Proton Block and Voltage Gating Are Potassium-dependent in the Cardiac Leak Channel Kcnk3*

Received for publication, March 9, 2000, and in revised form, March 27, 2000
Published, JBC Papers in Press, March 27, 2000, DOI 10.1074/jbc.M001948200

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Potassium leak conductances were recently revealed to exist as independent molecular entities. Here, the genomic structure, cardiac localization, and biophysical properties of a murine example are considered. Kcnk3 subunits have two pore-forming P domains and unique functional attributes. At steady state, Kcnk3 channels behave like open, potassium-selective, transmembrane holes that are inhibited by physiological levels of proton. With voltage steps, Kcnk3 channels open and close in two phases, one appears to be immediate and one is time-dependent (τ = ~5 ms). Both proton block and gating are potassium-sensitive; this produces an anomalous increase in outward flux as external potassium levels rise because of decreased proton block. Single Kcnk3 channels open across the physiological voltage range; hence they are “leak” conductances; however, they open only briefly and rarely even after exposure to agents that activate other potassium channels.

Leak currents are considered essential to normal electrical function in sympathetic ganglia (1, 2), myelinated axons (3–6), carotid body type 1 cells (7), and cardiac myocytes (8–12). Nonetheless, their existence as independent transport entities, rather than residual flux through other pathways, was controversial until the cloning of KCNK0 from Drosophila melanogaster (13). KCNK0 (previously ORK1), encodes a potassium channel subunit with two P domains and four predicted transmembrane segments (2P/4TM)1 (13). KCNK0 channels are open across the physiological voltage range, show no delay in current development with voltage steps, and “openly rectify,” that is, they operate like potassium-selective holes in an electric field (13).2 KCNK0 channels are tightly regulated; activation yields an open probability (P_o) close to 1, and inhibition produces channels that are almost always closed (63).3 Mammalian genes homologous to KCNK0, now enumerated KCNK1–9, are emerging rapidly. Like KCNK0, those that show function are potassium-selective leak conductances (16–24). Based on homology to KCNK0 we isolated Kcnk3 from a murine cardiac cDNA library (16, 24), localized it to chromosome 5B in mouse and 2p23.3-p24.1 in human (25), and called the predicted protein product OAT1. Two other groups cloned Kcnk3 concurrently and called the encoded subunit TASK1 (26) and TBAK1 (27). For clarity, we will now employ the Human Genome Organization nomenclature: KCNK3 gene and KCNK3 channel for human isolates and Kcnk3 and Kcnk3 for mice. Significant discrepancies exist between the findings of the three groups. Although all agree that Kcnk3 predicts 2P/4TM subunits that form pH-sensitive, openly rectifying potassium channels, there is no consensus as to tissue distribution (atria or ventricle), function (instantaneous or time-dependent, low or high open probability), or the predicted protein sequence.

In this report, five points are highlighted. First, the genomic sequence for murine Kcnk3 is determined to confirm the accuracy of the cDNA under study; this reveals an intron in the midst of the coding sequence for the signature motif (G Y G) of the first P loop (an arrangement seen to be conserved in the family from nematodes to humans). Second, Kcnk3 messenger RNA is localized to murine cardiac ventricle and, at lower levels, in the atria; Kcnk3 protein is then confirmed to have the same cardiac distribution. Third, half-maximal blockade of Kcnk3 channels by external protons is confirmed to be near physiological pH and shown to be sensitive to external potassium. Fourth, Kcnk3 currents are seen to develop with voltage changes in two phases; one appears to be immediate and one is time-dependent; the fraction of current in each phase is responsive to external potassium. Fifth, single Kcnk3 channels are shown to open only briefly (to one of two conductance levels) and rarely; although open probability increases with depolarization, it is not significantly augmented by a wide array of stimuli including activation or inhibition of protein kinase A or C, application of volatile anesthetics or metabolic poisons, changes in osmotic strength, or exposure to low oxygen tension.

Based on its location and similar functional attributes, we hypothesize Kcnk3 to be the correlate of a native cardiac current that remains active throughout the action potential plateau but whose molecular basis has been unclear, I_{Kp} or I_{Ksus} (8–12, 28). The findings support the idea that Kcnk3 channels link cardiac excitability to changes in acid-base status.

MATERIALS AND METHODS

Cloning of Kcnk3

cDNA—A homology search of NCBI data base using the BLAST program suite (29) and the coding sequence of the ORK1 channel

* This work was supported by a grant from the National Institutes of Health (to S. A. N. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1774 solely to indicate this fact.
‡ Supported in part by grants from the National Institutes of Health, the Yale University Children’s Health Research Center, and the March of Dimes Birth Defects Foundation.
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1 The abbreviations used are: 2P/4TM, two P domains and four predicted transmembrane segments; bp, base pair(s); kb, kilobase(s); MES, 4-morpholineethanesulfonic acid; PMA, phorbol 12-myristate 13-acetate.
2 N. Ilan and S. A. N. Goldstein, submitted for publication.
3 N. Zilberberg, N. Ilan, R. Gonzalez-Colaso, and S. A. N. Goldstein, submitted for publication.
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(KCNK3) as query (accession number U55321) identified expressed sequence tag W09160. Northern blot analysis using the 801-bp cDNA fragment in W09160 detected an abundant single message at ~3.8 kb in murine heart (24). The 801-bp cDNA fragment was used to screen a random primed and oligo(dT)-primed murine heart cDNA library in λgt11 (CLONTECH, Palo Alto, CA). Of 28 clones that hybridized to the probe, eight were purified and subcloned, and their ends subjected to automated DNA sequencing; three clones were sequenced in their entirety. This yielded a 5' untranslated sequence, an open reading frame, and a 3' untranslated sequence. An additional 160 bp of the upstream 5'-untranslated sequence was obtained by a rapid amplification of cDNA ends using 1 μg of total RNA prepared from murine cardiac muscle, as described (30) and primer A (5'-CACCAGGCAAGTCTGAAAG-3'). Single-stranded ligation and amplification were performed with primers D (5'-GGCAGGCTGCTGCGCCCGGA-3') and C with (5'-CTCACCGCGGGCCAGTCGCAGC-3'), respectively. Analyses of nucleotide and predicted amino acid sequences were performed utilizing GCG software from the University of Wisconsin (Madison, WI). The cDNA sequence is listed (accession number AB008537). The coding sequence was placed between the 5'- and 3'-untranslated regions of the Xenopus á-globin gene in pBP2 (a gift of Bernd Fakler,Tuebingen, DE) and cRNA produced using T3 RNA polymerase and a kit (Ambion, Austin, TX). Transcripts were quantified by spectrophotometry and compared with control samples separated by agarose gel electrophoresis. Our cDNA sequence (accession number AF065162) was verified by comparison with the genomic sequence (accession numbers AF241798 and AF242508) and varies from the partial sequence reported for murine TASK1 at amino acid residues 4 (Gln replaces Glu), 123 (Val replaces Ile), and 286 (where an additional Gly is added) (26) and murine TBAK1, which includes a 9-residue amino-terminal extension and a single residue difference at position 101 (Pro replaces Ala) (27). These differences do not coincide with known consensus sites in the Kcnk3 genomic clone (see below) for splice junctions or editing and are judged to be errors in the sequences reported by others.

Genomic—A murine genomic DNA library in bacteriophage P1 was screened with two oligonucleotide primers corresponding to the 3' end of the coding region of Kcnk3 cDNA as described (31). These primers, 5'-GCAGCCCTCCAGGCCGGCTGCTCCAGCTGAAAG-3' and 5'-GTCACCGCGGGCCAGTCGCAGC-3', amplify a 168-bp fragment from murine genomic DNA. Polymerase chain reaction-positive clones were purified and subcloned into pGEM-ZZ plasmid vectors (Promega Corp., Madison, WI). Subcloned fragments were analyzed by restriction endonuclease digestion, Southern blotting, and nucleotide sequencing.

Northern Blot and in Situ Hybridization Analyses

32P[ATP]-labeled probes used for Northern blots were the 801-bp fragment (W09160) and a ß-actin cDNA (Amersham Pharmacia Biotech) (32). In situ hybridization was performed with adult C57BL/6 mice (Jackson Labs, Bar Harbor, ME) using sense and antisense probes from the 801-bp Kcnk3 fragment, as described (33). [32P]UTP (Amersham Pharmacia Biotech) incorporation into the 801-bp Kcnk3 fragment was 70–85%. Heart sections (8 μm) were hybridized overnight, treated with ribonuclease-A, and dehydrated by soaking in 100% EtOH in 0.6 M concentration was varied by substitution of NaCl for KCl. Pipette resistance ranged from 3–5 MΩ for single channel recordings and 0.3–0.6 MΩ for macroscopic recordings. A murine genomic DNA library in bacteriophage P1 was screened with two oligonucleotide primers corresponding to the 3' end of the coding region of Kcnk3 cDNA as described (31). These primers, 5'-GCAGCCCTCCAGGCCGGCTGCTCCAGCTGAAAG-3' and 5'-GTCACCGCGGGCCAGTCGCAGC-3', amplify a 168-bp fragment from murine genomic DNA. Polymerase chain reaction-positive clones were purified and subcloned into pGEM-ZZ plasmid vectors (Promega Corp., Madison, WI). Subcloned fragments were analyzed by restriction endonuclease digestion, Southern blotting, and nucleotide sequencing.

Electrophysiology

Oocytes were isolated from Xenopus laevis frogs (Nasco, Atkinson, WI), subjected to collagenase treatment to ease removal of the follicle, and injected with 46 nl of sterile water containing 2–4 ng of Kcnk3 cRNA.

Whole Cell—Macroscopic currents were measured 1–4 days after cRNA injection by two-electrode voltage clamp using a Geneclamp 500 amplifier (Axon Instruments, Foster City, CA). Data were sampled at 4–20 kHz and filtered at 1–5 kHz. Data acquisition and analysis were performed using Pulse (Instrutech, Great Neck, NY) and Sigmaplot (Jandel Scientific, San Rafael, CA) software. Electrodess were made from 1.5-mm borosilicate glass tubes (Garner Glass Co., Claremont, CA), contained 3 M KCl, and had resistances between 0.3 and 1 MΩ. Oocytes were studied while perfused at 0.5–1 ml/min with 5 mM KCl bath solution 93 mM NaCl, 5 mM KCl, 1 mM MgCl2, 0.3 mM CaCl2, 5 mM HEPES, pH 7.4, with NaOH. In indicated cases, KCl was substituted for NaCl. For solutions at pH 6.0, MES replaced HEPES. Studies were performed at room temperature.

RESULTS

Kcnk3 Encodes a Two P Domain Subunit with Four Predicted Transmembrane Segments—Three independent cDNA clones for Kcnk3 isolated from a murine cardiac cDNA library were found to be identical (accession number AF065162) (24). Related to the predicted initiator methionine, the cDNA contains an A in position –3 and a termination codon 209 bp upstream with no additional ATG triplets in the intervening sequence. The open reading frame is 1227 bp, and secondary structure analyses predict a protein of 409 amino acids with two classical P domains (2P) bounded by hydrophobic segments that suggest the presence of four transmembrane segments (4TM) (Fig. 1A).
A 2P/4TM topology is consistent with the absence of a recognizable leader sequence, the external disposition of one consen-
sus site for N-linked glycosylation, and an internal location for
two sites for protein kinase A, two for protein kinase C, three for
calcium-calmodulin kinase II, one for tyrosine kinase, and a
carboxyl-terminal PDZ consensus motif.

Alignments of the P domains for subunits predicted to have
two P domains reveals that Kcnk3 is most similar to the open
and outward rectifiers (KCNKØ, KCNK2, Kcnk4, and
KCNK5), more distantly related to clones with as yet undefined
function (KCNK1, Kcnk6, Kcnk7, and KCNK8) and most dis-
tinct from the outward rectifier of yeast cells, TOK1 (Fig. 1B).
Homology among the genes is insignificant except for the P
domain segments where we can achieve ~30% identity.

We verified the predicted Kcnk3 cDNA sequence by compar-
ison to the genomic DNA sequence (accession numbers
AF241798 and AF242508). Three Kcnk3 genomic clones were
identified by polymerase chain reaction screening, and one was
studied in detail by restriction enzyme analysis, Southern blot-
ing, and limited nucleotide sequencing. The region of this
clone containing the Kcnk3 gene, including 5′ and 3′-untrans-
lated sequences and the coding region, were sequenced on both
strands. Comparison of cDNA and genomic sequences showed
that Kcnk3 is a two exon gene spread over ~21 kb (Fig. 1C).
Evaluation of the exon/intron boundaries revealed the AG:GT
rule was not violated and that no AG nucleotide pairs were
present in the 15 bp upstream of the 3′ (acceptor) splice junc-
tion. The single exon/intron boundary is located at a function-
ally critical position in the channel, in the midst of the selec-
tivity filter “signature sequence” (G YG) of the first P domain.

Kcnk3 mRNA Is Abundant in Murine Cardiac Ventricle—

Kcnk3 Protein Forms Potassium-selective, Openly Rectifying
Ion Channels—When Kcnk3 cRNA was injected into X. laevis
cocytes, a new current was observed by two-electrode voltage
clamp (Fig. 3A) that was not present in control cells. In
response to changes in voltage, the current rose to a new steady
state level. Once activated, inactivation was not observed (10 s
pulses; not shown). At physiological levels of bath potassium (5
mM) and pH (7.4), the channel passed large outward currents
with depolarizing voltage steps but only small inward currents
at hyperpolarized potentials (Fig. 3A, left panel). Increasing
external potassium concentration produced a shift in reversal
potential and large inward currents (Fig. 3, A and B). The
change in reversal potential indicated that the channel was
selective for potassium over sodium and chloride. Thus, in-
creasing external potassium levels from 5 to 100 mM (by iso-
tonic substitution of NaCl with KCl) produced a shift in rever-
sal potential of 56 ± 3 mV/10-fold change in potassium (Fig.
3C) in good agreement with the Nernst relation, which predicts
an ~58 mV change for a perfectly selective channel under these
conditions.

Changes in current-voltage relationships with altered exter-
nal potassium indicated that Kcnk3 channels were openly rec-
tifying. Thus, inward currents were smaller than outward cur-
rents when external potassium was low and increased to equal
magnitude when potassium levels were approximately the
same across the membrane (Fig. 3, A and B). This behavior was reasonably well approximated by the Goldman-Hodgkin-Katz relation (Equation 1) for current through across an ion-selective partition at differing transmembrane gradients of permeant ion (Fig. 3B). It was notable that Equation 1 failed to approximate the experimental data at 5 mM bath potassium because outward currents were smaller than predicted. This was subsequently explained by potassium-dependent proton inhibition of Kcnk3 channel currents (see below).

Kcnk3 channels exhibit an Eisenman type III permeability series (Fig. 3D). To assess relative permeability compared with potassium (the predominant internal permeant ion), a test cation replaced the sodium and potassium in the bath solution to achieve a pseudo bi-ionic condition in whole cell mode and Equation 2 was used. Permeability was highest for rubidium (1.1 ± 0.1, n = 12) and potassium (= 1), intermediate for cesium and ammonium (0.30 ± 0.02 and 0.23 ± 0.03, n = 8, respectively), and lowest for sodium and lithium (less than 0.031 ± 0.003 and 0.031 ± 0.002, n = 8, respectively). Although rubidium had a greater relative permeability than potassium its relative conductance was over 2-fold lower (Fig. 3D).

**Proton Inhibition of Kcnk3 Channels at Physiological pH Is Potassium-sensitive**—With 5 mM potassium in the bath, Kcnk3 currents were maximal at pH 8.0, significantly blocked at pH 7.4, and completely inhibited at pH 6.0 (Fig. 4A). Proton block was well fit to Equation 3 with a half-maximal blocking concentration (pH50) of 7.24 ± 0.03 and a Hill coefficient of 1.02 ± 0.06, suggesting that one proton was required to block (Fig. 4B). As external potassium levels rose, block by protons was diminished (Fig. 4C); at pH 7.0, the fraction of unblocked current at 30 mM in a potassium-free bath solution was 0.32 ± 0.02 and increased to 0.54 ± 0.02, 0.73 ± 0.02 and 0.94 ± 0.02 with 5, 20, and 100 mM bath potassium, respectively. Increasing proton levels inhibited Kcnk3 channels despite elevated potassium levels (not shown). The effect of potassium on proton inhibition explained the anomalous increase in outward current seen with elevation of external potassium (Fig. 3A); although increasing bath potassium decreased the outward driving force for potassium flux, it also diminished proton inhibition (Fig. 4C) leading to an overall increase in outward current.

**External Potassium Alters the Fraction of Kcnk3 Current That Is Time-dependent**—The rise and fall of Kcnk3 currents showed a phase that appeared immediate and another that was delayed. In whole cell mode, ~40% of activation was judged to be time-dependent with steps from −80 to 60 mV at physiological levels of pH (7.4) and potassium (5 mM) (Fig. 5A). Both raising external potassium from 5 to 100 mM (Fig. 5B, left panel) and decreasing protons from pH 7.0 to 8.0 (Fig. 5A, right panel) decreased the fraction of current that was time-dependent (Irel/I). Similarly, 55% of deactivating current was judged to be time-dependent with a step from 60 to −120 mV at pH 7.4 and 5 mM potassium (Fig. 5C), and raising external potassium (Fig. 5D, left panel) and lowering proton level (Fig. 5D, right panel) decreased the fraction of current that was time-dependent. Irel/I reflects the fraction of channels closed at rest; thus, higher potassium and lower proton in the bath increased the fraction of channels that were open before the test pulse. At physiological resting potentials and ionic conditions, roughly half the Kcnk3 channels that passed current upon depolarization were already open. The effects of external potassium were consistent with the theory that increased occupancy of the external pore by potassium (either by raising bath levels or decreasing proton block) favored the open channel state.

**Voltage Alters the Fraction of Time-dependent Kcnk3 Currents**—Kcnk3 currents in on-cell patches showed changes with voltage consistent with altered open probability (Fig. 6). Thus, the fraction of time-dependent current decreased with more positive holding potential (from −150 to −60 mV; Fig. 6A, middle panel) or test pulse (from 0 to 60 mV; Fig. 6A, right panel), indicating opening of channels by depolarization. Deactivation showed a similar dependence on voltage; activation at positive potentials (from 0 to 60 mV; Fig. 6B, middle panel) opened more channels, increasing the fraction of current that decayed upon subsequent hyperpolarization, and more positive deactivation potential (from −150 to −60 mV; Fig. 6B, right panel).
Voltage Influences the Kinetics of Time-dependent Kcnk3 Currents—Activation and deactivation were well approximated by single exponential relationships, and both rates showed a weak dependence on voltage. The rate of activation was greater at more positive voltages (Fig. 6A), although deactivation was faster at more negative potentials (Fig. 6B). With 5 mM external potassium at pH 7.4, the time constant (τ) for the rise in the current (with a step from −80 to 45 mV) was 4.4 ± 0.5 ms and showed an e-fold change per −250 mV over this voltage range (Fig. 6B). This rate of was largely insensitive to both external potassium and pH. Thus, τ with 20 mM potassium at pH 7.4 was 4.1 ± 0.2 ms (n = 3), whereas it was 4.4 ± 0.4 and 4.3 ± 0.6 ms at 5 mM potassium at pH 8.0 (n = 4) and pH 7.0 (n = 4), respectively.

The time constant for current decay was 5.0 ± 0.2 ms (n = 5) at −120 mV and changed e-fold per −500 mV over this range of potentials (Fig. 6D). Another measure of deactivation rate was

*Fig. 3.* Kcnk3 channels are openly rectifying and potassium-selective. Kcnk3 whole cell currents were assessed in oocytes by two-electrode voltage clamp with pulses from a holding voltage of −80 mV with 50 ms steps from −120 to 45 mV in 15 mV increments, followed by a 20 ms step to −120 mV. A 1-s interpulse interval was used. A, Kcnk3 currents at 5, 20, and 100 mM external potassium solutions at pH 7.4. Scale bars represent 2 μA and 20 ms. Arrows indicate the zero current level. B, steady state current-voltage relations in 5 (closed circle), 20 (open triangle), 50 (closed triangle), and 100 mM (open box) potassium solution for a single oocyte. The solid lines are drawn according to Equation 2. C, the reversal potential of Kcnk3 currents was determined with 5, 20, 50, and 100 mM potassium solutions (mean ± S.E., n = 6). Linear regression gave a shift of 56 ± 3 mV/10-fold change in potassium concentration. D, Kcnk3 steady state current-voltage relationships for groups of oocytes studied with 100 mM rubidium, potassium (filled circle), rubidium (filled square), cesium (open circle), or lithium (open square) in the external solution. The conclusion that permeability assessments were not significantly altered by native conductances was supported by the magnitude of Kcnk3 currents in cells expressing the channel and that absence of less selective pathways (C).

The time constant for current decay was 5.0 ± 0.2 ms (n = 5) at −120 mV and changed e-fold per −500 mV over this range of potentials (Fig. 6D). Another measure of deactivation rate was

*Fig. 4.* Kcnk3 channels are inhibited by protons at physiological pH. Kcnk3 currents were studied in whole cell mode at the indicated pH and potassium solutions. Oocyte membrane potential was held at −80 mV and pulsed from −120 to +45 mV in 15 mV voltage steps for 50 ms, followed by a 20 ms step to −120 mV. A 1-s interpulse interval was used. A, representative oocyte expressing Kcnk3 exposed to varying pH with 5 mM potassium bath solution. Scale bars represent 1 μA and 20 ms. B, dependence of Kcnk3 currents at 0 mV on bath pH (mean ± S.E.) for groups of five cells in 5 mM potassium solution normalized to pH 8.0. The solid line represents a fit of the data to Equation 3. A pK_{a} of 7.24 ± 0.03 and a Hill coefficient of 1.02 ± 0.06 were obtained. C, inhibition of Kcnk3 current by protons was potassium-dependent. Steady state current-voltage relationships for pH 8.0 (filled circle) and pH 7.0 (open circle) at indicated levels of bath potassium (in mM).
Potassium-sensitive Block and Gating

Single Kcnk3 channels studied in outside-out patch mode recapitulated observations made macroscopically; single channels were selective for potassium, openly rectifying, and sensitive to external pH (Fig. 8A). Thus, Kcnk3 channels were observed at both depolarized and hyperpolarized voltages in approximately symmetric 100 mM potassium with a reversal potential of −80 mV (Fig. 8A, left panel). When external potassium was lowered to 5 mM, no inward currents were observed, and the reversal potential was −80 mV, close to the equilibrium reversal potential for potassium (Fig. 8A, middle panel). Lowering pH from 7.4 to 6.0 at 5 mM potassium completely inhibited single-channel activity (Fig. 8A, right panel) in a reversible manner (not shown).

Latency to First Opening and Open Probability of Single Kcnk3 Channels—The response of single channels to voltage was assessed by holding outside-out patches at the reversal potential for potassium (−80 mV in this case) and repeatedly stepping to +40 and then −120 mV, test voltages with equal but opposite driving force for potassium. Most traces were null with no openings or closings observed (Fig. 8B, trace 5); others showed evidence for brief openings with depolarization (Fig. 8B, traces 1 and 3) or hyperpolarization (Fig. 8B, traces 2 and 4). The ratio of openings to the two conductance levels was similar to that observed in steady state recordings (−7.3, not shown).

In on-cell patches at −120 mV with approximately symmetric 100 mM potassium (Fig. 7A) open probability was −0.04 ± 0.01 at steady state. Dwell time for openings were well approximated by a single time constant of 0.3 ± 0.1 ms, suggesting that open times for the two conductance levels were similar (Fig. 7B, right panel). Close time distributions were best fit by two time constants representing closed states with mean dwell times of −5 and 65 ms, with relative frequencies of 19 and 81%, respectively (Fig. 7B, right panel).

Important sources of error in these estimates were uncertainty about whether more than one channel was present in a patch (in which case open probability estimates were too large) and missed brief openings as the minimal detectable event was 90 μs at the filter frequency employed (2 kHz) and mean open time was ~300 μs (tending to depress estimates of open probability). Errors because of unappreciated channels with open probability ~1 were ruled out because lowering pH had no effect on current base line. Finally, one of seven patches thought to have a single Kcnk3 channel showed an P<sub>o</sub> of −0.14; this patch was excluded from analyses but may have represented Kcnk3 channels in a different functional mode.

Modulation of Kcnk3 Currents—The presence of multiple regulatory consensus sites in the carboxyl-terminal segment of Kcnk3 and its low open probability suggested that the channel might be down-regulated in oocytes. Moreover, whole cell Kcnk3 currents tended to slowly “run down,” −15% over 30 min (Table I). Therefore, a variety of common potassium channel inhibitors and activators were evaluated in the presence of 20 mM external potassium to permit ready measurement of both inward and outward currents (Table I). Only depolarizing voltage steps were found to increase open probability significantly. Kcnk3 channels were found to be relatively insensitive to common blockers added to the bath (tetrathyamine, 4-aminopyridine, and glibenamid and sensitive to amiloride and barium). PMA (50 nM), an activator of protein kinase C, had no rapid effect (10 min) but decreased current by ∼1/3 over 30 min (Table I). Bisindolylmaleimide I (5 μM), an inhibitor of protein kinase C, slowly increased currents ∼1/3 over 30 min and suppressed PMA-induced down-regulation when the agents were applied together (Table I). Activation of protein kinase A by 20 μM forskolin and 1 mM IBMX decreased current by −40%, although PKA inhibitor H-89 at a standard dose (5 μM) had no effect (Table I). Small to moderate decreases in Kcnk3 currents were also observed with manipulations known to activate K<sub>ATP</sub> channels (3 mM sodium azide) (39), Kcnk4 channels (100 μM arachadonic acid) (17), and a native leak channel (decreased oxygen tension achieved by purging the bath solution with nitrogen) (7) (Table I). Minimal changes in current were observed when bath osmotic and ionic strength were increased, bath calcium and/or magnesium were increased or removed, calcium was eliminated and 5 mM EGTA added, ultra-pure potassium was employed as a sole source of external monovalent cations, or the volatile anesthetics halothane or chloroform were applied at supra-therapeutic levels (Table I). These manipulations did not significantly alter the fraction of time-dependent current at 60 or −120 mV (not shown).

DISCUSSION

The superfamily of genes encoding potassium channel subunits with two P domains has emerged with remarkable speed since the cloning of TOK1 (with a predicted 2P/8TM topology) (40) and KCNKØ (with a predicted 2P/4TM topology) (13). At present, more than 70 genes are listed in public data bases. Mammalian genes, now identified as KCNK1–9 by the Human Genome Organization, all have a predicted 2P/4TM topology. Thus far, KCNK channels that function as “leak” conductances; they have a non-zero open probability across the physiological voltage range. KCNK2 and KCNK5 are outward rectifiers, a phenotype first observed for TOK1 (40–42); under symmetric ionic conditions these pass large outward potassium currents and small inward potassium currents (16, 18, 43). KCNKØ, Kcnk3, and Kcnk4 are open rectifiers, showing linear
current-voltage relationships under symmetric conditions and Goldman rectification (35, 36) when potassium levels are unequal across the membrane (13, 17, 24, 26, 27, 44). Kcnk9 is an inwardly rectifying clone (23), whereas KCNK1, Kcnk6, Kcnk7, and KCNK8 do not show reproducible function (16, 19–22). Previously accessible only in native cells, cloned leak channels are now available for detailed evaluation under controlled conditions.

**Genomic Organization—Kcnk3**

Has an intron in the first P domain. This boundary is located at the same location in KCNK5, KCNK7, and KCNK8 as well as 20 predicted two P domain channel genes in the nematode Caenorhabditis elegans (45). Maintenance of this organization suggests either that this noncoding region serves an important regulatory role or that its natural deletion is infrequently propagated because random excision risks damage to the signature sequence residues that are required for function. That the genomic organization of Kcnk7, which is nonfunctional in experimental cells, is like that of Kcnk3 indicates the intron/exon boundary does not designate which KCNK isolates will show function.

The functional attributes we determine for Kcnk3 differ in a

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**Fig. 6.** Activation and deactivation of Kcnk3 channels are weakly voltage-dependent. **A**, activation. Left panel, Kcnk3 current traces in on-cell patches pulsed for 50 ms from −80 mV to 60 mV (followed by a step to −120 mV) with 5 mM potassium, pH 7.4 in the pipette. I_{TD} and I_{NTD} represent the time-dependent and non-time-dependent components of the current. The dotted line indicates zero current. Scale bars represent 100 pA and 10 ms. Middle panel, holding voltage alters the fraction of time-dependent Kcnk3 current measured at 40 mV for groups of 5 cells (mean ± S.E.). Steady state and initial currents were estimated using a single exponential fit of the data. Right panel, activation voltage alters the fraction of time-dependent Kcnk3 current for groups of 5 cells (mean ± S.E.). The open symbols are the ratios by the same protocol in whole cell mode. **B**, the command voltage alters the time course of activation. The time constant (τ) for activation at various voltages for groups of five cells studied as in **A** and fit with a single exponential of the form a + b*exp (−x/τ). **C**, deactivation. Left panel, Kcnk3 current traces in on-cell patches pulsed for 50 ms from a holding potential of 60 mV to −120 mV with 100 mM potassium, pH 7.4, in the pipette. Otherwise conditions are as in **A**. Scale bars represent 100 pA and 20 ms. Middle panel, prior activation voltage alters the fraction of time-dependent Kcnk3 current at −120 mV for groups of 5 cells (mean ± S.E.). Steady state and initial currents were estimated using a single exponential fit of the data. Right panel, deactivation voltage alters the fraction of time-dependent Kcnk3 current for groups of 5 cells (mean ± S.E.). The open symbols are the ratios by the same protocol in whole cell mode. **D**, the command voltage alters the time course of deactivation. Time constant (τ) for deactivation at various voltages for groups of five cells were studied as in the right panel in **C** and fit with a single exponential as in **B**. **E**, deactivation. The time course of recovery from prior activation was evaluated in on-cell mode by increasing the time between two successive 50 ms activating test pulses to 40 mV from −80 mV with 5 mM potassium, pH 7.4, solution in the pipette; the two pulses were separated by intervals of 2–100 ms, and traces are superimposed. Scale bars represent 100 pA and 20 ms.

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4 N. Zilberberg and S. A. N. Goldstein, unpublished observation.

5 D. Bockenhauer, M. A. Nimmakayalu, D. C. Ward, S. A. N. Goldstein, and P. G. Gallagher, submitted for publication.
number of respects from those reported by others; most notable, they did not observe time-dependent currents (26, 27, 44). Sequence differences, although minor, may explain functional discrepancies between the clones under study. Based on combined cDNA and genomic sequencing, it appears that the Kcnk3 variant we study is derived from the same gene as TASK1 (26) and TBAK1 (27). Alternative splicing cannot explain variations among the three cDNA sequences. Thus, these differences must result from RNA editing (46), or they are due to sequence errors introduced during reverse transcription or polymerase chain reaction.

Kcnk3 Channel Protein: a Potential Correlate of Cardiac IKp—Whereas we visualize Kcnk3 transcripts throughout the heart with a predominance in ventricle by in situ hybridization (Fig. 2B), others find message only in cardiac atria (26). Here we report ventricular localization by direct demonstration that Kcnk3 protein is also in ventricular tissue and at lower levels in atrial samples (Fig. 2C). The conclusion that Kcnk3 channels function in cardiac myocytes (rather than nonmuscular cells) is supported by a report that Kcnk3 message can be amplified from single cardiac myocytes by polymerase chain reaction (27), rare observation of single channels in rat ventricular myocytes with the same unitary conductance as murine Kcnk3 (47), and suppression of mouse ventricular currents by antisense oligonucleotides.6

Currents in native cells with attributes similar to those we delineate for Kcnk3 channels have been reported (8–12, 28). Like Kcnk3, IKp in guinea pig cardiac myocytes is prominent in ventricle, activates rapidly, and does not inactivate with sustained depolarization (8, 9). Moreover, IKp is insensitive to external tetraethylammonium and 4-aminopyridine and sensitive to external barium (8); the effect of low pH on guinea pig IKp has not been reported. Although differences between IKp and Kcnk3 in open probability and barium block argue against identity, species differences in homologous potassium channels are well recognized (48).

Leak channels remain active at all potentials in contrast to inwardly rectifying cardiac potassium channels (49, 50). Thus, Kcnk3 channels are expected to contribute not only to establishing resting membrane potential but to the height and length of action potentials and, therefore, the duration of myo-

6 C. M. B. Lopes, M. Apkon, and S. A. N. Goldstein, unpublished observation.
Potassium-sensitive Block and Gating

TABLE I
Effects of various agents on Kenk3 current magnitude

| Treatment (dose, cells studied) | Percent change ± S.E. |
|--------------------------------|-----------------------|
| None (n = 6)                   | −4 ± 2 (10 min); −14 ± 4 (30 min) |
| TEA (75 mM, n = 8)             | −23 ± 2 (z0 = 0.00 ± 0.01) |
| 4-AP (2 mM, n = 7)             | −36 ± 4 (z0 = 0.00 ± 0.09) |
| Amiodorone (10 μM, n = 4)      | +3 ± 1 |
| Barium (0.5 mM, n = 3)         | −43 ± 4 (z0 = 0.12 ± 0.03) |
| Proton (pH 7.3/5 mM KCl, n = 15) | −64 ± 5 (−120 mV) (z0 = 0.45 ± 0.01) |
| Calcium-free (n = 5)           | −52 ± 2 (0 mV) |
| Calcium added (2 mM, n = 4)    | −16 ± 6 |
| Magnesium-free (n = 4)         | +5 ± 3 |
| Magnesium added (2 mM, n = 4)  | −10 ± 4 |
| Calcium-free + EGTA (5 mM, n = 4) | +1 ± 5 |
| Calcium and magnesium-free (n = 4) | −12 ± 9 |
| Ultra-pure potassium (100 mM, n = 3) | +4 ± 1 |
| PMA (50 nM, n = 9)             | −13 ± 4 (10 min); −46 ± 5 (30 min) |
| Bisindolylmaleimide I (5 μM, n = 5) | +28 ± 3 (30 min) |
| Bisindolylmaleimide I + PMA (n = 4) | +9 ± 2 (30 min) |
| Forskolin (20 μM) + IBMX (1 mM, n = 3) | −43 ± 6 |
| H-89 (5 μM, n = 4)             | −28 ± 1 |
| H-89 + forskolin + IBMX (n = 3) | −46 ± 5 |
| Arachidonic acid (100 μM, n = 4) | −74 ± 3 |
| Sodium azide (3 mM, n = 6)     | −18 ± 3 |
| Hypoxia (O23 − 5 torr, n = 5)  | −19 ± 3 |
| Hypersomotic (100 mM added NaCl, n = 4) | +8 ± 7 |
| Halothane (0.35 mM, 3 MAC, n = 3) | +17 ± 2 |
| Chloroform (1.3 mM, 2 MAC, n = 3) | −15 ± 2 |

Note: *These agents were evaluated in 5 mM external potassium; all others were evaluated in 20 mM potassium solution ("Materials and Methods").

Cardiac contraction. Indeed, the function of **H**-sensitive leak potassium conductance has been recognized in native cardiac cells (51–53).

**Kenk3 Shows Potassium-dependent Proton Block**—A key property of Kenk3 channels is their sensitivity to changes in external **pH** in the physiological range. Here we show that proton block is more effective at lower external **pH** (Fig. 4). This served to explain the anomalous increase in outward current observed as external potassium level is elevated. The mechanism of potassium-dependent, proton block is the subject of another report.7

**Time-dependent Changes in Kenk3 Open Probability with Voltage Steps**—Classical voltage-gated potassium channels sense changes in membrane potential via a transmembrane segment that has multiple basic residues (54–57). Shaker channels show a half-maximal activation voltage of −90 mV and an e-fold change in open probability per −25 mV (these parameters are insensitive to potassium reversal potential or absolute level). Voltage-dependent changes in Shaker current magnitude take time because they result from changes in channel conformation. Immediate increases in current (seen when channels open before a voltage step) are rarely observed with voltage-gated channels in native cells for two reasons: resting membrane potential is usually below the voltage required for opening and voltage steps that open channels also favor their entry into a nonconducting conformation, the inactivate state.

Kenk3 manifests both immediate (τ < 0.25 ms) and time-dependent (τ = −5 ms) changes in current magnitude. Observation of immediate currents supports the conclusion that Kenk3 channels open at all voltages. The fraction of open channels is sensitive to external potassium and voltage (Figs. 5 and 6) and does not appear to result from release from block. Thus, current magnitudes were not significantly altered by either elevation or removal of bath calcium and/or magnesium, addition of EGTA, or formulation of solutions with 100 mM ultra-pure salts (Table I).

Kenk3 channels appear to reside in at least four states (at all voltages) and to undergo a reversible voltage-dependent gating process. A simple model must include two open states of different conductance (O1, O2) and two closed states that feed into the open states with time constants of 5 and 65 ms (C1 and C2). Single-channel recordings do not reveal whether channels traverse O1 to enter O2. That Kenk3 enters a closed conformation is suggested by observation of a latency to first opening for single channels (Fig. 8), time-dependent development of current (Fig. 3), and the absence of evidence for blockade as a cause for entry into the nonconducting mode (Table I). The closed to open transition is seen to be voltage-sensitive by the effect of potential on the fraction and kinetics of time-dependent current (Figs. 5 and 6); a single rate-limiting voltage-gated step is suggested by good approximation of current relaxation with a single exponential.

Although KCNK0 was used to isolate Kenk3 and is also a 2P/4TM open rectifier, it shows only instantaneous changes in current magnitude with voltage steps at both the single-channel and macroscopic level. Conversely, T0K1 (the 2P/8TM outward rectifier) shows weak voltage dependence and sensitivity to potassium (40, 42, 58). Recently, Loukin and Saimi (59) showed that T0K1, like Kenk3, visits two kinetically distinct closed states, a nearly open state (whose dwell time depends on membrane potential and potassium reversal potential), and a deeply closed state (responsive on voltage and external potassium). They observed that temperature had a significant effect on activation from the deep closed state but little effect on nearly open state. They concluded that the deep closed state reflected function of a channel gate, whereas the nearly open state was an effect of ions in the pore (59). A role for permeant ion occupancy of the pore in voltage-dependent gating is well recognized in chloride (60) and potassium chan-

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7 C. M. B. Lopes, F. Sesti, N. Zilberberg, M. E. Buck, and S. A. N. Goldstein, unpublished observation.
nals (54, 61, 62). Our results also suggest a role for occupancy of the pore by permeant ion in gating.

**Kcnk3 Channels in Oocytes Have a Low Open Probability**—
Kcnk3 channels had a low open probability despite numerous experimental manipulations known to alter the opening and closing of other potassium channels (Table I). The paucity of openings does not result from block by protons because channels show low Po above pH 8.0 where they are not inhibited (Fig. 5B); nor is there evidence found for block by magnesium, calcium, or heavy metal contaminants (Table I). Although activation of PMA increases the open probability of KCNKØ 

... increase the open probability of Kcnk3 channels.

**Acknowledgments**—We are grateful to C. Wong and R. Goldstein for expert technical assistance.

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(Sakmann, B., and Neher, E., eds) 2nd Ed., pp. 483–588, Plenum Press, New York