Determinants of the Anesthetic Sensitivity of Two-pore Domain Acid-sensitive Potassium Channels

**MOLECULAR CLONING OF AN ANESTHETIC-ACTIVATED POTASSIUM CHANNEL FROM LYMNAEA STAGNALIS**

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Certain two-pore domain K⁺ channels are plausible targets for volatile general anesthetics, yet little is known at the molecular level about how these simple agents cause channel activation. The first anesthetic-activated K⁺ current Iₖ(AAn) that was characterized was discovered in the mollusk *Lymnaea stagnalis* and is remarkable for both its sensitivity to general anesthetics and its stereoselective responses to anesthetic enantiomers (Franks, N. P., and Lieb, W. R. (1988) Nature 333, 662–664 and Franks, N. P., and Lieb, W. R. (1991) Science 254, 427–430). Here we report the molecular cloning of a two-pore domain K⁺ channel LyTASK from *L. stagnalis* and show that, when expressed in HEK-293 cells, it displays the same biophysical characteristics as the anesthetic-activated K⁺ current Iₖ(AAn). Sequence analysis and functional properties show it to be a member of the TASK family of channels with ~47% identity at the amino acid level when compared with human TASK-1 and TASK-3. By using chimeric channel constructs and site-directed mutagenesis we have identified the specific amino acid 159 to be a critical determinant of anesthetic sensitivity, which, when mutated to alanine, essentially eliminates anesthetic activation in the human channels and greatly reduces activation in LyTASK. The L159A mutation in LyTASK disrupts the stereoselective response to isoflurane while having no effect on the pH sensitivity of the channel, suggesting this critical amino acid may form part of an anesthetic binding site.

For more than 150 years, simple volatile organic molecules have been used as general anesthetics. How and where these agents act in the central nervous system to cause loss of consciousness and insensitivity to pain remains a mystery, although a great deal of progress has been made toward identifying plausible mechanisms and possible molecular targets (3–6). Although it is generally accepted that these drugs act by binding directly to protein targets, it is far from agreed what these targets are or whether the state of general anesthesia is due to effects at a relatively small number of critical molecular sites or due to the combined effects of small perturbations at a very large number of sites. The molecular targets that have most often been proposed to be responsible for the actions of volatile general anesthetics (3–6) are the γ-aminobutyric acid, type A receptor, the related glycine receptor, and certain members of a relatively recently discovered family of K⁺ channels, the two-pore domain K⁺ (2PK) channels, so named because their primary sequences contain two pore-forming segments.

The 2PK channels form a diverse and highly regulated superfamily of channels that are thought to provide baseline regulation of membrane excitability (7–12). The possibility that neuronal excitability might be reduced by the activation of K⁺ channels has been long considered to be a plausible mechanism for general anesthesia (13–15), and such an anesthetic-activated K⁺ current was first characterized in molluscan pacemaker neurons (1, 16). Subsequently, a family of 15 channels has been identified in mammals (7–12) with several members being activated by volatile anesthetics. Most work has been done on TASK and TREK channels. Not only are these channels sensitive to anesthetics (17), but recent work has also shown that genetically engineered animals that lack these channels have a diminished anesthetic sensitivity (18, 19). How anesthetics cause 2PK channel activation at the molecular level, however, is poorly understood.

Using a combination of electrophysiology and molecular genetics, some key anesthetic determinants have been identified for both TASK and TREK channels (20, 21). The identification of specific amino acids that are essential for anesthetic action provides a powerful tool for investigating the importance of the target in the effects of anesthetics in the whole animal. If...
Anesthetic Determinants of TASK K⁺ Channels

an amino acid can be identified whose mutation affects only anesthetic sensitivity with little or no effect on other channel properties, then such a mutation can in principle be tested in whole animals to assess the importance of that channel for the in vivo anesthetic phenotype (22, 23).

This approach requires two, ideally closely related, isoforms of the channel with very different anesthetic responses (usually differing sensitivities). One difference that has yet to be exploited in this regard is the fact that many general anesthetics show stereoselectivity in their actions (24). For the inhalational anesthetics this was first shown using isoflurane with the anesthetic-activated potassium current Iₖ(Aan) in Lymnaea stagnalis (2). S(+)-Isoflurane was twice as effective at activating this current as the R(−)-enantiomer. We reasoned that knowledge of the amino acid sequence of the channel from Lymnaea might provide novel information that would help to identify the location of the anesthetic binding sites.

In this report we describe the cloning of an anesthetic-activated channel from L. stagnalis and show how its unusual properties can be used to provide information that differentiates between the location of anesthetic binding sites and regions of the channel responsible for transducing anesthetic binding to changes in gating. We exploit the fact that hTASK channels differ greatly in the extent they are activated by the volatile agent chloroform. Using a combination of chimeric constructs (between hTASK-1 and hTASK-3 channels and hτASK-1 and LyTASK channels) and site-directed mutagenesis, we identify a specific amino acid that determines anesthetic sensitivity, and we use stereoselectivity as a criterion to determine its likely role as part of an anesthetic binding site.

EXPERIMENTAL PROCEDURES

Isolation of Total RNA from the Central Nervous System of L. stagnalis—Adult L. stagnalis were obtained from Blades Biological, Ltd. (Edenbridge, Kent, UK) and kept in tanks of water at 22 °C until use. The circumsophageal ganglia from 3–4 animals were rapidly removed and placed into snail Ringers (see below). Total RNA was then extracted using TRIzol reagent (Invitrogen) together with Phase Lock Gel Heavy (Eppendorf, Cambridge, UK) to facilitate the separation between the organic and aqueous phases, and Pellet Paint CO-precipitant (Novagen, Nottingham, UK), a dye-labeled carrier, which allowed easy visualization and localization of the RNA pellet. DNA was removed by treatment with Amplification Grade DNase I (Invitrogen) (1 unit/g of RNA). The DNase I was then removed by a phenol:chloroform:isoamyl alcohol extraction (25:24:1) followed by a chloroform:isoamyl alcohol (24:1) extraction (25).

Degenerate Reverse Transcription-PCR—Total RNA from Lymnaea central nervous system (400 ng) was reverse transcribed with 50 ng of random hexamers in a 20-μl reaction volume using the SuperScript First-strand Synthesis System (Invitrogen). To exclude the contribution of genomic DNA to the final PCR step, incubations with and without reverse transcriptase were run simultaneously. The first strand cDNA was used as a template for PCR amplification using degenerate primers designed against stretches of conserved amino acid residues at the start and including part of the TM2 region (forward primer: 5’-CGS GGG AAR GGC WTT YTG YAT GTT CTA YG-3’; where S = C/G, R = A/G, W = A/T, and Y = C/T) and within the P2 region (reverse primer: 5’-CRW ART CRC CRA ARC CDA TWG TNG TYA R-3’; where D = A/G/T and N = A/C/G/T) of predicted and cloned 2PK channels. 2 μl of first strand cDNA was amplified (2 μM primers) using the HotStar-Taq Master Mix (Qiagen). PCR reaction mixtures were resolved on a 1.5% agarose gel and visualized with ethidium bromide. A distinct band of the expected size of ~300 bp for the putative 2PK channel cDNA was observed. The PCR reaction mixture was purified using QIAquick columns (Qiagen), directly subcloned into the pCRII TOPO vector (Invitrogen) by TA cloning and sequenced on both strands using the universal M13 primers. Sequencing was done by MWG-Biotech (Ebersberg, Germany). Databases were searched for similar sequences using the BLAST alignment program (26).

Rapid Amplification of cDNA Ends—5’-RACE and 3’-RACE PCR reactions were performed according to the manufacturer’s instructions using the BD SMART RACE cDNA amplification kit (Clontech, St Germain en Laye, France) to obtain the complete 5’- and 3’-ends of the target LyTASK cDNA. The partial sequence of the LyTASK was used to design specific primers for 5’- and 3’-RACE. The primer used for the 5’-RACE was 5’-AGG AGA ATA TGG CCG CCC CCG ACG TGAG-3’. The primer used for the 3’-RACE was 5’-AGA GCC TGC GCG AGC GCC TCA AC-3’. Thermal cycling was performed using touchdown PCR. RACE products were isolated from a 1.2% agarose gel using a Nucleotrap gel extract kit (Clontech), subcloned into pCRII TOPO vector and sequenced on both strands. The whole coding region of LyTASK cDNA was obtained by PCR using primers designed from the extreme 5’-end (5’-CGG AGA AGA GCA CAC ATG CCT CCA-3’; sense) and 3’-end (5’-TAA ATC TAA TCA GCA ACT TGT GTT-3’; antisense) of LyTASK cDNA with the BD Advantage 2 PCR enzyme system (Clontech) and the 5’-RACE ready cDNA as template. Amplified products were subcloned into pCRII TOPO and sequenced on both strands.

Sequence Analysis—L. stagnalis amplicons were used for translated blast searches (tBLASTx) of the NCBI non-redundant nucleotide sequence data base (www.ncbi.nih.gov/BLAST/). The TMpred program (27), which predicts membrane-spanning regions and their orientation using a data base of known transmembrane proteins, was used to predict the topology of the cloned LyTASK. Potential N-linked glycosylation sites were predicted using NetNGlyc 1.0. KinasePhos was used with a 95% prediction specificity to predict putative phosphorylation sites for protein kinases. ClustalW was used to align the deduced amino acid sequence of the cloned LyTASK with 2PK channels from other species. ALIGN was used with default settings to perform pairwise alignments of the deduced amino acid sequence of the cloned LyTASK with 2PK channels from other species. Phylogenetic trees were generated using ClustalW.

Expression Vectors—For transfection into modified HEK-293 cells (tSA 201), 1.1-kb cDNA containing the entire coding region for LyTASK was subcloned into the mammalian expression vector pcDNA3.1(+) (Invitrogen) via non-directional cloning by ligating into the EcoRI sites after cutting LyTASK/
pCRII TOPO (Invitrogen) with EcoRI. Expression vectors with LyTASK cDNA in the correct orientation were identified by restriction analysis with AgeI and XhoI. pcDNA 3.1(β/H11001) expression vectors with cDNA encoding human TASK-1 (GenBank™ accession number NM 002246) and human TASK-3 (GenBank™ accession number NM 016601) were a gift from Dr A. Mathie (Imperial College London).

Site-directed Mutagenesis of TASK Channels—Site-directed mutagenesis was performed on TASK cDNA using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Complementary primers were designed so as to contain the desired mutation together with a silent mutation introducing a diagnostic restriction site. Mutant DNA constructs were verified by restriction digests and sequencing to confirm the introduction of the correct mutated bases (MWG-Biotech).

Chimeric Constructs—Chimeras between the different TASK channels were generated by PCR using the overlap-extension method. The cDNAs encoding the chimeric channels were subcloned into pcDNA 3.1 and confirmed by sequencing.

Cell Culture and Transfection into HEK-293 Cells—The HEK-293 cells were maintained under standard conditions in growth media (minimum essential media Eagle with Earle’s salts, L-glutamine and sodium bicarbonate, supplemented with 1% non-essential amino acids, 1% penicillin (10,000 units/ml), streptomycin (10 mg/ml), and 10% heat-inactivated fetal bovine serum) in a 5% CO₂:95% air, humidified incubator at 37 °C. For electrophysiological studies, cells were plated on glass coverslips previously coated with poly-D-lysine. Cells were transiently cotransfected with TASK/pcDNA3.1 and green fluorescent protein/pcDNA3.1 using the calcium phosphate precipitation method. Transfected cells were identified for electrophysiology using a Nikon microscope with an epifluorescence attachment. Electrophysiological recordings were performed within 24–48 h of transfection.

Whole Cell Patch Clamp Recording from HEK-293 Cells—All chemicals and reagents were obtained from Sigma (Poole, Dorset, UK) or VWR International Ltd. (Lutterworth, Leicestershire, UK), unless otherwise stated. The composition of the normal control extracellular solution was (in mM) 140 NaCl, 2.5 KCl, 2 MgCl₂, 10 HEPES, 10 D-glucose, 1 CaCl₂, titrated to pH 7.4 with NaOH. Extracellular solutions containing elevated K⁺ were made by substituting NaCl with KCl. When the extracellular pH was a variable, the extracellular solution was titrated with either NaOH or HCl. For the experiments with zinc and copper, stock solutions of 1 mM ZnCl₂ and 500 mM CuCl₂, respectively, were prepared in extracellular solution and diluted to the desired test concentrations on the day of the experiment. For experiments with arachidonic acid a stock solution of 100 mM was made in ethanol, kept under nitrogen at −20 °C for no more than 3 days, and was diluted in extracellular

transmembrane domains (boxed) and the pore forming regions (shaded) are indicated. Consensus sites for N-glycosylation (#), phosphorylation by protein kinase A (filled circle), protein kinase C (*) and casein kinase II (filled square) are shown. B, transmembrane topology of the LyTASK channel predicted using Tmpred (27) shows four potential transmembrane segments (TM1–TM4). The two potential pore-forming regions (P1 and P2) are evident, as are the long extracellular loop between TM1 and TM2 and the long C-terminal domain.
solution to the test concentration (10 μM) before the experiments. In experiments with arachidonic acid, the control solution contained the same concentration of ethanol (1.7 mM) as the test solution. Patch electrodes were pulled from thick-walled borosilicate glass capillaries (GC150F-10, Harvard Apparatus, Edenbridge, Kent, UK) using a two-stage vertical puller (PP-830, Narishige, Tokyo, Japan), and their tips were fire-polished. Patch electrodes were back-filled with 0.2-μm-filtered intracellular solution, which consisted of (mM): 120 KCH₃SO₄, 4 NaCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES, 10 EGTA, 3 Mg-ATP, 0.3 Na₂-GTP, titrated to pH 7.20 with KOH. Electrode resistances ranged from 3 to 5 MΩ. Voltage clamp recordings were performed in the whole cell configuration using a patch clamp amplifier (Axopatch 200A, Axon Instruments, Union City, CA). Series resistance was compensated by 80% and monitored during the recording. Voltage commands were applied, and currents were recorded using pClamp 6 software (Axon Instruments). Cells were held at −80 mV and a linear voltage ramp from −120 mV to −80 mV in 2 s followed by a return ramp from 0 mV to −80 mV in 0.5 s, applied at 10-s intervals. Recordings with a positive holding current at −80 mV (indicating a negligible leak conductance) and an access resistance of <20 MΩ were included in the analysis. The output of the patch clamp amplifier was filtered at 100 Hz (−3 dB) using an 8-pole Bessel filter (Frequency Devices 902, Lyon Instruments, Ltd., UK) before being digitized at 500 Hz and recorded on a computer hard disk via an analog to digital converter (Digidata 1200, Axon Instruments). All experiments were performed at room temperature (22 ± 1 °C), and solutions were applied by a gravity-fed perfusion system. Currents were usually measured from the ramps at −50 mV where the contribution of endogenous currents from HEK-293 cells was negligible.
Two-electrode Voltage Clamp Recording from *L. stagnalis* Neurons—Circumesophageal ganglia from *L. stagnalis* were excised, and the loose connective tissue overlying the right parietal ganglion was removed. This ganglion was then dissected away intact and held by its nerve root using light suction. The tough endoneurium was removed after softening in Pronase (≈2 mg/ml) for 10–15 min exposing the cluster of light yellow neurons used in these experiments (in the ventral part of the ganglion). The ganglion was held in a small chamber and continuously perfused with normal snail Ringers, which consisted of (in mM): 50 NaCl, 2.5 KCl, 4 CaCl2, 4 MgCl2, 10 HEPES, 5 glucose titrated to pH 7.4 with NaOH. Electrodes were pulled from 1-mm borosilicate glass capillaries and filled with 2.5 M KCl and had resistances between 10 and 30 MΩ. Following impalement, the chosen cell was carefully removed from the ganglion, and all experiments were performed on completely isolated neurons. A two-electrode voltage-clamp amplifier was used (Axoclamp 2A, Axon Instruments), with the current record being filtered (50 Hz, −3 dB) by an 8-pole Bessel filter. Data were digitized at 200 Hz and transferred to a computer for analysis. Steady-state I-V curves were constructed by slowly ramping the membrane potential (usually at 3 mV s⁻¹) in the depolarizing direction. When the extracellular pH was a variable, the extracellular solution was titrated with either NaOH or HCl and the pH dependence of the anesthetic-activated potassium current I_{K\text{An}} was measured from cells clamped at −40 mV. This minimized the small contribution of an anesthetic-activated inward current, which is present to varying degrees in *Lymnaea* right-parietal ganglion neurons and which reverses at 0 mV (data not shown). Zinc- and copper-containing solutions were prepared as described above.

Anesthetic Solutions—Anesthetic-containing solutions were made on the day of the experiment, and care was taken throughout to minimize losses of volatile anesthetics. Anesthetic solutions at a chosen concentration were prepared as volume fractions of a saturated aqueous solution. The concentration of the saturated solutions were taken to be 17.5 mM for halothane (28), 66.6 mM for chloroform (29), and 15.3 mM for halothane (28). Anesthetic-containing solutions were used (Anaquest Inc. (Murray Hill, NJ). Chemical purities were 99.0% for S(+) isoflurane and 99.1% for R(−) isoflurane; the corresponding optical purity ratios were 99.5%/0.5% and 99.0%/1%, respectively.

Statistics and Data Analysis—Data are expressed throughout as means ± S.E., unless otherwise stated. In the figures, when the error bars are not shown they are smaller than the size of the symbol. Concentration-response curves were usually fitted with Hill equations of the form (Equation 1),

\[ I = I_{\text{max}} \frac{c^{n_{I}}} {c^{n_{I}} + EC_{50}} \]  

(Eq. 1)

where \( I \) is the response, \( c \) the concentration, \( I_{\text{max}} \) the maximum response, \( EC_{50} \) the concentration for a 50% effect, and \( n_{I} \) is the Hill coefficient. Any percentage change was calculated relative to the average of controls taken just before and just after a test application. Statistical significance was assessed using the Student’s t test.

RESULTS

Molecular Cloning and Sequence of LyTASK—Degenerate oligonucleotides were designed to hybridize in PCR reactions with conserved regions of 2PK channel cDNAs derived from total RNA extracted from *L. stagnalis* CNS. Comparison with the databases using tblastx identified a cDNA fragment for which the derived amino acid sequence was similar to the region extending from the start of the second transmembrane domain to the end of the second pore domain of previously cloned mammalian 2PK TASK channels. The whole coding region for the channel was obtained using 5′- and 3′-RACE PCR, followed by reverse transcription-PCR using specific primers based on the predicted N and C termini. The cDNA sequence contained an open reading frame of 1083 bases that encoded a 361-amino acid polypeptide with a calculated molecular mass of 41 kDa (Fig. 1A).

Four transmembrane segments (TM1 to TM4) were predicted (see Fig. 1B) using the Tmpred program (27). Two pore-forming regions (P1 and P2) containing the T\(g\)X(Y/I/F)G consensus motif of potassium channels (see Fig. 1A) also scored highly using this algorithm. The putative K⁺ channel subunit exhibits a short intracellular N-terminal domain, a large extracellular loop between TM1 and P1, and a large intracellular carboxyl domain (see Fig. 1B), consistent with other known 2PK channels (7–9). The *Lymnaea* subunit may potentially undergo several post-translational modifications (see Fig. 1A). One putative N-linked glycosylation site is present in the TM1-P1 loop (Asn-53). Putative intracellular phosphorylation sites for several protein kinases were identified, including two for protein kinase A at Ser-10 and Thr-152; one for protein kinase C at Ser-338; and two for casein kinase II at Thr-334 and Ser-340.

An alignment of the amino acid sequence of the *Lymnaea* subunit with sequences of mammalian 2PK channel members

![Figure 2. Sequence comparison of LyTASK and mammalian TASK channels. A, alignment of amino acid sequences of LyTASK with mammalian TASK channels. Transmembrane regions are shaded and pore-forming regions are indicated by a line. (Accession: hTASK-1, gi|2465542; TASK-1, gi|15431283; HTASK-3, gi|7706135; rTASK-3, gi|14583127; hTASK-5, gi|11641275; rTASK-5, gi|3993570; rTASK-1, gi|39930507; TASK-5, gi|4504848; TRESK, gi|16507967; THIK-1, gi|11177515; THIK-2, gi|11177513; TWI, gi|15451900; TWI-2, gi|4574321; TALK-1, gi|16753201; TALK-2, gi|13926110; TRESK, gi|32469494.](image-url)
Anesthetic Determinants of TASK K⁺ Channels

FIGURE 3. Volatile general anesthetics halothane and chloroform strongly activate LyTASK and hTASK-3, but while hTASK-1 is activated by halothane, it is inhibited by chloroform. A, concentration-response curve for LyTASK current activation by halothane. The top left inset shows the time course of activation of LyTASK by a range of concentrations of halothane (1.8, 0.53, 0.18, and 0.05 mM) applied as indicated by the black bars. B, concentration-response curve for LyTASK current activation by chloroform. The top left inset shows the time course of activation of LyTASK by a range of concentrations of chloroform (0.20, 1.98, 0.66, and 6.6 mM). C, concentration-response curve for hTASK-3 current activation by halothane. The top left inset shows the time course of activation of hTASK-3 by a range of concentrations of halothane (1.8, 0.53, and 0.18 mM). D, concentration-response curve for hTASK-3 current activation by chloroform. The top left inset shows the time course of activation of hTASK-3 by a range of concentrations of chloroform (0.66, 0.20, and 0.06 mM). E, concentration-response curve for hTASK-1 current activation by halothane. The top left inset shows the time course of activation of hTASK-1 by a range of concentrations of halothane (0.05, 0.18, and 0.53 mM). F, concentration-response curve for hTASK-1 current inhibition by chloroform. The top left inset shows the time course of inhibition of hTASK-1 by a range of concentrations of chloroform (6.6, 1.98, and 0.66 mM). Each data point in the concentration-response curves is the mean percentage activation or inhibition (average number of cells, n = 5) measured at −50 mV from voltage ramps recorded every 10 s (see “Experimental Procedures”). The current-voltage relationship in each panel is in the absence and the presence of 0.53 mM halothane (panels A, C, and E) or in the absence and the presence of 1.98 mM chloroform (panels B and D) or 6.7 mM chloroform (panel F).

of the TASK subfamily is shown in Fig. 2. The Lympneae subunit shares ~47% overall amino acid identity with human and rat TASK-1 and TASK-3 and ~41% with human and rat TASK-5. The Lympneae subunit (which we refer to as LyTASK) has a lower degree of similarity to the other cloned mammalian 2PK channels with 20–27% amino acid identity. Sequence identity of the Lympneae subunit to TASK-1, TASK-3, and TASK-5 is much higher in the core region comprising the transmembrane domains, the extracellular loop, and the pore regions (60% amino acid identity with hTASK-1 and hTASK-3) rather than in the C-terminal domain (24% amino acid identity with hTASK-1, 30% with hTASK-3). The alignment (Fig. 2A) reveals a number of features that are common to TASK-1, TASK-3, TASK-5, and the Lympneae subunit. It also highlights several features that are not shared between them. The two amino acid motifs P1 and P2 that form the selectivity filter of a 2PK channel are conserved in LyTASK, TASK-1, TASK-3, and TASK-5. LyTASK N-linked glycosylation site (Asn-53) is conserved in the mammalian TASK-1, TASK-3, but not in TASK-5 (see Fig. 2A). TASK-1, TASK-3, TASK-5, and LyTASK all contain a histidine residue immediately downstream of the “GYG” pore motif in the P1 domain (His-98). The protonation of this His-98 has been shown to be important for both normal pH sensitivity and K⁺ selectivity of TASK-1 (31). This would suggest that the Lympneae protein is an acid-sensing K⁺ channel (see below). In contrast to mammalian TASK-3, which contains a glutamic acid at position 70, which confers sensitivity to zinc (33, 34), LyTASK has a valine in this position indicating that it may be zinc-insensitive (see below).

A dendrogram (Fig. 2B) was constructed from the amino acid sequences of all the currently cloned human 2PK channels together with LyTASK. This tree shows that TASK-1, TASK-3, TASK-5, and LyTASK form a separate cluster among the 2PK channels.

Anesthetic Activation of LyTASK K⁺ Channel—The full-length LyTASK cDNA was subcloned into the mammalian expression vector pcDNA 3.1(+) and expressed in HEK-293 cells. Cells transfected with LyTASK cDNA displayed outwardly rectifying whole cell currents (see control I–V traces, lower right insets in Fig. 3, A and B). The size of the current varied considerably from cell to cell, with an average outward current (at −50 mV) of 0.6 ± 0.7 nA (mean ± S.D.). Volatile anesthetics markedly potentiated the whole cell current. The activation was rapid and sustained, persisted for as long as the anesthetic was present, and rapidly reversed upon removal of the anesthetic (see upper left insets of Fig. 3, A and B). The concentration-dependent activation of LyTASK by volatile general anesthetics is illustrated in Fig. 3 (A and B) for halo-
Anesthetic Determinants of TASK K⁺ Channels

FIGURE 4. Biophysical properties of the halothane-activated LyTASK current in HEK-293. A, The whole cell current-voltage relationships of the anesthetic-activated current were well fitted by the Goldman-Hodgkin-Katz constant-field current equation (35) for free electrodiffusion through an open ion-selective pore. In 2.5 mM extracellular K⁺, the reversal potential was accurately predicted by the Goldman-Hodgkin-Katz equation with the channel permeability being the only adjustable parameter. B, the reversal potential was accurately predicted by the Nernst equation. The line is a least-square fit to the data points (means from, on average, 5 cells) with a slope constrained to be equal to the theoretical value of 58.6 mV/decade.

LyTASK Currents and IK(An) in Lymnaea Are Sensitive to Extracellular pH—Because the LyTASK-predicted amino acid sequence contained a histidine at position 98 that is known to confer acid sensitivity in mammalian TASKs (30–32), we investigated the sensitivity of LyTASK and IK(An) in Lymnaea to extracellular pH. Fig. 5A shows the time course of the inhibition of the anesthetic-activated LyTASK current by extracellular acidification. For extracellular pH ranging from 4.4 to 5.4 a strong inhibition of the anesthetic-activated LyTASK current was observed, whereas more alkaline pH, ranging from 6.9 to 8.4, had little effect on the anesthetic-activated LyTASK current. The pH dependence of the anesthetic-activated LyTASK current in HEK cells is characterized by a pK value of 6.38 ± 0.02 (Fig. 5B). A very similar pH dependence was observed for the anesthetic-activated TASK current in Lymnaea. This is illustrated by the current-voltage curves for IK(An) shown in Fig. 6A. Halothane caused a strong activation of IK(An) at pH 7.4, but this activation was considerably reduced at more acidic pH. Indeed, at pH 5.4, IK(An) was completely blocked, and halothane caused no significant activation of the current. The pH dependence of the anesthetic-activated current IK(An) in Lymnaea is characterized by a pK value of 6.33 ± 0.02 (Fig. 6B). The inset to Fig. 6B shows that, for a neuron clamped at −40 mV, a switch into halothane at pH 7.8 caused a large outward current, whereas a switch into halothane at pH 5.8 had little effect. Note the small inhibition in the baseline current in the absence of halothane when the pH is changed from 7.8 to 5.8, indicating a very low basal activation of IK(An).

Pharmacological Properties of LyTASK and IK(An)—To further characterize LyTASK channels and determine whether they share similar pharmacological properties to the mammalian TASK channels and the anesthetic-activated current IK(An) in Lymnaea, the sensitivity of the whole cell current to various pharmacological agents was examined. Experiments were carried out in the presence of halothane (0.18 mM) to activate LyTASK, and inhibition values were determined at −50 mV. Because mammalian 2PK channels are differentially sensitive to

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zinc (33, 34), and because LyTASK lacks the glutamic acid residue (position 70) critical for TASK-3 zinc sensitivity, we first tested the sensitivity of LyTASK to this divalent cation. We found that 100 μM zinc had no significant effect on the halothane-activated LyTASK current, inhibiting the response by 1 ± 2% (n = 7). We also determined the sensitivity of LyTASK to copper, because mammalian 2PK channel are also differentially sensitive to this cation (34). LyTASK was also insensitive to copper; 50 μM copper caused only a slight (7 ± 1%; n = 5) reduction in current. Similarly, the anesthetic-activated current I_{K(An)} in Lymnaea displayed the same insensitivity to zinc and copper as LyTASK with 50 μM zinc and 50 μM copper producing only 4 ± 1% (n = 5) and 3 ± 5% (n = 3) inhibition, respectively. The LyTASK-mediated current was also insensitive to 10 μM arachidonic acid, which caused a negligible change in current (0.3 ± 0.7%, n = 5).

Isoflurane Isomers Stereoselectively Activate LyTASK—The LyTASK current was strongly activated by isoflurane, which allowed the stereoselectivity of the effect to be studied. Fig. 7A shows the time course of LyTASK activation by 0.46 mM racemic isoflurane and its individual enantiomers. The current induced by the S(+)-isomer was substantially greater than that induced by the R(−)-isomer. The current induced by the racemate was intermediate between those induced by the isomers. This stereoselective activation of isoflurane on LyTASK was invariably observed. The histograms in Fig. 7B represent the activation by racemic isoflurane and its enantiomers averaged over several cells. A more precise measure of the degree of stereoselectivity is obtained by determining the ratio of activation of the S(+)-isomer to that of the R(−)-isomer for a given cell and then averaging this ratio for a number of cells. This stereoselectivity ratio was determined to be 1.66 ± 0.11 (mean ± S.E., n = 7), which is significantly different from unity (p < 0.01). Because isoflurane enantiomers show identical partition-
Anesthetic Determinants of TASK K⁺ Channels

FIGURE 7. The volatile anesthetic isoflurane stereoselectively activates LyTASK, but human TASK channels display little or no stereoselectivity. A, time course of activation of LyTASK by racemic isoflurane (Rac) and its individual optical isomers, S(+) and R(−)-isoflurane (0.46 mM). The S(+) isomer is more effective than the R(−)-isomer at activating LyTASK. Each data point is the anesthetic-activated current measured at −50 mV from voltage ramps recorded every 10 s. B, summary showing the differential effects of the racemate, the S(+) and the R(−)-isomers in activating LyTASK. The stereoselectivity ratio provides a more precise measure of the degree of stereoselectivity and is the percentage activation induced by the S(+) isomer divided by the percentage activation induced by the R(−)-isomer determined for individual cells. The stereoselectivity ratio (mean ± S.E.) was 1.66 ± 0.11 (n = 7). C, time course of activation of human TASK-3 by racemic isoflurane (Rac) and its individual optical isomers S(+) and R(−)-isoflurane (0.46 mM). The activation of human TASK-3 by the S(+) isomer is similar to the activation observed with the R(−)-isomer. D, summary data showing the similar effects of the racemate, the S(+) and the R(−)-isomers in activating hTASK-3. The stereoselectivity ratio (mean ± S.E.) was 1.23 ± 0.08 (n = 11). E, time course of activation of human TASK-1 by racemic isoflurane (Rac) and its individual optical isomers S(+) and R(−)-isoflurane (0.46 mM). The activation of human TASK-1 by the S(+) isomers is not significantly different (p > 0.2) to the activation observed with the R(−)-isomer. F, summary data showing the similar effects of the racemate, the S(+) and the R(−)-isomers in activating hTASK-1. The stereoselectivity ratio (mean ± S.E.) was 0.90 ± 0.08 (n = 5).

ing into lipid bilayers (36), these results are consistent with isoflurane binding directly to the channel to exert its effects.

Human TASK Channels Show Little or No Stereoselectivity—With the construction of chimeric channels in mind (see below), we determined whether isoflurane also displayed stereoselectivity when activating human TASK channels. These data are illustrated in Fig. 7, which show that isoflurane displayed little or no stereoselectivity, with hTASK-3 (Fig. 7, C and D) showing modest stereoselectivity (stereoselectivity ratio 1.23 ± 0.08, n = 11), which is significantly different to unity (p < 0.05) and hTASK-1 (Fig. 7, E and F) showing no significant stereoselectivity (p > 0.2). Moreover, the percentage activation of hTASK channels by isoflurane was considerably smaller (~80% for hTASK-3 and ~60% for hTASK-1 at 0.46 mM isoflurane) than that observed with LyTASK (~250% at 0.46 mM isoflurane).

Truncated Channels and Chimeric Constructs—To identify regions of the channel necessary for anesthetic sensitivity of LyTASK, LyTASK and hTASK-1 subunits were used to make chimeric constructs, because their anesthetic sensitivities and stereoselectivity for isoflurane differ so greatly. Previous work has shown that the C-terminal domain of the channel is not necessary for anesthetic activation of TASK-1, and yet it plays a significant role in the anesthetic activation of TASK-3 (20, 21). To determine whether the C-terminal domain of the channel is necessary for anesthetic activation of LyTASK, this domain was removed by introducing a stop codon after the fourth transmembrane domain at position 249. Deletion of the C-terminal domain of LyTASK did not appear to affect the basal activity of the channel. It also had no effect on anesthetic activation. Fig. 8A shows that the activation of the truncated channel, LyTASK249, by isoflurane (0.46 mM), was not significantly different (p > 0.1) to that of the full-length channel (compare with Fig. 7B). This was also the case for chloroform and halothane (data not shown). The truncated channel also retained stereoselectivity for isoflurane (Fig. 8A) with a stereoselectivity ratio of 1.52 ± 0.10 (n = 8), which was not significantly different (p > 0.3) to that of the full-length channel (see Fig. 7B).

As a corollary to this experiment, we constructed a chimera that consisted of the core region of the channel being hTASK-1 and the C-terminal domain being LyTASK. This construct hTASK-1242LyTASK361 (Fig. 8B) essentially displayed the properties of the hTASK-1 channel with regard to isoflurane sensitivity, with no stereoselectivity and relatively low percentage activations (compare Fig. 7, C and D).

Because a stretch of 6 amino acids (243–248) at the beginning of the C-terminal domain of TASK channels has previously been shown (21) to be necessary for both activation by halothane and inhibition by thyroid-releasing hormone, we investigated the equivalent region in the LyTASK channel. By mutating two amino acids (I243V and L247M) we converted this stretch of 6 amino acids in the LyTASK channel to that of hTASK-1 (see Fig. 2A). We found that the percentage activation of LyTASK242hTASK-1248 by isoflurane was markedly reduced (Fig. 8C), but the stereoselectivity for isoflurane remained large, with a stereoselectivity ratio of 1.76 ± 0.13 (n = 8). The percentage activations by chloroform and halothane
were also reduced to less than half those seen with LyTASK_{248} (data not shown).

In addition to constructing chimeras of hTASK-1 and LyTASK we followed a parallel strategy, which was to make chimeras between hTASK-1 and hTASK-3, to identify regions that determined chloroform activation, which differed greatly between the channels (see Fig. 3, D and F). Our hypothesis here was that this difference is due to subtle differences in a binding site that could accommodate several different inhalational anesthetics.

The results using this approach are shown in Fig. 9. This shows the chloroform sensitivity of various hTASK-1/hTASK-3 chimeras in which the N terminus is hTASK-1, and the junction with hTASK-3 is moved progressively toward the C terminus. The first chimera (hTASK-1_{169}hTASK-3_{374}), with the junction in the first transmembrane domain, showed an anesthetic phenotype that was indistinguishable from hTASK-3 (Fig. 9A). This was also the case with the next chimera (hTASK-1_{113}hTASK-3_{374}) where the junction was just after the second transmembrane domain (Fig. 9B). However, when the junction was moved to the middle of the third transmembrane domain (hTASK-1_{169}hTASK-3_{374}), the anesthetic phenotype changed dramatically and became more like that of hTASK-1 (Fig. 9C). The next chimera, with the junction at the start of the large C-terminal domain (hTASK-1_{34}hTASK-3_{374}), also displayed an hTASK-1 phenotype (Fig. 9D). These results imply that key anesthetic determinants lie within the region between the cytoplasmic end of TM2 and the middle of TM3.

We next performed alanine scanning in this region to investigate the effects of changing each of the amino acids in LyTASK that differed from both of the hTASK channels. The logic here was to try to identify amino acids which conferred the extra sensitivity, and stereoselectivity, on LyTASK, with the hope that this would identify amino acids critical for anesthetic activation. A single amino acid (Leu-159) in this region caused a large reduction in the anesthetic activation of LyTASK when mutated to alanine (Fig. 10A), reducing the anesthetic activation by a factor of 5–6 for halothane and isoflurane and by a factor of ~2.5 for chloroform. The equivalent mutation of hTASK-3 (M159A) had similar effects, virtually eliminating the anesthetic activation (Fig. 10B), with chloroform becoming slightly inhibitory. In hTASK-1, the M159A mutation converted the activation observed in the wild-type channel for halothane and isoflurane to an inhibitory response (Fig. 10C) and enhanced the inhibition observed with chloroform.

The histograms show the similar effects of racemic isoflurane (Rac) and its individual optical isomers S\textsubscript{(+)}- and R\textsubscript{(+)}-isoflurane (0.46 mM) in activating the chimeric construct LyTASK\textsubscript{242}LyTASK\textsubscript{361}. The stereoselectivity ratio (mean ± S.E.) was 1.10 ± 0.04 (n = 6). The inset shows the time course of activation of the chimeric construct by the racemate, the S\textsubscript{(+)}- and R\textsubscript{(+)}-isomers. C substitution of six amino acids (243–248) from LyTASK truncation with the corresponding region of hTASK-1 does not affect the stereoselectivity of the channel for isoflurane. The histograms show the differential effects of racemic isoflurane (Rac) and its individual optical isomers S\textsubscript{(+)}- and R\textsubscript{(+)}-isoflurane (0.46 mM) in activating LyTASK\textsubscript{242}hTASK\textsubscript{34} truncation. The S\textsubscript{(+)}-isomer was more effective than the R\textsubscript{(+)}-isomer at activating LyTASK\textsubscript{242} by the racemate, the S\textsubscript{(+)}- and R\textsubscript{(+)}-isomers. B, substitution of LyTASK core region with the corresponding region of human TASK-1 suppresses the stereoselectivity of the channel for isoflurane.
Although the anesthetic sensitivity of LyTASK(L159A) was greatly reduced compared with the wild-type channel, it was still sufficient to determine if the remaining activation by isoflurane retained its stereoselectivity. The results of these experiments are shown in Fig. 11 (A and B), which show that the stereoselectivity observed with the wild-type channel (see Fig. 7, A and B) is eliminated by the L159A mutation. We also tested the pH dependence of the mutated channel and found that its sensitivity to pH (Fig. 11, C and D) was essentially the same as that of the wild-type channel (Fig. 5, compare A and B) with a pK of 6.32 ± 0.03.

DISCUSSION

The properties of the anesthetic-activated channel (1, 2) in Lymnaea (its insensitivity to tetraethylammonium and 4-aminopyridine and its lack of voltage gating) suggested that it might be a member of the 2PK channel family (16). We therefore designed degenerate primers based on this hypothesis. The channel we cloned has a sequence that clearly identifies it as a 2PK channel (Fig. 1). Four transmembrane domains are readily identifiable, together with the characteristic pair of pore-forming domains. The Lymnaea channel is most closely related to the subfamily of mammalian TASK channels (Fig. 2) with 47% identity at the amino acid level with human TASK-1 and TASK-3. No other 2PK channel has been cloned from Lymnaea, and only two others are known from mollusks, both from Aplysia. One appears to be specific for mollusks with no mammalian counterpart (37), whereas the other (38) is related to the THIK family, so-named because of its inhibition by halothane (39).

It seems very likely that the channel we have cloned is the same as that responsible for the anesthetic-activated potassium current IK(An) in Lymnaea. They have several characteristic features in common. Both IK(An) in Lymnaea and the current passed by LyTASK in HEK cells are well described by the Goldman-Hodgkin-Katz constant-field equation (Fig. 4 and references 1 and 40). More importantly, both show a similar anesthetic sensitivity to halothane, isoflurane and chloroform (Figs. 3 and 7 and reference 16) with a similar stereoselectivity for the isoflurane enantiomers (Fig. 7 and reference 2). The LyTASK current is also insensitive to arachidonic acid, in common with IK(An) in Lymnaea (1). The primary sequence of LyTASK suggested that it would be insensitive to blockage by zinc but be inhibited by increasing extracellular acidity. We confirmed that this was the case for LyTASK in HEK-293 cells and then performed the same experiments on IK(An) in Lymnaea. Once again an excellent correspondence was observed (compare Figs. 5 and

Figure 9. hTASK-1/hTASK-3 chimeras show the region between the cytoplasmic end of TM2 and the middle of TM3 is involved in determining anesthetic sensitivity. Each panel shows the effects of chloroform (data points and black lines) on a chimeric construct in which the N terminus is hTASK-1 and the C terminus is hTASK-3 with the junction at the indicated position. The gray lines show the activation of hTASK-3 (concentration-response curve from Fig. 3D) and the inhibition of TASK-1 (concentration-response curve from Fig. 3F) by chloroform for the purposes of comparison. A, the chimera hTASK-1_85hTASK-3_374 showed an anesthetic phenotype that was indistinguishable from hTASK-3. B, the chimera hTASK-1_121hTASK-3_374 also responded to chloroform in a similar manner to hTASK-1. C, the chimera hTASK-1_169hTASK-3_374 responded to chloroform in a similar manner to hTASK-1. D, the chimera hTASK-1_240hTASK-3_374 also displayed a hTASK-1 phenotype. These data imply that key anesthetic determinants lie within the region 132–169.

JULY 20, 2007• VOLUME 282 • NUMBER 29 • JOURNAL OF BIOLOGICAL CHEMISTRY 20987
For completeness, we also showed that both LyTASK and $I_{K(An)}$ in 	extit{Lymnaea} were insensitive to copper (see “Results”). In addition, we note that LyTASK in 	extit{Lymnaea} must be nearly closed under normal resting conditions, because, at acidic pH values that block the channel (see Fig. 6B), we observed only small changes in the baseline current (see inset to Fig. 6B). Either the closing of this small basal level of $I_{K(An)}$ is sufficient to modulate neuronal behavior, or the current can be opened by an endogenous activator. Although it is probable that LyTASK underlies $I_{K(An)}$ in 	extit{Lymnaea}, the importance of having cloned the channel lies in its relatively high sequence identity to human TASK channels yet its very different sensitivity to general anesthetics. The LyTASK current is 4–5 times more sensitive to inhalational anesthetics than its human equivalent and, more importantly in the context of this report, it displays a marked stereoselectivity to isoflurane. When two channels share a high sequence identity yet display very different phenotypes, the construction of chimeric channels is a powerful approach to determining which parts of the primary sequence are responsible for the phenotype in question. The C-terminal region of 2PK channels has been shown to be important in their modulation by a variety of different factors (8, 9, 20). Because this region of the channel is also the most variable in terms of primary sequence between the various members of the family, we began by deleting the C-terminal domain of LyTASK (i.e. the removal of amino acids 249–361) to determine its effect on anesthetic sensitivity and stereoselectivity. This deletion had no effect on either the sensitivity or the stereoselectivity of the channel (Fig. 8). Clearly this implies that the determinants of anesthetic sensitivity must lie within the core region of the channel (amino acids 1–248). A corollary to this experiment was to test the anesthetic sensitivity of a chimera, which consisted of the first 242 amino acids being those of hTASK-1 and with the C-terminal domain (243–361) being those of LyTASK. This had the lower anesthetic sensitivity and lack of stereoselectivity of the human channel (Fig. 8). This is again consistent with the core region of the channel determining anesthetic sensitivity.

A stretch of 6 amino acids near the start of the C-terminal domain (amino acids 243–248) has been shown to be critical for anesthetic activation (21). Mutations in this region were shown to not only reduce the activating effects of anesthetics but also reduce inhibition caused by the neurotransmitter thyroid releasing hormone. It was not possible to determine whether this region of the channel formed part of an anesthetic binding site or was necessary for transducing binding into gating (21). The stereoselective response of LyTASK to isoflurane allows us to distinguish between these two possibilities. When these six amino acids in LyTASK were mutated to be the same as those of hTASK-1, this construct showed a relatively low anesthetic sensitivity characteristic of the human channel, yet with the marked stereoselectivity that is characteristic of the 	extit{Lymnaea} channel (Fig. 8C). From this we conclude that these six critical amino acids are indeed important in determining the sensitivity of the channel to anesthetics but are unlikely to form part of an anesthetic binding site. Because of our observation that the human TASK channels differed qualitatively in their responses to chloroform (see Fig. 3, D and F), we adopted a parallel strategy in addition to constructing the hTASK-1/LyTASK chimeras discussed above. We constructed a series of hTASK-1/hTASK-3 chimeras and investigated their sensitivities to chloroform to further define amino acids which determine anesthetic activation. This approach showed that the region between the end of TM2 and the middle of TM3 must contain critical determinants of anesthetic sensitivity.
this amino acid in LyTASK to alanine, or making the equivalent mutation in the human channels (M159A), either greatly reduced, or completely eliminated anesthetic activation (Fig. 10).

One of our motivations behind cloning the potassium channel from *Lymnaea* was that we hoped to use its stereoselective responses to isoflurane as a means of distinguishing between amino acids that form part of an anesthetic binding site from those that are involved in transducing anesthetic binding to changes in channel gating. We therefore determined whether the mutation of Leu-159 to alanine in LyTASK, in addition to greatly reducing anesthetic activation, also disrupted the stereoselective response of the channel to isoflurane. We found that this was indeed the case with the mutation completely eliminating the stereoselectivity that is observed with the wild-type channel. The most parsimonious explanation is that amino acid 159 in TASK channels forms part of an anesthetic binding pocket and that subtle changes in the binding environment are sufficient to disrupt the stereoselectivity. The fact that this mutation alters the responses of the TASK channels, both quantitatively and qualitatively, to halothane, isoflurane, and chloroform, adds some further support for this interpretation. Nevertheless, it is conceivable that the mutation at 159 could lead to an allosteric change at a distant binding site.

An attractive experiment would be to introduce this mutation into animals to assess the importance of TASK channels to general anesthesia. As mentioned in the introduction, this approach really requires a mutation that is “silent,” in the sense that it only affects the response of the channel to anesthetics and has no effect on any other channel properties. The one property that characterizes, indeed defines, the TASK channels is their inhibition by acid pH. Our observation that the mutation of Leu-159 to alanine has no significant effect on the pH sensitivity (Fig. 11D) gives some reassurance that the mutation is indeed silent, although single-channel analysis is required before this can be confirmed, and before this mutation can be introduced into animals with some confidence that the interpretation might be straightforward.

In conclusion, we have cloned an anesthetic-activated potassium channel LyTASK from *L. stagnalis* and shown that it has the same biophysical properties as the anesthetic-activated current *IK*(an) (1, 2). The channel is closely related to human TASK two-pore domain channels and, using a strategy involving chimeric constructs and site-directed mutagenesis, we have identified a novel determinant of anesthetic sensitivity (amino acid 159). When this amino acid is mutated to alanine, anesthetic activation is greatly reduced and stereoselective responses to

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**FIGURE 11.** Mutation of LyTASK at amino acid position 159 disrupts isoflurane stereoselectivity but does not affect pH dependence. A, the anesthetic activation that remains in LyTASK(L159A) shows no significant stereoselectivity, in contrast to the wild-type channel. Time course of activation of LyTASK(L159A) by *S*(+)- and *R*(−)-isoflurane (0.46 mM). The activation of LyTASK(L159A) by the *S*(+) isomer is the same as that observed with the *R*(−)-isomer. B, summary data showing the similar effects of the racemate, the *S*(+)−, and the *R*− isomers in activating LyTASK(L159A). The stereoselectivity ratio (mean ± S.E.) was 0.90 ± 0.05 (n = 8). C, LyTASK(L159A) shows a very similar pH sensitivity to that of the wild-type channel. These data show the effects of changing the extracellular pH on the halothane-activated (0.53 mM halothane) current in HEK-293 cells transfected with LyTASK(L159A). D, pH dependence of the halothane-activated *K* current of LyTASK(L159A). The data points (means ± S.E.; from an average of five cells) are fitted to a Hill equation with a *pK* of 6.32 ± 0.03. The open circle represents the *pK* of the wild-type channel (data from Fig. 5B).
Anesthetics are eliminated. This suggests that this amino acid may form part of an anesthetic binding site.

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