Identity of a Novel Delayed Rectifier Current From Human Heart With a Cloned K⁺ Channel Current


In human myocardium, the nature of the K⁺ currents mediating repolarization of the action potential is still speculative. Delayed rectifier channels have recently been cloned from human heart, and stably incorporated into a human cell line for the first time. The myocyte current amplitude was 3.6 ± 0.2 pA/pF (at +20 mV, n=15) and activated with a time constant of 13.1 ± 2 milliseconds at 0 mV (n=15). The half-activation potential (V1/2) was -6 ± 2.5 mV (n=10) with a slope factor (k) of 8.6 ± 2.2 (n=10). The heterologously expressed HHK current amplitude was 136 pA/pF (at +20 mV, n=9) with an activation time constant of 11.8 ± 4.6 milliseconds at 0 mV; V1/2 was -4.1 ± 2.4 mV (mean ± SEM, n=8); and k was 7.0. The conductance of single HHK channels was 16.9 picosiemens in 5 mM bath K⁺. Both native and cloned channel currents inactivated partially during sustained depolarizing pulses. Both currents were blocked by micromolar concentrations of 4-aminopyridine and were relatively insensitive to tetraethylammonium ions and class III antiarrhythmic agents. They had a half blocking concentration (Kb) for block by 4-aminopyridine of ~50 μM but were relatively resistant to clofilium (Kb was 60 ± 13 μM, n=6 for HHK). The strong correspondence between the properties of the two currents provides the first demonstration that a specific K⁺ channel produces a rapid delayed rectifier current in human cardiac tissue. (Circulation Research 1993;73:210-216)

Key Words • potassium channels • delayed rectifier currents • human hearts

The first analysis of delayed rectification in heart by Noble and Tsien identified two currents, I₁ and I₂, with distinctly different kinetics and voltage dependence. Recent pharmacological and electrophysiological data have revealed multiple, often species-specific, cardiac delayed rectifier currents with varying conductances and kinetic properties. Advances in the cloning of ion channels have simplified the study of the complex mixture of ionic currents present during cardiac action potential repolarization. The isolation and expression of individual channel components has led to the description of delayed rectifier channel currents from rat[9] and mouse[10] heart and also the description of HK2,11 a rapidly activating delayed rectifier current from adult human ventricular myocardiun. No information exists on repolarizing delayed rectifier currents in human heart myocytes, so the significance of cloned channels has remained unclear. We have cloned

Received January 20, 1993; accepted April 16, 1993.
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a rapidly activating delayed rectifier channel, expressed it in a human cell line, and correlated it with novel currents recently identified in human atrium.12 The similarities between the cloned channel currents and the native current in atrium are striking and establish the importance of cloned channels in the study of human cardiac repolarization.

Materials and Methods

Detection and Expression of K⁺ Channel Clone

A pool of six rat brain K⁺ channel cDNAs was random prime-labeled and used to screen a human fetal heart cDNA library (Clontech catalog No. HL1114a). Six positive clones, isolated from 1×10⁵ recombinant clones screened, were subcloned into the Bluescript II vector (Stratagene Inc, La Jolla, Calif) and found to encode portions of the K⁺ channel clone isolated from human heart (HHK). The longest (1936-bp) cDNA constituted the majority of HHK with the exception of 60 bp of coding sequence at the 5' end of the gene. The missing 60-bp segment was obtained from a 500-bp overlapping clone isolated by screening the library with oligonucleotides from the 5' end of the HHK cDNA. The complete cDNA for HHK was made by attaching synthetic oligonucleotides coding for the first 60 bp (including a consensus sequence for translation initiation) onto the 5' end of the 1936-bp nearly full-length cDNA clone. The entire coding region was then subcloned into
the mammalian expression vector pRc/CMV for transfection into the human embryonic kidney cell line, HEK-293. fHK-pRc/CMV (1 μg) plasmid was linearized with Sal I before transfection to facilitate recombination of the plasmid DNA with the HEK chromosomal DNA. The linearized DNA (1 μg) was mixed with 50 μg lipofectin (Bethesda Research Laboratories) and used to transfet 1×10⁶ HEK cells. Forty-eight hours after transfection, G418 (0.5 mg/mL) was added to the media to select for resistant clones. Antibiotic-resistant clones were selected after 2 weeks of growth and maintained in minimum essential medium, 10% fetal bovine serum, penicillin-streptomycin-fungizone (Bethesda Research Laboratories), and G418 (0.5 mg/mL).

Electrophysiology

Specimens of atrial appendages were obtained from the hearts of 10 patients undergoing aortocoronary bypass surgery, ranging in age from 48 to 72 years old. All patients had normal P waves on electrocardiography, and no patient had a history of supraventricular arrhythmias. The protocol for tissue procurement was approved by the ethics committee of the Montreal Heart Institute. The cell isolation procedure was according to the method of Fermi et al in 1992, which was based on a technique described by Escande et al. Only quiescent rod-shaped cells showing clear cross striations were used, and they were superfused at 3 mL/min with a Tyrode's solution containing (mM) NaCl, 126; KCl, 5.4; MgCl₂, 0.8; CaCl₂, 1.0; NaH₂PO₄, 0.33; HEPES, 10, and glucose, 5.5; pH was adjusted to 7.4 with NaOH. The bath temperature was 23° to 25°C for all data presented here. Embryonic kidney cell lines incorporating fHK were maintained at 37°C in a 95% O₂–5% CO₂ incubator in 35-mm Petri dishes on glass coverslips coated with poly-D-lysine. Coverslips were removed from the incubator before the experiments and placed in dishes containing the experimental solution at 23° to 25°C. Whole-cell currents and cell-attached patch-clamp recordings were made using variations of the patch-clamp technique. Electrodes of 1- to 5-MΩ resistance were pulled from Corning 7052 glass (thick wall, Corning Glass Inc, Corning, NY) on a horizontal Flaming-Brown micropipette puller, fire-polished, and filled with a standard internal solution for whole-cell recording that contained (mM) potassium aspartate, 120; KCl, 20; Na₂-ATP, 4.0; HEPES, 5.0; MgCl₂, 1.0; and EGTA, 10; pH was adjusted to 7.2 with KOH. For cardiac myocytes, a modified internal solution was used that contained (mM) KCl, 130; MgCl₂, 1.0; HEPES, 10; EGTA, 5.0; Mg-ATP, 5.0; and Na₂-citrate phosphate, 5.0; pH was adjusted to 7.2 with KOH. An EPC-7 (List Electronic) or an Axopatch 1D (Axon Instruments, Foster City, Calif) amplifier was used for voltage-clamp measurements; data were filtered at 5 to 10 kHz before digitization via a Labmaster DMA interface. The pCLAMP suite of programs was used for data acquisition and analysis. Analog capacity compensation and 50% to 70% series resistance compensation was used during all whole-cell measurements. Isolated single embryonic kidney cells used for electrophysiological analysis had a mean cell capacitance of 21.8±2.9 pF (mean±SEM, n=20). Capacitance was measured by integration of the uncovered capacity transient. The external solution contained (mM) NaCl, 130; KCl, 5.0; sodium acetate, 2.8; MgCl₂, 1.0; HEPES, 10; glucose, 10; and CaCl₂, 1.0; pH was adjusted to 7.4 with 1N NaOH. HEK cells possessed a small endogenous delayed rectifier current that rarely exceeded 100 to 200 pA in amplitude at the most positive potentials studied and failed to inactivate during long voltage-clamp depolarizations. The overexpression of fHK at 50 to 100 times the level of endogenous current permitted a clear delineation of fHK current in whole-cell and macropatch measurements (eg, see Fig 3D). For myocytes, special steps were taken to ensure that current recordings were not contaminated by other ionic currents. Recordings were filtered at 1 kHz, series resistance was compensated, and leak current was subtracted. Sodium current was inactivated by holding the membrane potential positive to −50 mV or by isotonic replacement with choline chloride for NaCl. CoCl₂ was always present to block Ca²⁺ and Ca²⁺-activated currents. To minimize contamination from other K⁺ currents in myocytes, 10 mM tetrathylammonium (TEA), 1 mM BaCl₂, and 100 nM atropine were present in the bathing medium. Chemicals were from Sigma Chemical Co, St Louis, Mo. TEA was included in the external bath solution by equimolar replacement of NaCl. Clofibrium was dissolved in dimethyl sulfoxide or Tyrode's solution to make a 0.1-M stock solution and diluted in saline to form final bath concentrations of 1 to 100 μM.

Results

We isolated fHK, a novel clone from human heart, and its amino acid sequence is compared in Fig 1 with other K⁺ channels expressed in human heart. fHK differs from hPCN1, originally cloned from a human insulinoma, in only three N-terminal amino acid residues. The nucleotide sequences are identical except for these three codons, but at these codons, fHK is identical to HK2 cloned from human ventricle. This suggests the possibility of cloning errors in the hPCN1 sequence. Except for two striking differences, fHK is highly homologous to HK2. HK2 lacks a unique stretch of 11 amino acids in the putative extracellular region between domains S1 and S2. Also, fHK has two potential protein kinase A phosphorylation sites in the C terminus, whereas HK2 has only one. Conversely, HK1, also from human ventricle, is quite different from fHK, particularly in the N and C termini. In the standardized nomenclature, fHK, hPCN1, and HK2 might be Kvl.5 a, b, or c, respectively.

fHK is expressed in both the atrium and ventricle of adult human heart (Fig 2). Reverse transcriptase–polymerase chain reaction using total RNA from adult human atrium and ventricle results in the amplification of a 403-bp fHK fragment. Since one of the polymerase chain reaction primers comes from the unique fHK S1-S2 region, this reaction specifically detects fHK message and not HK2. Because the reverse transcriptase–polymerase chain reaction protocol is not strictly quantitative, we cannot compare the level of expression of fHK in atrium and ventricle other than to say that the gene is expressed at easily detectable levels in both tissues. The presence of fHK transcripts in human atrial tissue was confirmed by the Northern blot shown in Fig 2B.

In view of the lack of information regarding delayed rectifiers in human heart, an electrophysiological comparison was made between fHK stably expressed in a
FIG 1. Peptide sequence alignments of fHK with other cloned human K⁺ channels. The deduced amino acid sequence of the novel fetal cardiac clone fHK is compared with a clone from a human insulinoma cell line (hPCNI10) and the HK2 and HK1 channels from human heart. The phosphorylation sites in the subunit. The two filled circles represent the two potential protein kinase A phosphorylation sites in fHK.
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**Fig 2.** K⁺ channel clone (fHK) expression in human heart total RNA. Panel A: Reverse transcriptase (RT)-polymerase chain reaction (PCR) detection of fHK sequences in adult human heart total RNA. First-strand cDNA prepared from adult human atrium (A) total RNA (lanes 1 and 2) or adult human ventricle (V) total RNA (lanes 3 and 4) was the template in a PCR using fHK-specific oligonucleotides as primers. Lanes labeled “+RNase” were products of reactions pretreated with RNase to detect the presence of contaminating genomic DNA. Lane 5 shows the expected 403-bp PCR product of this primer pair using cloned fHK cDNA template. Total RNA was prepared from adult human atrium or ventricular muscle (obtained from hypertrophic hearts removed at transplant) with the guanidinium thiocyanate-based RNeagents kit (Promega Corp, Madison, Wis). Total RNA (1 µg) (with or without preincubation in the absence of 20 µg/mL DNase-free RNase [Promega] for 30 minutes at 37°C) was used as template in a first-strand cDNA synthesis reaction (RT-PCR kit, The Perkin-Elmer Corp, Norwalk, Conn) primed with random hexamers. The two oligonucleotides that were chosen as PCR primers allowed the specific amplification of fHK and not HK2 sequences. Primer 1 (5’ gggcgagccgcccacgccct 3’) is from the S1-S2 linker region unique to fHK, and primer 2 (5’ gcccgactccctgctggagc 3’) is downstream in the S5 domain and common to both fHK and HK2. The PCR reactions were done after protocols outlined in the RT-PCR kit using 10 pmol of each primer and 35 cycles: 94°C, 30 seconds; 60°C, 30 seconds; and 72°C, 30 seconds. Ten microliters of each PCR was electrophoresed on a 1% agarose gel and visualized with ethidium bromide. In a control reaction with the fHK plasmid as template, this primer pair resulted in the amplification of a 403-bp fragment, which is the expected size based on the cloned sequence. Panel B: Northern blot analysis of fHK expression in human atrium. Human atrial total RNA (10 µg) was fractionated on a 1% agarose-methylmercury gel and transferred to nitrocellulose. The fHK-pRe/CMV plasmid was linearized with BstEII, and 32P-labeled runoff antisense transcripts corresponding to the 400 bp of coding sequence at the 3’ end of the cDNA were made with SP6 polymerase using the MAXIScript RNA synthesis kit (Ambion, Inc, Austin, Tex). Hybridization was performed at 65°C overnight with rapid hybridization buffer (Amersham Corp, Arlington Heights, Ill) at a probe concentration of 10⁶ cpm/mL. The blot was washed twice for 10 minutes in 2× standard saline citrate and 0.1% sodium dodecyl sulfate at room temperature, followed by two 15-minute washes in 1× standard saline citrate and 0.1% sodium dodecyl sulfate at 65°C. Autoradiography was for 20 hours. Two bands are visible, a major one at 2.5 kb and a less intense one at 1.5 kb. This is the same pattern that was observed when human atrial total RNA was probed with HK2. Thus the mobilities of the RNA size markers are noted on the left.
K⁺ channels with single-channel conductances ranging from 5.4 to 16 picosiemens have been suggested to mediate delayed rectification in heart tissue from a number of species, although none were human.⁴ ⁷ ⁹ Yue and Marban have described a rapidly activating K⁺ channel (14 picosiemens) similar to the macroscopic rectifiers in rat heart.⁵ ⁷ ⁹ Because of the high expression density of fHK channels in HEK cells, it was difficult to obtain single-channel patches. Patches with multiple channels (2 to 10) could be obtained relatively easily, and transition amplitudes similar to those shown in Fig 4 could be seen. Ensemble currents inactivated during step depolarizations of 0.2 to 1 second in duration. Single-channel patches could sometimes be obtained using high-resistance electrodes (<10 MΩ). During 70-mV depolarizations, such single-channel patches (Fig 4A) revealed openings of fHK channels of approximately 1.2 pA in size. Mean slope conductance for patches from four cells was 16.9 picosiemens (Fig 4D). The ensemble average produced a rapid outward current (Fig 4B) that relaxed slightly toward the end of the pulse, consistent with the kinetics of whole-cell currents.
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Discussion

This study constitutes the first demonstration that a delayed rectifier current in native myocytes from human heart has properties identical with current carried by a cloned K⁺ channel from human heart and expressed in a human cell line. The atrial current can provide important control of repolarization in human heart, and this K⁺ channel must now be considered as a target for antiarrhythmic drug research and therapies. The very rapid activation of this delayed rectifier K⁺ current indicates that it is time to reevaluate the mechanisms of termination of human heart action potentials.

Acknowledgments

This study was supported by grants from the National Institutes of Health and Marion Merrell Dow (Dr Brown); the Heart and Stroke Foundation of Ontario (Dr Fedida); and the Medical Research Council of Canada, Quebec Heart Foundation, Fonds de Recherche de l’Institut de Cardiologie de Montreal (Drs Nattel and Fedida).

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Identity of a novel delayed rectifier current from human heart with a cloned K+ channel current.
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Circ Res. 1993;73:210-216
doi: 10.1161/01.RES.73.1.210
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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