Volatile Anesthetics Activate the Human Tandem Pore Domain Baseline K⁺ Channel KCNK5

Andrew T. Gray, M.D., Ph.D.,* Byron B. Zhao, Ph.D.,† Christoph H. Kindler, M.D.,‡ Bruce D. Winegar, Ph.D.,§ Matthew J. Mazurek, B.A.,‖ Jie Xu, B.A.,† Raymond A. Chavez, Ph.D.,† John R. Forsayeth, Ph.D.,# C. Spencer Yost, M.D.**

Background: Previous studies have identified a volatile anesthetic-induced increase in baseline potassium permeability and concomitant neuronal inhibition. The emerging family of tandem pore domain potassium channels seems to function as baseline potassium channels in vivo. Therefore, we studied the effects of clinically used volatile anesthetics on a recently described member of this family.

Methods: A cDNA clone containing the coding sequence of KCNK5 was isolated from a human brain library. Expression of KCNK5 in the central nervous system was determined by Northern blot analysis and reverse-transcription polymerase chain reaction. Functional expression of the channel was achieved by injection of cRNA into Xenopus laevis oocytes.

Results: Expression of KCNK5 was detected in cerebral cortex, medulla, and spinal cord. When heterologously expressed in Xenopus oocytes, KCNK5 currents exhibited delayed activation, outward rectification, proton sensitivity, and modulation by protein kinase C. Clinical concentrations of volatile general anesthetics potentiated KCNK5 currents by 8–30%.

Conclusion: Human KCNK5 is a tandem pore domain potassium channel exhibiting delayed activation and sensitivity to volatile anesthetics and may therefore have a role in suppressing cellular excitability during general anesthesia. (Key words: Background channel; leak current; mammalian; outward rectifier; pH.)

POTASSIUM (K⁺) channels have important functions in many tissues. In nonexcitable tissues, K⁺ channels modulate the resting membrane potential, K⁺ homeostasis, and signal transduction. In excitable tissues, K⁺ channels also control action potential initiation, frequency, and duration. At this time the largest mammalian families of cloned K⁺ channels are voltage-gated channels (Kv series) and inward rectifiers (Kir series). These channels contain one pore-forming domain within their primary amino acid sequence and form functional ion channels as tetrameric complexes.¹

Recently, K⁺ channel subunits with two pore domains in tandem have been cloned.² This structural feature may allow ion channels to form as homodimers with four pore domains lining the ion conduction pathway.³ The basic structure of mammalian tandem pore domain K⁺ channels includes four membrane-spanning domains and a relatively large extracellular segment (50–70 amino acids) preceding the first pore region.¹ Although the genome of Caenorhabditis elegans contains a large number (≥ 40) of tandem pore domain K⁺ channel subunits,¹ only seven mammalian subunits of this type have been reported.

The functional properties of mammalian tandem pore domain K⁺ channels are diverse.⁵ The family includes the weak inward rectifiers TWIK-1 and TWIK-2, the outward rectifier TREK-1, and the open rectifiers TASK-1 and TRAAK (fig. 1A). Open rectification implies that currents satisfy the Goldman-Hodgkin-Katz current equation for an open channel. Although TWIK-1, TWIK-2, TREK-1 and TASK-1 are expressed in many tissues,
TRAAK is exclusively expressed in the central nervous system. Tandem pore domain K⁺ channels are not gated by physiologic ligands or voltage and therefore serve as baseline (or background) channels.

Physiologic baseline K⁺ channels have been described in a number of mammalian tissues, including pancreas, corneal endothelia, sympathetic ganglia, carotid bodies, and thin myelinated axons. Electrophysiologic properties of cloned outward and open rectifier baseline K⁺ channels closely resemble baseline K⁺ currents that underlie the resting membrane potential of vertebrate myelinated nerves. Thus, these channels likely contribute to the resting membrane conductance of myelinated nerves that limits action potential duration.

Baseline K⁺ channels also may have a role in mediating effects of volatile anesthetics. The concept that volatile anesthetics enhance neuronal K⁺ permeability has received increasing experimental support over the past 20 yr. Hyperpolarization induced by volatile anesthetics has now been described in a wide variety of cortical, hippocampal, and spinal neurons. Most recently, Sirois et al. found that hypoglossal motor neurons display an increase in K⁺ conductance with application of clinical concentrations of volatile anesthetics. The cloning of this K⁺ channel and its basic physiology were previously described. This channel has been named KCNK5 because it is the fifth member of the mammalian tandem pore domain K⁺ channel family isolated (potassium channel subfamily K).

**Methods**

Isolation of a cDNA Clone

A 360-base pair expressed sequence tag from human prostate epithelium (accession AA533124) was identified with homology to the second pore domain of mammalian tandem pore potassium channels by tBLASTn query. These BLAST (Basic Local Alignment Search Tool) queries consisted of protein sequences of previously cloned mammalian tandem pore domain potassium channels used to identify homologous nucleotide sequences of expressed sequence tags. To obtain full-length clones, we screened a human lung cDNA library from Gibco BRL (Gaithersburg, MD) and a human brain cDNA library from Edge Biosystems (Gaithersburg, MD) with modified solution hybridization techniques. One full-length clone was found from the BRL library, and multiple clones were found from the Edge library. Both strands of one clone from the Edge library were sequenced and determined to be full length. This clone was therefore chosen for expression studies, and its insert was subcloned into the POX vector to enhance efficiency of expression in oocytes. Sequence analysis and alignments were performed with LaserGene (DNASTAR Inc., Madison, WI) and the ExPaSy server (University of Geneva, Geneva, Switzerland) to search the Prosite database.
Northern Blot Analysis and Reverse-Transcription Polymerase Chain Reaction

A 500-bp base pair restriction fragment was excised from the expressed sequence tag clone (AA533124) by EcoRI and AatII. This DNA fragment was labeled with the RediPrime DNA labeling system (Amersham, Arlington Heights, IL) and hybridized against commercially available human multiple brain region Northern blots (Clontech, Palo Alto, CA) by standard methods and exposed to x-ray film at -80°C.

To examine KCNK5 expression in the spinal cord and peripheral nervous system, reverse-transcription polymerase chain reaction (RT-PCR) was performed on adult male Sprague-Dawley rat tissues. Messenger RNA was reverse transcribed with reverse transcriptase (1st Strand cDNA Synthesis kit; Boehringer-Mannheim, Indianapolis, IN). PCR primers based on the human KCNK5 sequence were used:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-CTGCTACACTCGGCCATCAC-3'</td>
<td>5'-GCTCTAGACCTACACCG-GCAGCGGTCCTGTCG-3'</td>
</tr>
</tbody>
</table>

PCR conditions were as follows: 30 s at 94°C, 30 s at 55°C, and 90 s at 72°C for 30 cycles. The resultant PCR product was gel-purified and confirmed to be KCNK5 by sequencing. Control reactions were performed under the same PCR conditions with primers for cyclic adenosine monophosphate (AMP) response element-binding protein (CREB): 5'-ACTGTACCTTAAGTCACGGTCCCGCCAGAAAG-GTGCCT-3'.

Site-directed mutagenesis was performed on the KCNK5 clone with the QuikChange kit according to the instructions of the manufacturer (Stratagene, La Jolla, CA). Nucleotide changes in the mutagenized cDNA were verified by sequencing.

Transcript Preparation and Oocyte Electrophysiology

cRNA transcripts were synthesized from linearized cDNA templates using T3 or T7 RNA polymerases (mMessage mMachine; Ambion, Austin, TX). Defolliculated Xenopus laevis oocytes were injected with 1-15 ng cRNA using standard methods for oocyte preparation and maintenance.7 One to 4 days after injection, two-electrode voltage clamp recordings were performed at room temperature (GeneClamp 500B; Axon Instruments, Foster City, CA). Most voltage pulse protocols were applied from a holding potential of -80 mV using 1-s voltage pulse steps ranging from -140 to +40 mV in 20-mV increments, with 1.5-s interpulse intervals. Except where noted, all two-electrode voltage clamp experiments were performed using frog Ringer's solution (composition: 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl2, 10 mM HEPEs, pH 7.6) or high K+ frog Ringer's solution (composition: 2.5 mM NaCl, 115 mM KCl, 1.8 mM CaCl2, 10 mM HEPEs, pH 7.6) as perfusate. Recordings were obtained in a 25-μl recording chamber at flow rates of 1-4 μl/min. Signals were filtered using a four-pole low-pass Bessel filter set at a 50-100 Hz cutoff before sampling at 100-1,000 Hz. Water-injected oocytes were used as controls, undergoing the same treatments as transcript-injected oocytes.

For volatile anesthetic studies, a full-length TRAAK clone6 was isolated from a mouse brain cDNA library with PCR (primers 5'-CGAATTCCTGCCTGGAAG-GACCATGCGGACG-3' and 5'-GCTCTAGACCTACACCG-GCACGGGCTTGTGTCG-3'). The PCR conditions of the first cycle were as follows: 94°C, 30 s; 64°C, 30 s; 72°C, 90 s. The annealing temperature was then decreased 1°C per cycle until it reached 56°C, and PCR was continued for 20 more cycles. The PCR product of expected size was gel-purified and subcloned into the pOX vector at the EcoRI and Xbal sites. The clone identity was confirmed by sequencing.

Racemic isoflurane (Anaquest, Madison, WI), halothane with 0.01% thymol (wt/wt; Ayerst Laboratories, Inc. Philadelphia, PA and Halocarbon Laboratories, Augusta, SC), and desflurane (Ohmeda, Liberty Corner, NJ) were used for experiments. The nonimmobilizer 1,2-dichlorohexafluorocyclobutane (Lancaster Synthesis Inc., Windham, NH) was also studied.27 Methods for volatile anesthetic delivery and analysis have previously been described.28

Statistical Analysis

Except where noted, data are reported from at least three oocytes. Normalized response is defined as current measured for the -80 to +40 mV pulse during the treatment condition relative to the control condition. For quantitation of steady state response, currents were averaged during the last 100 ms of the 1-s voltage pulses. Fractional instantaneous currents were defined as instantaneous currents divided by steady state currents. Mean values are expressed ± SE with n values indicating the number of oocytes studied. Statistical significance is defined by P < 0.05.

Results

KCNK5 Sequence

KCNK5 encodes a 499-amino acid protein with a calculated molecular weight of 55.1 kd. Hydropathy
A mammalian volatile anesthetic-activated K⁺ channel

Fig. 2. Analysis of KCNK5 distribution. Northern blots of adult human tissues were probed at high stringency with a 500-base pair restriction fragment excised from the expressed sequence tag clone (GenBank accession AA533124). (A) Brain region specific Northern blots. (B) KCNK5 expression in the spinal cord and peripheral nervous system of adult rat tissues. Reverse-transcription polymerase chain reaction products for cortex and medulla, as well as cervical ventral (CV), cervical dorsal (CD), thoracic ventral (TV), thoracic dorsal (TD), lumbar ventral (LV), and lumbar dorsal (LD) spinal cord sections. Lanes for lumbar sympathetic ganglia (LSG) and lumbar dorsal root ganglia (DRG) are also shown. Control reactions showing amplification of cyclic adenosine monophosphate response element-binding protein (CREB) sequence are shown in the same tissues.

Analysis predicts that KCNK5, like other mammalian tandem pore domain K⁺ channels, contains four transmembrane domains and two pore loops (fig. 1A). Figure 1B shows the relationship of the KCNK5 sequence to the previous isolated tandem pore domain K⁺ channels based on amino acid homology. KCNK5 has distant protein sequence homology with these other channels (20–24% overall similarity). However, KCNK5 has well-conserved pore and postpore domains, with the highest homology to the other family members found in the second pore region (56% identity).

Tissue Distribution

A human central nervous system Northern blot detected KCNK5 expression in spinal cord (fig. 2A). The band migrated with the 4.4-kb RNA standard. No signal was detected in whole-brain polyA⁺ RNA, although the full-length cDNA was cloned from a human brain cDNA library. A human multiple tissue Northern blot also showed KCNK5 mRNA expression in several tissues outside the central nervous system, including kidney, liver, pancreas, and placenta.

Figure 2B shows the distribution of KCNK5 expression in the rat central and peripheral nervous systems by RT-PCR. KCNK5 expression was found throughout the spinal cord in both ventral and dorsal sections. In addition, trace levels were detected in cortex, medulla, and lumbar dorsal root ganglia. KCNK5 expression was not observed in lumbar sympathetic ganglia.

Electrophysiology

Xenopus oocytes injected with 1–15 ng of KCNK5 cRNA manifested large (2–25 pA) outward currents at positive pulse potentials (fig. 3A). In high extracellular K⁺, both inward and outward currents could be observed (figs. 3B and 3C). Unlike previously described mammalian tandem pore domain K⁺ channels, KCNK5 currents did not entirely activate instantaneously; time-dependent gating associated with voltage jumps followed a slow time course (τ ≈ 150 ms for −80 to +40-mV pulse in frog Ringer’s solution). KCNK5 currents showed no inactivation during voltage pulses as long as 5 s. KCNK5 tail currents were observed after voltage prepulses to 0 mV and reversed near the expected reversal potential for K⁺, implying slow deactivation of potassium currents.

KCNK5 passes little inward current in 2.5 mM extracellular K⁺ (fig. 3C, filled circles), and the current-voltage curves exhibited pronounced outward rectification. In high extracellular K⁺ (fig. 3C, open circles), the zero-current potential shifted with K⁺ equilibrium potential as predicted for a K⁺ selective channel (55 ± 1 mV shift per 10-fold change in extracellular potassium concentration at room temperature).

Pharmacology

KCNK5 currents were reversibly inhibited by extracellular protons in a range relevant to physiologic pH (IC₅₀ at approximately pH 7.6; Hill coefficient = 1.1). Proton inhibition was not significantly voltage-dependent. 2,4-Dinitrophenol inhibits oxidative phosphorylation, thereby producing intracellular acidosis. 2,4-Dinitrophenol (1 mM) strongly inhibited KCNK5 currents (normalized response: 0.15 ± 0.01; n = 3) after 6 min of perfusion (fig. 3D). Another pharmacologic treatment known to lower intracellular pH (5% carbon dioxide in frog Ringer’s solution, adjusted to pH 7.6 with bicarbon-
Fig. 3. Biophysical properties of KCNK5 currents in *Xenopus* oocytes studied with two-electrode voltage clamp. (A and B) Whole-cell currents of KCNK5 cRNA-injected oocytes perfused with either frog Ringer's solution (FR) or high-potassium frog Ringer's solution (HK). Voltage pulses (1 s duration) are from −140 to +40 mV from a holding potential of −80 mV (inset). (C) Steady state current–voltage curves for FR (filled circles) and HK (open circles). (D) Intracellular pH. Current–voltage curves in the presence or absence of 1 mM DNP.

KCNK5 currents were tested for sensitivity to a number of known pharmacologic modulators of tandem pore domain K⁺ channels. Barium (1 mM) inhibition of KCNK5 was strongly voltage-dependent with significant inhibition observed only within the range of potentials from −40 to +20 mV (normalized responses, 0.48 ± 0.04 and 0.89 ± 0.02, respectively; Woodhull δ = 0.71). The divalent cation zinc (100 μM) produced only slight voltage-dependent inhibition of KCNK5 currents (0.74 ± 0.01 at −20 mV vs. 0.81 ± 0.01 at +40-mV pulses; n = 3). The antiarrhythmic quinidine (100 μM) inhibited KCNK5 currents (0.43 ± 0.02; n = 5) with similar potency to that reported for the weak inward rectifier TWIK-1.30 Ethanol 17 mM (0.1%) potentiated KCNK5 currents (1.18 ± 0.04; n = 6).

The protein kinase C activators phorbol-12,13-dibutyrate (500 nM) and phorbol-12-myristate-13-acetate (50 nM) potentiated KCNK5 currents (phorbol-12,13-dibutyrate: 1.81 ± 0.14, n = 6; phorbol-12-myristate-13-acetate: 1.37 ± 0.12, n = 3). The inactive phorbol ester 4-phorbol-12,13-dibutyrate (1 μM) had no effect on KCNK5 currents. Pharmacologic treatments known to increase intracellular cyclic AMP levels and thereby activate protein kinase A (forskolin 10 μM with 3-isobutyl-1-methylxanthine 1 mM; or 8-bromo-cyclic AMP 300 μM) had no effect on KCNK5 currents.
A mammalian volatile anesthetic-activated K⁺ channel

Fig. 4. Volatile anesthetic sensitivity of KCNK5. (A) Whole-cell currents of KCNK5 cRNA-injected oocytes perfused with either frog Ringer's solution (FR) or FR with halothane (270 µM). (B) Concentration-dependence of halothane and isoflurane potentiation of KCNK5 currents as quantified by the normalized response. Four to nine oocytes were studied in each treatment group.

The following pharmacologic treatments had minimal effect (< 10%) on KCNK5 currents: cesium chloride (1 mM), tetraethylammonium (10 mM), magnesium (1 mM), arachidonic acid (10 µM), tetrodotoxin (100 nM), and 4-aminopyridine (1 mM). Clotrimazole (1 µM), which is known to inhibit Lynnaea volatile anesthetic-activated potassium currents, had no effect on KCNK5 currents.

Volatile Anesthetic Sensitivity

Volatile anesthetics potentiated whole cell currents of KCNK5-expressing oocytes (figs. 4A and 4B). This modulation of KCNK5 currents was not significantly voltage-dependent. Application of halothane and isoflurane caused a concentration-dependent increase in KCNK5 currents over a range overlapping the minimum alveolar concentration needed to prevent movement in response to a surgical stimulus in 50% of subjects. Volatile anesthetic (halothane 570 µM) potentiation of inward currents passed by KCNK5 was observed in high-potassium (115 mM) perfusate (~80 mV holding current potentiated 41 ± 5%; n = 4). Other volatile anesthetic agents (desflurane, enflurane, and chloroform), in concentrations that ranged from one to two times the approximate minimum alveolar concentration, also potentiated KCNK5 currents (fig. 5). The volatile nonimmobilizer 1,2-dichlorohexafluorocyclobutane slightly inhibited KCNK5 currents at concentrations greater than its minimum alveolar concentration value predicted by the Meyer-Overton hypothesis (normalized response: 0.80 ± 0.02; n = 3). Isoflurane (500–1,000 µM) did not potentiate the currents of other cloned mammalian tandem pore domain potassium channels expressed in oocytes (normalized responses: 0.86 ± 0.06, 0.95 ± 0.08, 0.99 ± 0.02, and 0.87 ± 0.02 for TWIK-1, TREK-1, TASK-1, and TRAAK, respectively). Volatile anesthetic agents also did not potentiate control currents of water-injected oocytes. The halothane preservative thymol (0.1 µM) had no effect on currents of KCNK5- or water-injected oocytes.

KCNK5 fractional instantaneous currents were not altered by volatile anesthetic treatment. In contrast, treatment of KCNK5-expressing oocytes with extracellular...
alkalinity both increased steady state currents and increased fractional instantaneous currents. Chloroform caused hyperpolarization of KCNK5-injected oocytes and had no effect on water-injected oocytes (fig. 6). Halothane (380 and 680 μM) hyperpolarized KCNK5-injected oocytes (3.4 ± 0.4, n = 6; and 4.4 ± 0.5, n = 9; mean ± SE) and did not hyperpolarize water-injected oocytes. Site-directed mutagenesis was used to introduce termination codons into the C-terminal coding region. KCNK5 C-terminal truncation mutants (at amino acid positions 252 or 278) did not exhibit spontaneous or volatile anesthetic-evoked activity.

Discussion

Volatile Anesthetic Activation of KCNK5

Despite more than 150 yr of clinical use, the basic mechanisms underlying volatile anesthetic action are not yet understood. In the current study we have shown that the function of a member of a mammalian ion channel family responsible for baseline K⁺ currents (KCNK5) can be enhanced by volatile anesthetics. Previously, we reported that these anesthetic agents potentiate currents from the yeast tandem pore domain K⁺ channel TOK1. Patel et al. subsequently reported that the archaic volatile anesthetic chloroform potentiated currents through a mammalian tandem pore domain K⁺ channel, TREK-1, when expressed in cultured COS cells. The potentiation of KCNK5 currents by volatile anesthetics shown here provides another example of the potentiating effects of volatile anesthetics on members of this family and additional evidence for the hypothesis that volatile anesthetic action may relate to an anesthetic-induced increase in baseline K⁺ conductances. Volatile anesthetic modulation of tandem pore domain potassium channels may complement concomitant effects on other ion channels, such as enhancement of inhibitory γ-aminobutyric acid-mediated currents or reduction of excitatory glutamatergic currents.

In our studies, chloroform did potentiate TREK-1 currents in Xenopus oocytes. However, we did not observe isoflurane potentiation of currents of several other mammalian tandem pore domain potassium channels (TWIK-1, TREK-1, TASK-1, and TRAAK) when expressed in the same system. This finding contrasts with the recent data of Patel et al., who found potentiation of TASK-1 and TREK-1 by clinical volatile anesthetics. The discrepancy between these results may relate to differences in the expression systems (Xenopus oocytes vs COS cells), causing different cell-specific modifications or accessory subunit assembly.

Structural determinants of volatile anesthetic modulation of tandem pore domain potassium channels have recently been investigated. The last 48 amino acids of the C-terminus of TREK-1 are essential for volatile anesthetic activation but not for basal activity. However, unlike TREK-1, C-terminal truncations of KCNK5 did not result in functional channels. Although this finding suggests different mechanisms of volatile anesthetic activation among mammalian tandem pore domain potassium channels, truncations of KCNK5 could also alter its trafficking and assembly by elimination of consecutive positively charged amino acids in the C-terminus.
Recent studies have suggested that volatile anesthetics prevent movement in response to noxious stimuli through their actions at the spinal cord. This action seems separate from the mechanisms that lead to anesthesia or unconsciousness. Although the molecular site of action within the spinal cord is unclear, volatile anesthetics hyperpolarize both sensory and motor neurons in spinal cord preparations, which suggests the role of an increased baseline potassium conductance. The highest level of KCNKS expression in the central nervous system was found in the spinal cord, but KCNKS could also be detected in cerebral cortex and brainstem tissues by the more sensitive method of RT-PCR. Because KCNKS expression was observed in dorsal and ventral sections of spinal cord tissue, anesthetic modulation of the channel may have significant effects on both sensory and motor pathways.

Modulation by Protons

KCN5 currents are highly sensitive to extracellular pH. Some level of tonic proton inhibition is present at physiologic pH, as higher extracellular pH potentiated KCN5 currents. Extracellular or intracellular acidity from tissue ischemia can depolarize a wide variety of cells. Acid-sensitive baseline K+ channels, such as KCN5, may partly mediate this depolarization. Changes in both intracellular and extracellular pH also can occur with neuronal activity.

Of the previously isolated tandem pore K+ channels, TOK1, TWIK-1, and TWIK-2 are inhibited by intracellular acidity. Inhibition of TOK1 by intracellular acidity has been observed in excised patches, whereas for TWIK-1 this mechanism is thought to be indirect. We observed inhibition of KCN5 currents after treatment of oocytes with 2,4-dinitrophenol or carbon dioxide, suggesting that KCN5 is also inhibited by intracellular acidity. However, other consequences of these treatments (e.g., altered intracellular adenosine triphosphate levels, ionized calcium levels, etc.) could also be responsible for this effect.

In summary, we have isolated a human cDNA encoding a tandem pore domain potassium channel that, when heterologously expressed, exhibits sensitivity to volatile anesthetic agents. Among excitable tissues, the expression of this channel is most prominent in spinal cord. The modulation of channel activity by anesthetic agents closely resembles that of physiologic baseline potassium channels; therefore, further studies of cellular localization will help to determine effects of channel modulation on excitability in vivo.

The authors thank Nozomi Hyodo for help with the electrophysiologic studies, Douglas Bayliss for mouse TRIK-1, Shanna Downey for excellent technical assistance, and Winfried von Ehrenburg for editorial assistance.

References

35. Rampil IJ: Anesthetic potency is not altered after hypothermic spinal cord transection in rats. Anesthesiology 1994; 80:606–10