Abstract—The minK gene encodes a 129-amino acid peptide the expression of which modulates function of cardiac delayed rectifier currents ($I_{K_r}$ and $I_{K_s}$), and mutations in minK are now recognized as one cause of the congenital long-QT syndrome. We have generated minK-deficient mice in which the bacterial lacZ gene has been substituted for the minK coding region such that $\beta$-galactosidase expression is controlled by endogenous minK regulatory elements. In cardiac myocytes isolated from wild-type neonatal mice, $I_{K_s}$ is rarely recorded, while $I_{K_r}$ is common. In minK (−/−) myocytes, $I_{K_r}$ is absent and $I_{K_s}$ is significantly reduced and its deactivation slowed; these results further support a role for minK in modulating both $I_{K_s}$ and $I_{K_r}$. Despite these changes, ECGs in (+/+ ) and (−/−) animals are no different at adult and at neonatal stages. ECG responses to isoproterenol are also similar in the 2 groups. $\beta$-Galactosidase staining in postnatal minK (−/−) hearts is highly restricted, to the sinus-node region, caudal atrial septum, and proximal conducting system. Moreover, as early as embryonal day 11, segmentally restricted $\beta$-galactosidase expression is observed in the portions of the sinoatrial and atroventricular junctions that are thought to give rise to the conducting system, thereby implicating minK expression as an early event in conduction system development. More generally, the restricted nature of minK expression in the mouse heart suggests species-specific roles of this gene product in mediating the electrophysiological properties of the heart. (Circ Res. 1999;84:146-152.)

Key Words: $K^+$ current • conducting system • development • delayed rectifier

The congenital long-QT syndrome (LQTS) is characterized by prolongation of cardiac repolarization, ventricular arrhythmias, and sudden death.1-2 LQTS is most commonly caused by mutations in HERG or KvLQT1, which encode the structural (α) subunits for the channels underlying the cardiac delayed rectifier currents $I_{K_r}$ and $I_{K_s}$, respectively.3-5 These 2 pharmacologically and physiologically distinct currents were originally described in guinea pig heart6 and have since been observed in human heart.7,8 The rapidly activating component ($I_{K_a}$) is sensitive to specific blockers such as dofetilide or E4031, and the slowly activating one ($I_{K_s}$) is augmented by catecholamines.5,9

The minK (or Isk) gene encodes a small protein (129 amino acids in mouse and human) that modifies the currents resulting from expression of HERG or KvLQT1. The potassium currents resulting from expression of KvLQT1 alone are small and activate very rapidly, but $I_{K_s}$ is reconstituted when minK is coexpressed with KvLQT1.10,11 Whereas $I_{K_a}$ can be recapitated by expression of HERG alone,4,12 both antisense and coexpression studies suggest that minK augments $I_{K_s}$ (HERG-mediated current) without altering its gating.13,14 Mutations in the minK gene have been reported as a rare cause of LQTS.15

One phenotype of mice in which the minK gene has been disrupted is a striking movement disorder attributed to a defect in endolymph transport in the inner ear.16 Drici et al17 have recently reported that in these mice, QT interval prolonged to a greater extent at slow rates (seen with prolonged anesthesia) compared with that in wild-type (wt) mice. The present experiments were conducted in mice in which the minK gene was disrupted and the lacZ gene was included in the targeting vector, such that staining for $\beta$-galactosidase expression in minK (−/−) animals would report the pattern of minK expression. Our results indicate normal ECGs at physiological and isoproterenol-stimulated rates in the knockout mouse and suggest unexpectedly restricted minK expression in the heart as an underlying mechanism.

Materials and Methods

Isolation of the minK Genomic Clone

Four independent overlapping clones covering ≈20 kb of the minK locus were isolated by screening 107 plaques from a 129SV genomic library in Lambda Fix II (Stratagene) under high-stringency conditions using as a probe the minK cDNA sequence in plasmid T21 (a gift of Dr Mike Tamkun, Colorado State University, Fort Collins, Colo). Library
screening, DNA purification, restriction enzyme mapping, and subcloning were performed using standard procedures.16

Construction of the Targeting Vector

A 6.2-kb EcoRI/XhoI fragment covering exon 2 (which includes the complete coding region of the minK gene19) was subcloned into the Bluescript II KS+ vector (Stratagene). The XbaI site was deleted from the polylinker of the vector after digestion by Klenow fragment and 2 unique XbaI sites were introduced flanking the minK coding exon with the use of the Kunkel method of site-directed mutagenesis.20 The oligonucleotide used to introduce the upstream site (5’-CGTCAAGGTTCCCCGGATCTAGAGCAAAAAC-TCC-3’) is located 28 bases 5’ of the initiator ATG of the minK gene. The oligonucleotide used to introduce the downstream XbaI site (5’-CCGCTTGTCA CTCTAGAGT GTGGGGTTCA CGAC-3’) is located 11 bases 3’ of the stop codon. Bases that mutate the minK sequence are underlined. With introduction of these sites, the minK coding region could then be excised in its entirety (total deletion of 427 nt) by digesting the plasmid with XbaI. Religation created a plasmid (pSKII120) that included a unique XbaI site that was then used to accept the lacZ-neo cassette described below. Consensus splice sites of exon 2 from plasmid pPNT21 via EcoRI digestion. In its place, the minK gene 19 was subcloned into the pSKII147/4 containing the thymidine kinase (TK) gene at the 3’ end (a 3’-I site that was then used to accept the lacZ-neo cassette described below. The PGK (phosphoglycerate kinase-1)-neo-pA gene was excised from plasmid pPD46.21, which has an initiator site 5’-AACAGGCTG TGCTGGGTAC-3’ located 28 bases 5’ of the minK gene. A single 7.8-kb fragment was expected, if targeting occurred correctly.

Genomic Southern Analyses

DNA obtained from ES cells20 was digested with XbaI, Southern blotted using standard protocols,18 and probed. Mice were genotyped for correct targeting by preparing tail DNA of 3-week-old weanlings.23 The 3’ probe consisted of a 600-bp restriction fragment that immediately abuts the XhoI site 5’ of the targeting locus (Figure 1A). With this probe, hybridization is predicted to yield a 8-kb XhoI fragment from the wt locus and a 5.8-kb fragment for the correctly targeted allele. The 3’ probe consisted of the 400-bp XhoI/XhoI fragment immediately 3’ of the targeting locus (Figure 1A). With this probe, the same 8-kb band is expected in the wt case, while a 2-kb band is expected for the targeted allele.

To verify that there was only a single insertion event per genome, a Southern blot of mouse genomic DNA cut with EcoRI was probed with an internal probe consisting of a 265-bp PCR fragment from the 5’ end of the lacZ gene. A single 7.8-kb fragment was expected, if targeting occurred correctly.

RNase Protection Analysis

RNase protection was performed using standard methods.13 The riboprobe was complementary to the mouse minK cDNA, lacking the 5’ 70 coding nucleotides.

ES Cell Microinjection and Mouse Husbandry

Targeted ES cell clones from 129/Sv mice were microinjected into the blastocoe l cavity of embryos derived from natural matings of C57BL/6 mice. The resulting chimeras were bred to 2 different strains of mice, Black Swiss and 129/Sv (Taconic). The studies reported here were all conducted using the inbred 129/Sv mice; however, we have observed similar findings with the other strain. Mice were housed in microisolator cages on a 12-hour light/12 hours dark cycle and were specific pathogen free. Animal care principles were followed as outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

ECGs

Adult animals were anesthetized with ketamine (30 mg/kg) and pentobarbital (38 mg/kg), as described by Berul et al.24 A jugular vein was cannulated for administration of isoproterenol. In neonatal animals, ECGs were obtained in the drug-free state from littersmates resulting from (+/-) x (+/-) matings; these animals were then euthanized and genotyped as described above. Animals were kept on a 12-hour light/12-hour dark cycle and were specific pathogen free. Animal care principles were followed as outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Figure 1. Generation of minK (−/−) mice with lacZ knockin. A. Targeting vector showing the locations in light gray of exons 1a and 1b (upstream) and exon 2, within which the entire coding region for minK is contained. Indicated are restriction sites and the sizes of fragments predicted following XbaI or EcoRI digestion of wt DNA and DNA that has undergone a homologous recombinant event. The locations of probes used in Southern analyses described in the text are also indicated. NLS indicates nuclear localization signal. B, RNase protection of mRNA isolated from heart (H), kidney (K), brain (B), and liver (L) in wt (+/+) and minK null (−/−) animals. No minK mRNA was detected in (−/−) animals. RNA indicates yeast RNA control; cyc cyclophilin.
each ECG, 3 consecutive complexes were analyzed in lead aVR, in which the onset and end of the intervals are easiest to distinguish.

**Neonatal Mouse Cardiac Myocyte Dissociation**

Neonates were deeply anesthetized with isoflurane and euthanized by cervical dislocation. Hearts were removed and placed in prewarmed ADS buffer. Each heart was washed free of blood, and the atria and ventricles were dissected into 2- to 3-mm pieces. Buffer was removed by brief centrifugation, and fresh buffer with collagenase type II (0.5 mg/mL) and pancreatin (1 mg/mL) was then added. Digestion was performed at 37°C for 15 minutes, after which tubes were quick spun and the supernatant was transferred to DMEM supplemented with 10% horse serum and 5% FBS and placed in a 5% CO2 incubator.

**Electrophysiological Study Procedures**

Standard methods were used to record whole-cell potassium currents at room temperature during and following 0.5- to 5-s pulses to a range of depolarizing potentials. The extracellular solution was Tyrode's solution with 1 μmol/L nisoldipine added to block L-type calcium current and depolarized holding potentials (−40 mV) to inactivate sodium current and T-type calcium current (which we have previously observed in mouse atrial cells).13,25 $I_{K_C}$ was readily recognized as a rapidly activating outward current displaying prominent inward rectification and large, deactivating tail currents.6,25,26 $I_{K_S}$ was defined as slowly activating and deactivating outward current recorded in the presence of dofetilide and displaying ohmic or outwardly rectifying properties. All currents were normalized to cell size, which was determined by recording the capacitative current (before compensation) elicited by a voltage-clamp step from −80 to −70 mV. Individual cell capacitance was then calculated as $Q/V = \int I_{dt}/V$, where $Q$ is charge, $I$ is current, and $V$ is the magnitude of the voltage step (10 mV).

**Staining for β-Galactosidase (lacZ) Activity**

Organs were fixed at 4°C for 1 hour in 4% paraformaldehyde made up in 0.1 mol/L phosphate buffer (pH 7.3), 5 mmol/L EGTA (pH 8.0), and 2 mmol/L MgCl2 (fixative) and then washed for 15 minutes at 4°C with PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4, and 0.24 g KH2PO4 to 1 L in H2O, pH 7.4). They were then permeabilized in 0.1 mol/L phosphate buffer (pH 7.3), 2 mmol/L MgCl2, 0.01% sodium deoxycholate, and 0.02% NP-40 in 3 steps of 15 minutes each, at room temperature. Samples were then stained in the permeabilization solution plus 1 mg/mL X-Gal, 5 mmol/L potassium ferrocyanide, and 5 mmol/L potassium ferrocyanide in the dark at room temperature. Staining times varied with sample size. Samples were then briefly
Connexin40 (Cx40) Staining of lacZ-Stained Mouse Hearts

β-Galactosidase–stained mouse hearts were dehydrated through 50%, 70%, 95%, and 100% ethanol and then paraffin embedded. The hearts were then sliced into 5-μm sections and deparaffinized through 2 changes of xylene and 1 change each of 100%, 70%, and 50% ethanol for 2 minutes each. Slides were then washed in water followed by boiling in 10 mmol/L citrate buffer, pH 6.0, in a microwave oven 3 times for 2 to 3 minutes at an 80% power setting. They were then incubated for 30 minutes at room temperature in blocking buffer (1% BSA [essentially globulin free; Sigma], 0.3% Triton X-100, and 3% goat serum). Anti-Cx40 antibody (a gift of Dr Jeffrey Saffitz, Washington University, St. Louis, Mo) was diluted 1:200 into blocking buffer and reacted with the slides overnight at 4°C. The slides were then rinsed extensively with PBS followed by a 2-hour incubation with a 1:400 dilution of Cy3-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, Pa). Samples were analyzed on a Leica microscope with standard epifluorescence.

Results

Gene Targeting

Southern blotting of genomic DNA derived from ES cells selected under G418 and ganciclovir as well as from offspring of chimeric (and later-generation) animals demonstrated single inserts of the appropriate size and thus germline transmission. RNase protection analysis demonstrated that minK mRNA was absent in (–/–) animals (Figure 1B).

ECGs and K+ Currents

Homozygous null animals displayed the movement disorder previously reported.16 ECGs in adult wt and minK (–/–) mice (Figure 2A) showed no difference in any interval; specifically, the QT interval was identical in the 2 groups. Moreover, with infusion of isoproterenol, the increase in heart rate (shortening of R-R interval) and shortening of QT intervals were also similar in the 2 groups. In adult mouse heart (unlike in humans), the major repolarizing current is \( I_{\text{Kr}} \) (as discussed further in the text) \( I_{\text{Kr}} \) deactivation is slower in the minK (–/–) animals.

Whereas \( I_{\text{K}_0} \) was readily recorded in neonatal cells from both wt and (–/–) animals (Figure 3A), it was less frequently recorded in the knockout (11/48 [23%] versus 39/48 [81%] cells; \( P = 0.004 \)). The example shown in Figure 3A suggests that \( I_{\text{K}_0} \) deactivation was slower in (–/–) compared with wt animals. Indeed, in wt animals, the deactivating tail was best fit by 2 exponentials (eg, 97 ± 7 and 594 ± 33 ms after a pulse to +20 mV); by contrast, deactivation was best fit by a monoexponential (648 ± 65 ms) in the (–/–) animals. Current-voltage relations, presented in Figure 3B for cells in which surface area was also recorded, demonstrate that the amplitude of \( I_{\text{K}_0} \) in minK (–/–) mice was also significantly smaller than that in the wt animals. \( I_{\text{K}_0} \) was very infrequently recorded in acutely disaggregated neonatal mouse myocytes (4 of 45 cells, 9%) and was not recorded in >50 cells studied from (–/–) animals. Unlike \( I_{\text{K}_0} \) and \( I_{\text{Kr}} \), the amplitude of the transient outward current \( (I_{\text{To}}) \), which was detected in all (16 of 16) neonatal myocytes subjected to depolarizing clamp steps from a holding potential of −80 mV, was similar in the 2 groups (eg, 14.4 ± 2.2 pA/pF at +40 mV in wt animals versus 12.3 ± 2.5 in the [–/–] animals; \( n = 8 \) each, \( P = \text{NS} \)). \( I_{\text{To}} \) inactivation was biexponential, and the time constants were also no different (48.1 ± 5.5 versus 45.1 ± 9.4 ms \( [\tau_1] \); 116 ± 27 versus 124 ± 31 ms \( [\tau_2] \); wt versus [–/–] respectively).

β-Galactosidase Staining

In adult animals, there was virtually no β-galactosidase staining in the ventricles (Figure 4A). However, consistent areas of dense staining were observed in the sinus-node region, on the caudal aspect of the right atrial septum and in the subaortic region of the left atrial septum, in the region of the atrioventricular node, and in the proximal conducting system (Figure 4A through 4D). Ventricular endocardial β-galactosidase staining colocalized with immunostaining against a connexin isoform (Cx40) expressed specifically in the

Therefore, we recorded ECGs in neonatal animals and recorded outward currents in acutely disaggregated cardiac cells isolated from 2- to 3-day-old mice. As shown in Figure 2B, neonatal ECGs were no different in wt and (–/–) animals. QT intervals were longer in neonates (85 to 93 ms) than in adults (60 to 81 ms) regardless of phenotype, consistent with previously reported action-potential–duration data.27
mouse conducting system (Figure 4E and 4F) and not in working myocytes. The staining patterns were similar in (+/-) animals, and no staining was observed in wt animals.

In the day 11 embryo, β-galactosidase staining was most prominent in heart, although some staining in the developing brain was also observed (Figure 5A). However, it is apparent that even at this stage, minK expression in the heart was already restricted, with especially intense β-galactosidase staining in the outflow tract and in the interventricular junction (Figure 5B). Less intense staining was observed in the upper regions of both left and right ventricles, and only very little staining was observed in the apical regions of the embryonic ventricles. No surface staining was observed in the atria at this stage of development. Histological inspection of the serial cardiac sections showed staining circumferentially around the atrioventricular canal (Figure 5C), staining in the sinoatrial region (ie, staining in the left and right venous valves and in the septum spurium), and staining in the leading edge of the primary interatrial septum. Although most of the stained cells were myocytes, blue nuclei were also occasionally observed in endocardial cells (in particular in the distal outflow tract) and in mesenchymal cells in the endocardial cushions.

**Discussion**

In humans, mutations in minK have been associated with prolongation of the QT interval. However, we did not observe any difference in baseline QT (73±11 ms versus 73±16 ms; wt versus minK [-/-]) or R-R intervals (134±11 versus 138±29), nor was there any difference in QT when heart rate was increased by isoproterenol (Figure 2B). By contrast, at similar
R-R intervals, Drici et al.\textsuperscript{17} found slightly longer QT intervals in wt animals (\textasciitilde90 versus \textasciitilde70 ms) and a greater difference as heart rate slowed during prolonged anesthesia; like us, they also reported no difference in QT intervals at fast rates after isoproterenol, which they administered intraperitoneally. However, both we and Drici et al.\textsuperscript{17} found \(I_{K}\) in only \textasciitilde10\% of cultured neonatal cells from wt animals and in no cells from \(minK (+/–)\) mice. This is consistent with the restricted expression of \(minK\) that we have now reported. Since the currents determining total repolarization time in adult mice are different from those in humans,\textsuperscript{27} we believe our ECG data are consistent with the idea that, by birth, \(I_{K}\), and \(I_{Kr}\) do not play an important role in determining repolarization in mice; it may be that inactivation of the very prominent L-type calcium channel is the dominant factor.\textsuperscript{28} Thus, the mechanism underlying the ECG findings of Drici et al.\textsuperscript{17} will require further study; possibilities include a role for \(I_{Ks}\) or another \(minK\)-mediated current (in only a minority of cells) in rate dependence of repolarization or a differential effect of anesthesia. The baseline differences between the 2 studies may reflect in part the different high-pass filters (3 versus 0.1 Hz) used.

In cultured mouse atrial cells, anti-\(minK\) antisense oligonucleotides reduced the amplitude (but not the gating) of \(I_{K}\),\textsuperscript{13} and coexpression of \(minK\) with \(HERG\) has been shown to increase \(I_{K}\) amplitude without modifying its gating.\textsuperscript{14} Thus, the reduction in \(I_{K}\) amplitude we observed in \(minK (+/–)\) mice is consistent with the hypothesis that \(minK\) interacts with the murine \(HERG\) product to modify \(I_{K}\) in a subpopulation of cells. Further, the slowing of \(I_{K}\) deactivation that we observed in \(minK (+/–)\) animals (while not observed in previous experiments)\textsuperscript{13,14} also lends strong support to the concept that \(minK\) modifies \(HERG\) function. Drici et al.\textsuperscript{17} did not report a difference in \(I_{K}\) amplitude or gating in \(minK (+/–)\) mice, although the examples presented do suggest slower deactivation in the knockout mice (their Figure 6). The mechanism underlying such a difference in deactivation is unknown but is highly reminiscent of that observed when a cardiac-specific murine \(HERG\) splice isoform is expressed with the originally described cDNA\textsuperscript{22,23}; thus, suppression of expression or function of this splice variant may be occurring in the \(minK\) knockout mice. Studies to examine this hypothesis are in progress; it is worth noting that previous work, in an atrial cell line and in Xenopus oocytes, could not reveal this difference, because the relevant splice isoforms are not part of those experimental paradigms. Failure of expression of a cardiac-specific isoform of \(ERG\) might also underlie the reduction in proportion of cells expressing \(I_{K}\); another possibility is that the lack of \(minK\) associated with \(ERG\) channels results in a failure of the channel complexes to reach the cell surface.

\(minK\) expression in the developing mouse heart shows a pronounced segmental pattern, with predominance in those regions of the heart that flank the future atria and ventricles (Figure 5). These areas have previously been identified as cardiac segments with molecular phenotypes and electrophysiological characteristics that distinguish them from the “ordinary” myocardial segments\textsuperscript{34–36} and are recognized as supporting specialized (slow) conduction in the developing heart.\textsuperscript{37} These segments either become incorporated into atrial and ventricular working myocardium and lose their specialized characteristics or develop into the conduction system, including the sinoatrial node, the atrioventricular node, and the bundle branches and distal conduction system. The segments stained by \(\beta\)-galactosidase in the \(minK (+/–)\) mice appear similar to the primary ring tissue identified in the developing human heart by the G1N2 antibody,\textsuperscript{38} which identifies regions that develop into the conduction system. To date, no marker has been described that allows the study of the development of the analogous flanking segments and conducting system in the mouse. Thus, further studies to identify elements regulating restriction of \(minK\) to these areas of the heart should provide important new information of the development of the conducting system.

The staining in the sinus-node region in adult heart is consistent with reports that \(I_{Ks}\) may contribute to pacemaker function\textsuperscript{39} and also with the finding that \(minK\) mRNA is more abundantly expressed in the sinus-node region than in other regions in the ferret heart.\textsuperscript{40} Interestingly, the lower right atrial septum is a region of which the electrophysiological properties are increasingly recognized as playing a role in common reentrant arrhythmias, such as atrioventricular nodal reentrant tachycardia and common atrial flutter.\textsuperscript{41,42} The extent to which restricted \(minK\) expression in this region might contribute to development of these reentrant circuits requires further study. More generally, while the functional consequences of \(minK\) disruption in humans are likely to be different from those found in this mouse system, the electrophysiological and histological findings here are consistent with the concept that \(minK\) forms heteromultimers with multiple gene products to modulate cardiac ion currents.

Acknowledgments
This work was supported in part by grants from the United States Public Health Service (HL46681, HL49989, HL03727, HL52813, and
Recombination of the minK Gene With lacZ

CA68485). Microscopic imaging and color plates were generated in part through the use of the Vanderbilt University Medical Center Cell Imaging Core Resource, supported by grants CA68485 and DK20593. K.D.N. was supported by the Medical Scientist Training Program (GM07347). M.E.A. is supported by an award from the Cardiac Arrhythmia Research and Education Foundation, Inc. D.M.R. is the holder of the William Stokes chair in Experimental Therapeutics, a gift from the Dai-içi Corp. The expert advice provided by Al George and by Maxine Tamkum at all points during the development of this project is greatly appreciated. We also appreciate the superb technical assistance of Holly Waldrop, Dana King, Nancy Sugg, and Aimee Phelps and the secretarial assistance of Cynthia Tillman.

References


19. Labosky PA, Barlow DP, Hogan BL. Mouse embryonic germ (EG) cell lines: transmission through the germline and differences in the methylation imprint of insulin-like growth factor 2 receptor (Ig2r) gene compared with embryonic stem (ES) cell lines. Development. 1994;120:3197–3204.


Replacement by Homologous Recombination of the minK Gene With lacZ Reveals Restriction of minK Expression to the Mouse Cardiac Conduction System
Sabina Kupershmidt, Tao Yang, Mark E. Anderson, Andy Wessels, Kevin D. Niswender, Mark A. Magnuson and Dan M. Roden

Circ Res. 1999;84:146-152
doi: 10.1161/01.RES.84.2.146

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/84/2/146

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/