NotI and electroporated into 1 × 10⁹ R1 embryonic stem (ES) cells obtained from Samuel Lunenfeld Research
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Institute, Toronto, Canada. G418/ganciclovir- (mouse line A) and G418 (mouse line B)-resistant clones were screened by Southern blot analysis. Analysis of 311 G418-resistant clones revealed two clones (77 and 94 in mouse line B) that carried the floxed Neo/HSV-TK cassette and the third loxP site at the correct genomic region. 1 × 10^7 ES cells of clones 77 were electroporated with 6 μg of a Cre-expressing plasmid.

Cells were plated at different dilutions and were selected with ganciclovir. Ganciclovir-resistant clones in which the Neo/HSV-TK cassette and exons 14 and 15 had been excised were identified by Southern analysis. ES cells carrying the disrupted allele (line A) or the Cav1.2 and exons 14 and 15 had been excised were identified by Southern hybridization using tail DNA. The generation of the Ca,1.3(−/−) mice has been described recently (18).

RNA Isolation and First Strand cDNA Synthesis—Total RNA was isolated from 12.5-day-old mouse embryos using TRIZOL LS Reagent (Life Technologies, Inc.). For the first strand synthesis, 4 μg of total RNA were used according to the manufacturer’s instructions. Primer pairs for the detection of the other calcium channels were as follows: D41- (5′-CGT GGT GAA CTC CTC GCC TGC-3′) and D42- (5′-AAA AGG TGA TGG AGA TTC TAT TAT-3′) to amplify a 312-bp fragment of Cav1.1, Cav1.3, ISHSK1 (5′-CCG GGA TTC ATC TAT TTC GGC ACC ATC TC-3′) and D43- (5′-AAA AGG TGA TGG AGA TTC TAT TAT-3′) to amplify a 525-bp fragment of Cav1.1, and D41- (5′-TAG GGA GCC CCC GTG ATG ATC AC-3′) and D42- (5′-CCG GGT ACC GTA CCA GGC CCC CAT CCA-3′) to amplify a 525-bp fragment of Cav1.4.

Dissection and Cell Culturing of Marine Embryonic Cardiomyocytes—Individual embryos were obtained after breeding of heterogeneous Ca,1.2 (+/−) mice at day 9.5 p.c. or later. Cardiac myocytes were isolated as described (8) at day 12.5 p.c. or later. Myocytes were plated on plastic coverslips and cultured in Dulbecco’s modified Eagle’s medium (8) supplemented with 10% fetal calf serum and 5% penicillin/streptomycin (stock 10 mg/ml). Pharmacological tests were done in normal Tyrode’s solution containing (in mM) 140 NaCl, 5.4 KCl, 1.8 CaCl2, 1.0 MgCl2, 10 glucose, 5 HEPES, pH 7.4. Genotyping of embryos was done by PCR.

Electrophysiology—Whole-cell currents were recorded at room temperature 18–48 h after plating using fire-polished electrodes with resistances of 2–3 MΩ. Pipettes were filled with (in mM) 60 CsOH, 50 aspartic acid, 68 Ca(OH)2, 1 MgCl2, 5 K-ATP, 1 CaCl2, 10 HEPES, 11 EGTA, pH 7.4. Extracellular solution for sealing and recording of solute currents (INa) was (in mM) 130 NaCl, 5.4 KCl, 0.5 BaCl2, 5 glucose, 5 HEPES, pH 7.4. To isolate barium currents (INa), the solution was changed to (in mM) 130 N-methyl-D-glucamine, 4.8 CsCl, 5 BaCl2, 5 glucose, 5 HEPES, pH 7.4. The holding potential (HP) was −80 mV. Trains of test pulses were to −40 mV for INa or to 0 mV for INa of L-type calcium channel applied once every 10 s for 40 ms. Data were collected and stored at an EPC-9 computer under control of Pulse software (HEKA electronics). Total cell membrane capacitance was determined by compensation mechanisms of the EPC9 computer and used as a measurement of membrane area. (+/−), (−/+), and (−/−) cardiomyocytes had similar capacities of 30 ± 2.6 (n = 60), 26 ± 2.3 (n = 58), and 26 ± 2 (n = 53) pF, respectively. Inactivation curves were fitted by a Boltzmann relation as follows: HINa = (1 − A)/(1 + exp(V − V50)/k), where I is the current, INa mod the maximal current at the beginning of the experiment, V is the potential, V50 is the midpoint of the curve, k is the slope factor, and A is the non-inactivating part. Ba2+/Ca2+ selectivity of the current was determined by a 100-ms pulse from −80 mV (HP) to 0 mV for (+/−) and (+/−) and to −10 mV for (−/−) cardiomyocytes at 0.2 Hz. Five mM Ba2+ was exchanged for 5 mM Ca2+ in the bath solution. In some experiments the sequence was reversed.

Cumulative dose-response curves were recorded using 2–3 different nisoldipine concentrations per cell. The number of experiments was 4–9 for each concentration. The stoichiometries and apparent affinities of nisoldipine were determined by fitting the averaged dose-inhibition points to the Hill equation: HINa = 1/(1 + ([nisoldipine]/IC50)^h), where [nisoldipine] is the concentration of nisoldipine, IC50 is the half-blocking concentration, and H is the Hill coefficient, I is the averaged current measured, INa mod is the averaged current measured, and INa 50 is the maximal current measured in the absence of nisoldipine. To obtain apparent affinities for complex dose-inhibition relations, sums of Hill terms similar in form to that described above were fitted to the data.

Stock solutions were as follows: Bay K 8644 10 μM in ethanol; nisoldipine 20 μM in ethanol; isoproterenol + ascorbic acid 10 μM each in H2O, tetrodotoxin citrate (TTX) 1 mM in H2O. When required, stock solutions were freshly diluted to the indicated concentrations with the used extracellular solution. Data are shown as mean ± S.E. with the number of cells in parentheses. Graphics and statistical data analysis using Student’s t test were carried out using ORIGIN software (Microcal).

RESULTS

Genotype—Two different mouse lines were generated in which the Ca,1.2 calcium channel gene was disrupted. In mouse line A, a neomycin resistance cassette was inserted into the third exon of the Ca,1.2 gene (Fig. 1A) in the opposite orientation. 5′ to the Neo cassette stop codons were inserted into each reading frame of Ca,1.2. The generation of functional channels is highly unlikely because the introduced modification leads to a truncated non-functional protein also in the case of an incorrect splicing, i.e. even if exon three is skipped, because of an early stop codon in exon four. Southern hybridization (Fig. 1B) and RT-PCR (Fig. 1C) with different primer pairs confirmed the germ line transmission of the Ca,1.2 gene mutation in mouse line A. In mouse line B, exons 14 and 15 were deleted using the Cre/lox recombination system (Fig. 2A). Deletion of exons 14 and 15 destroyed the transmembrane segment II5 and the pore in repeat II of the Ca,1.2 channel. In addition, the Neo/HSV-TK cassette was removed to avoid non-specific effects produced by the cassette and the products of the cassette. Southern hybridization (Fig. 2B) and RT-PCR (Fig. 2C) with different primer pairs confirmed the germ line transmission of the Ca,1.2 gene mutation in mouse line B. Deletion of the exons 14 and 15 was verified by sequencing of the RT-PCR product obtained with primer pair VS9 and VS18 (Fig. 2B). The primary transcript was spliced from exon 13 to an intron sequence directly upstream of exon 16 (Fig. 2D). The newly splicing event caused a frameshift resulting in an early stop codon. In agreement with these results, Western analysis with antibodies against the Ca,1.2 and the Ca,v1.1 protein yielded no specific bands in (−/−) embryonic hearts.

Identical results were obtained with both knockout lines. All experiments were at least repeated once in the other knockout line. Heterozygous Ca,1.2 (+/−) mice were indistinguishable from wild type (+/+) mice in shape, development, and behavior. The mating of heterozygous mice led to viable (+/+) and (+/−) pups. The genotype and number of newborn mice was (+/+) 363 and (+/−) 546 for line A and (+/+) 33 and (+/−) 79 for line B. No viable knockout (−/−) mice were born. Examination of various gestation stages showed that viable embryos were present at day 12.5 p.c. at approximately Mendelian ratio ((+/+) 100, (+/−) 171, and (−/−) 91 in line A and (+/+) 12, (+/−) 55, and (−/−) 22 in line B). No viable (−/−) embryos were detected at day 14.5 p.c.

Phenotype—Visual inspection suggested that Ca,1.2 (+/−), and (−/−) embryos developed normally up to day 12.5 p.c. Hearts contracted with the same frequency at day 12.5 p.c. (Fig. 3A). After day 14.5 p.c., the beating frequency of the remaining (+/+) and (+/−) embryos increased. Cardiac cells from day 12.5 p.c. (+/+), (+/−), and (−/−) embryos could be cultured for more than a week. During this time, the frequency of spontaneous contractions increased in each genotype from about 30 to around 160 beats/min (Fig. 3B) suggesting that the Ca,1.2 gene is not necessary for rhythmic activity or is compensated during embryonic development but is required after day 13 p.c. A previous report (9) indicated that the spontaneous contractions of cardiomyocytes from stage II embryos were caused by oscillations of intracellular [Ca2+]i and did not require the influx of extracellular Ca2+ during beat. To confirm these results, (+/−) and (−/−) heart cells were superfused with Ca2+-free normal Tyrode’s solution. Within 6 s, all cardiomyocytes stopped contracting. Addition of 1.8 mM Ca2+ restored beating in 2–5 s (n = 7 experiments for each
Maximal inward currents with Ca\(^{2+}\) as charge carrier were reduced in each genotype, and current inactivation was increased to 90% in the presence of Ca\(^{2+}\) (Fig. 4, D and E) suggesting Ca\(^{2+}\)-dependent inactivation of the channel in each genotype.

Superfusion of individual cells with the calcium channel agonist Bay K 8644 (1 μM) increased \(I_{\text{Ba}}\) and induced a shift of the \(I-V\) relation to hyperpolarized potentials in all three genotypes. The calcium channel blocker nisoldipine (1 μM) inhibited \(I_{\text{Ba}}\) in each cell line but with less efficiency in \((-/-)\) cardiomyocytes (Fig. 4). The same results were obtained in mouse line A and B (Fig. 4, A and B). However, the current amplitude differed significantly between the three genotypes (Fig. 4C). \(I_{\text{Ba}}\) increased slightly in the \((+/-)\) cells after day 14.5 p.c. and was equal to \(I_{\text{Ba}}\) of \((+/-)\) cells. The difference in current densities was not due to distinct cell sizes or differences in \(I_{\text{Na}}\). The \(I_{\text{Na}}\) amplitudes were similar with 251 ± 19 (\(n = 57\)) for \((+/-)\), 219 ± 18 (\(n = 54\)) for \((-/-)\), and 208 ± 16 (\(n = 61\)) pA/pF for \((-/-)\) cells. This analysis suggested that embryonic cardiac cells from two independent Ca\(_{1.2}\) knockout mouse lines expressed a bona fide L-type calcium channel. An alternative explanation for this phenotype was that the observed L-type channel was the so-called slip-mode sodium conductance channel (19). This channel is blocked by TTX with an IC\(_{50}\) of 0.1 μM and allows permeation of calcium in the presence of cAMP kinase or after activation of the β-adrenergic receptor. In support, \(I_{\text{Na}}\) was stimulated in each cell line 1.8–2.0-fold (\(n = 8\) to 20 cells) by isoproterenol. A similar adrenergic stimulation of \(I_{\text{Na}}\) has been reported for day 9.5 p.c. mouse embryonic heart cells (10). However, \(I_{\text{Ba}}\) was not affected at all by 10 μM TTX, whereas \(I_{\text{Na}}\) was blocked reversibly in each cell line (not shown). Therefore, it was concluded that the slip-mode channel did not cause the observed DHP-sensitive \(I_{\text{Ba}}\) in Ca\(_{1.2}\)(−/−) cardiac cells.

DHP Sensitivity of \(I_{\text{Ba}}\)—The experiments shown in Fig. 4 indicated that \(I_{\text{Ba}}\) of the Ca\(_{1.2}\)(−/−) cells was less sensitive to nisoldipine than that of the cells with a wild type or heterozygous genotype. Therefore, the extent of channel block was tested by superfusion of the \((+/-)\), \((-/-)\), or \((-/-)\) cells with 1 μM nisoldipine at the HP of −80 mV with trains of test pulses (Fig. 5A). Nisoldipine reduced \(I_{\text{Ba}}\) of \((+/-)\) and \((-/-)\) cardiomyocytes to 23 ± 3.7% (\(n = 10\)) and 27 ± 3.3% (\(n = 8\)), respectively. In contrast, \(I_{\text{Ba}}\) of \((-/-)\) cells was reduced only to 65 ± 3.3% (\(n = 11\)) of the control. A shift of the HP from −80 to −40 mV reduced \(I_{\text{Ba}}\) to zero in all three genotypes. \(I_{\text{Ba}}\) recovered to 80% of the previous value in each cell line after reversal of the HP from −40 to −80 mV. The voltage-dependent reversibility of the block indicated that nisoldipine bound preferentially to the inactivated state of the channel, a phenomenon described extensively for the Ca\(_{1.2}\) channel (see Refs. 3, 5, and 20 and references cited therein). The affinity of nisoldipine to block \(I_{\text{Ba}}\) at a HP of −80 mV was determined from dose-inhibition curves (Fig. 5B) that were fitted to the Hill equation. The calculations yielded a low (˜0.1 μM) and a high (˜3 μM) IC\(_{50}\) value for \((+/-)\) and \((-/-)\) cells and only a high (1.1 μM) IC\(_{50}\) value for \((-/-)\) cells (Table I). The inhibition curves were well fitted with a Hill coefficient of 1.0 suggesting that nisoldipine blocked two independent currents in the \((+/-)\) and \((-/-)\) cells. Considering the relative variability introduced by the calculation method, we suggest that the high IC\(_{50}\) values were not different between the three genotypes and that the real high IC\(_{50}\) value is close to 1 μM.

L-type Ca\(_{1.2}\) channels have been reported to be blocked by nisoldipine with IC\(_{50}\) values around 10 nm (21) suggesting that day 12.5 p.c. wild type myocytes expressed a relative low affinity L-type Ca\(_{1.2}\) channel. This low affinity is apparently a
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myocytes from the three genotypes ((A and B, respectively. Left column,
current voltage (I-V) relations in the absence (control) and presence of 1 nM Bay K

8644 (/) or 1 nM nisoldipine (O). The HP was ~80 mV. Cells were
depolarized to potentials between ~80 and +30 mV with 10-mV increments for 100 ms at 0.2 Hz (n = 5 cells per genotype). Center column, genotypes. Right column, representative current traces in the absence (control) and presence of 1 nM Bay K 8644 (Bay K) or 1 nM nisoldipine (nis).

Depolarization was from ~80 to 0 mV. The horizontal line represents 0 mV (scale bars, 20 ms and 20 pA/pF). C, I_\text{max} densities of cardiac myocytes from the three genotypes (+/+; filled column; +/−; striped column; −/−, open column) at day 12.5 p.c. and days 14.5–16.5 p.c. 100-ms pulses were from ~80 mV to the peak of the I-V relations. Number of cells are given within each column. Barium-(D) and calcium-dependent (E) inactivation of current in 12.5 p.c. (+/+, filled column), (+/−, striped column), and −/−, open column) cardiomyocytes. Maximum peak I_\text{max} or I_{\text{Ca} (\text{Ca})} (I_{\text{max}}) and the sustained I_{\text{Ca} (\text{Ca})} after 100 ms (I_{\text{Ca} (\text{Ca})}) were determined. Inset in D, representative current traces of a (−/−) cardiomyocyte in the presence of either 5 mM barium or 5 mM calcium. The horizontal line represents 0 mV (scale bars, 20 ms and 100 pA).

FIG. 4. Kinetics and pharmacology of cardiac L-type I_\text{Ca}A and B show results obtained with cardiac myocytes isolated at day 12.5 p.c. from embryos of mouse line A and B, respectively. Left column, current voltage (I-V) relations in the absence (control) and presence of 1 nM Bay K 8644 (/) or 1 nM nisoldipine (O). The HP was ~80 mV. Cells were depolarized to potentials between ~80 and +30 mV with 10-mV increments for 100 ms at 0.2 Hz (n = 5 cells per genotype). Center column, genotypes. Right column, representative current traces in the absence (control) and presence of 1 nM Bay K 8644 (Bay K) or 1 nM nisoldipine (nis). Depolarization was from ~80 to 0 mV. The horizontal line represents 0 mV (scale bars, 20 ms and 20 pA/pF). C, I_\text{max} densities of cardiac myocytes from the three genotypes (+/+, filled column; +/−, striped column; −/−, open column) at day 12.5 p.c. and days 14.5–16.5 p.c. 100-ms pulses were from ~80 mV to the peak of the I-V relations. Number of cells are given within each column. Barium-(D) and calcium-dependent (E) inactivation of current in 12.5 p.c. (+/+, filled column), (+/−, striped column), and −/−, open column) cardiomyocytes. Maximum peak I_\text{max} or I_{\text{Ca} (\text{Ca})} (I_{\text{max}}) and the sustained I_{\text{Ca} (\text{Ca})} after 100 ms (I_{\text{Ca} (\text{Ca})}) were determined. Inset in D, representative current traces of a (−/−) cardiomyocyte in the presence of either 5 mM barium or 5 mM calcium. The horizontal line represents 0 mV (scale bars, 20 ms and 100 pA).

FIG. 5. Nisoldipine block of I_\text{Ca} from (+/+), (+/−), and (−/−) cardiomyocytes. A, time course and voltage dependence of block. Filled and open symbols represent I_\text{max} of a (+/+), and (−/−) cardiomyocyte under control conditions (⁄ and 〇), in the presence of 1 nM nisoldipine at HP of ~80 mV (● and ○), and at HP of ~40 mV (▲ and △). Current was determined as current amplitude plus nisoldipine divided by the amplitude in the absence of nisoldipine. Inset, representative current traces (scale bars, 10 ms and 200 pA). B, concentration-inhibition relations. Data points are the mean ± S.E. (n = 4–9 per point). The lines are the fits obtained by the Hill equation. Data for (−/−) cardiomyocytes were fitted with a one-component Hill equation, whereas the data for (+/+ ) and (+/−) cardiomyocytes, the (+/+ ) cells in the late stage (day 15.5 p.c.), and the Ca 1.3 knockout cells at day 12.5 p.c. were fitted with a two-component Hill equation. , Ca 1.2 +/− cells at day 12.5 p.c.; ▲, Ca 1.2 −/− cells at day 12.5 p.c.; ■, Ca 1.2 +/− cells at day 15.5 p.c.; ●, Ca 1.2 −/− cells at day 12.5 p.c.; ■, Ca 1.2 +/− cells at day 15.5 p.c.; ▲, Ca 1.3 −/− cells at day 12.5 p.c.

TABLE I

Summary on relative fraction of current and IC_{50} values for nisoldipine

Values were obtained from the experiments shown in Fig. 5B by the calculations given under “Experimental Procedures.” IC_{50} values are in μM and relative fraction of current is given in parentheses.

| Genotype | IC_{50} (fraction) |
|----------|--------------------|
| Ca 1.2 cardiac myocytes | 12.5 p.c. (+/+) | 12.5 p.c. (+/−) | 12.5 p.c. (−/−) | 15.5 p.c. (+/+) |
| Ca 1.3 cardiac myocytes | 12.5 p.c. (−/−) | 0.003 (0.77) | 0.7 (0.23) |

Developmental property of the mouse heart, since day 15.5 p.c. wild type myocytes were blocked by nisoldipine with IC_{50} values of 10 nM and 5.7 μM (Fig. 5B and Table I). The high (10 nM) and intermediate (100 nM) DHP sensitivity was most likely caused by the expression of different splice variants of the Ca_{1.2} gene (21–25). Ca_{1.2} channels that contain the sequence of exon 21 have a lower affinity for DHPs than those containing the alternatively used exon 22 (23, 25). In agreement with the increase in the affinity between day 12.5 and 15.5 p.c., the relative abundance of exon 22 mRNA increased and that of exon 21 decreased in wild type cardiomyocytes.
between these days. These results confirmed that embryonic cardiomyocytes express L-type Ca\textsubscript{v}1.2 channels with different DHP sensitivity in the nanomolar range. In addition to the Ca\textsubscript{v}1.2 channel, embryonic cardiomyocytes express a second L-type like calcium channel which is also present in the Ca\textsubscript{v}1.2 (−−) cells and has a nisoldipine affinity in the μmolar range. The current of (+/+) and (−/−) cells was blocked by high concentrations of mibebradil with IC\textsubscript{50} values of 4.4 and 3.2 μm (n = 3–4 cells for each genotype), respectively. Mibebradil blocks current through the Ca\textsubscript{v}3.1 T-type channel (26) and the Ca\textsubscript{v}1.2 L-type channel (27) at 0.2 and 4.3 μm, respectively.

Expression of Other L-type Calcium Channels in the Embryonic Heart—The genes for four L-type calcium channels have been identified as follows: the skeletal muscle Ca\textsubscript{v}1.1 (α\textsubscript{1B}), the cardiac Ca\textsubscript{v}1.2 (α\textsubscript{1C}), the neuro-endocrine Ca\textsubscript{v}1.3 (α\textsubscript{1D}), and the retinal Ca\textsubscript{v}1.4 (α\textsubscript{1Y}) channel. RT-PCR amplification of these channels showed that wild type embryonic murine heart expressed the mRNA for Ca\textsubscript{v}1.1, Ca\textsubscript{v}1.2, and Ca\textsubscript{v}1.3. Amplification with L-type channel-specific primers allowed the amplification of the deleted exon 14 and 15 mRNA in (−−/−−) cardiomyocytes yielded Ca\textsubscript{v}1.1- and Ca\textsubscript{v}1.3-specific amplicons in a ratio of 1:10. No specific amplicon was obtained for Ca\textsubscript{v}1.4. In addition, Ca\textsubscript{v}1.4 mRNA was detected in the adult eye but not the heart of embryos by in situ hybridization with a Ca\textsubscript{v}1.4-specific probe (data not shown). The expression of Ca\textsubscript{v}1.1 has been reported previously in embryonic heart cell lines (28, 29).

However, the possibility that Ca\textsubscript{v}1.1 caused the rhythmic activity was rejected, since no specific protein band was detected by Western blot and the fast activation kinetics of the low affinity L-type like channel were not in line with those of a skeletal muscle calcium channel (see Fig. 4). Adult hearts express the Ca\textsubscript{v}1.3 channel (13–15). Deletion of the Ca\textsubscript{v}1.3 gene caused cardiac arrhythmia (18). Therefore, day 12.5 p.c. Ca\textsubscript{v}1.3 (−−/−−) heart cells were analyzed. These cells had a regular twitch and Table I). However, these cells had still the second I\textsubscript{Ba} that was blocked half-maximally at 3 nm nisoldipine (Fig. 5B and Table I). These cells had still the second I\textsubscript{Ba}, that was blocked half-maximally at 0.7 μm nisoldipine, which value is very close to the IC\textsubscript{50} value of Ca\textsubscript{v}1.2 (−−/−−) cells. These findings strengthen the hypothesis that the low affinity L-type like I\textsubscript{Ba} was caused by a channel not identified so far.

DISCUSSION

Five conclusions can be drawn from this study as follows. (a) Mouse embryos develop apparently normal in the absence of the Ca\textsubscript{v}1.2 gene up to day 12.5 p.c. (b) An intact Ca\textsubscript{v}1.2 gene is required for embryo development after day 13 p.c. It is unknown why the Ca\textsubscript{v}1.2 channel is needed for development.

Other HVA calcium channels (Ca\textsubscript{v}1.1, Ca\textsubscript{v}1.3, Ca\textsubscript{v}1.4, Ca\textsubscript{v}2.1, and Ca\textsubscript{v}2.3) are not necessary for embryonic and fetal development (18, 30–34). (c) Hearts contract in the absence of an intact Ca\textsubscript{v}1.2 gene at day 12.5 p.c. (d) Contraction requires influx of Ca\textsuperscript{2+} through an unidentified L-type like calcium channel. (e) This channel is not the Ca\textsubscript{v}1.3 L-type channel. These conclusions are supported by two independent mouse lines in which different exons of the Ca\textsubscript{v}1.2 gene were destroyed and a mouse line in which the Ca\textsubscript{v}1.3 gene was inactivated.

The L-type like channel cannot be an alternatively spliced product of the Ca\textsubscript{v}1.2 gene. In mouse line B, exons 14 and 15 were deleted that code for part of the channel pore. Thus, a hypothetical channel would not contain a pore and should, therefore, be non-conducting. Furthermore, the aberrant RNA splicing caused a frameshift that would yield a truncated channel with no pore region. In mouse line A, an unexpected splicing event from exon 2 to exon 4 would lead to a frameshift and stop in exon 4. The L-type like channel is also not coded for by the Ca\textsubscript{v}1.3 gene, since it was still present in Ca\textsubscript{v}1.3 knockout embryos. The mRNA of the skeletal muscle Ca\textsubscript{v}1.1 channel was detected in embryonic mouse cardiac cells. Several lines of evidence suggest that the L-type like channel was not the Ca\textsubscript{v}1.1 channel. 1) The activation kinetics of this channel were much faster than those reported for the calcium channel of developing murine skeletal muscle (30). 2) Mice in which the Ca\textsubscript{v}1.1 gene is disrupted die at birth but develop normally until birth (30). Therefore, it is very unlikely that the L-type like current was caused by the Ca\textsubscript{v}1.1 channel. This current was also not caused by the Ca\textsubscript{v}1.4 channel. This channel has been reported to be specifically expressed in the retina (33, 34). In line with this expression pattern, the mRNA of the Ca\textsubscript{v}1.4 gene was not detected in the embryonic heart. Its sensitivity against nisoldipine distinguishes the L-type like channel from the brain channels Ca\textsubscript{v}2.1, Ca\textsubscript{v}2.2, and Ca\textsubscript{v}2.3. Furthermore, disruption of the Ca\textsubscript{v}2.1 and Ca\textsubscript{v}2.3 gene leads to viable pups (31, 32) arguing against an essential role of these channels for cardiac rhythm generation.

The low affinity for nisoldipine would be in line with reports that some T-type calcium currents are blocked at high concentrations by several DHPS (35). Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 are expressed in the heart (36). However, the Ca\textsuperscript{2+}-dependent and slow inactivation of the L-type like channel has not been observed with T-type channels. Furthermore, the expressed Ca\textsubscript{v}3.1 channel is inhibited at submicromolar concentrations of mibebradil (37), is not activated by Bay K 8644, and is inhibited marginally by 1 μm (+)-isradipine or 10 μm nifedipine (37). Similarly, amlodipine blocked the expressed Ca\textsubscript{v}3.2 channel with an IC\textsubscript{50} of 31 μm (38). These considerations strengthen the notion that the L-type like current was caused by an unknown calcium channel.

The preliminary characterization of the new current showed that it has many properties of a classical HVA L-type calcium channel. The current differs from the classical L-type calcium channel by its low affinity for the DHP nisoldipine at negative membrane potentials but resembles Ca\textsubscript{v}1.2 channels by the voltage dependence of the block (see Fig. 5A). This property was responsible for the inhibition of cardiac contraction by 1 μm nisoldipine, since at depolarized membrane potentials which are necessary for channel opening the affinity for nisoldipine increased significantly. The affinity of the Ca\textsubscript{v}1.2 channel for DHPS is lowered 100-fold by mutation of Tyr-1485, Met-1486, and Ile-1493 in the IVS6 segment (39) and is lost upon mutation of Thr-1061 in the IIISHS segment (see Refs. 3, 5, and 20). It is possible that the new channel has an altered IVS6 segment but retained other parts of the DHP-binding site.

In conclusion, the present study shows that early cardiac rhythm generation required an unidentified L-type like calcium channel. This channel has many properties of the well characterized Ca\textsubscript{v}1.2 channel. Presumably, this similarity has prevented so far its identification in embryonic cells. The channel was also present in fetal hearts and may be present in adult hearts. Its functional significance beyond embryonic development remains to be established and will require the identification of its structure. The Drosophila melanogaster and Caenorhabditis elegans genome contain calcium channel genes of unknown function (40). One may speculate that the L-type like channel is encoded by a similar gene in the mouse.

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