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Two-Pore-Domain K^+ Channels Are a Novel Target for the Anesthetic Gases Xenon, Nitrous Oxide, and Cyclopropane

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ABSTRACT

Nitrous oxide, xenon, and cyclopropane are anesthetic gases that have a distinct pharmacological profile. Whereas the molecular basis for their anesthetic actions remains unclear, they behave very differently to most other general anesthetics in that they have little or no effect on $GABA_A$ receptors, yet strongly inhibit the *N*-methyl-D-aspartate subtype of glutamate receptors. Here we show that certain members of the two-pore-domain K^+ channel superfamily may represent an important new target for these gaseous anesthetics. TREK-1 is markedly activated by clinically relevant concentrations of nitrous oxide, xenon, and cyclopropane. In contrast, TASK-3, a member of this family that is very sensitive to volatile anesthetics, such as halothane, is insensitive to the anesthetic gases. We demonstrate that the C-terminal cytoplasmic domain is not an absolute requirement for the actions of the gases, although it clearly plays an important modulatory role. Finally, we show that Glu306, an amino acid that has previously been found to be important in the modulation of TREK-1 by arachidonic acid, membrane stretch and internal pH, is critical for the activating effects of the anesthetic gases.

Nitrous oxide, or "laughing gas," was one of the very first general anesthetics to have been used clinically, yet its molecular mode of action has puzzled pharmacologists ever since its introduction during the 1840s. The pharmacological profile of nitrous oxide is not dissimilar to that of the "inert" gas xenon, another inorganic anesthetic gas whose mechanism of action has also been the subject of much speculation. However, only relatively recently has a plausible molecular target been proposed (Franks et al., 1998; Jevtovic-Todorovic et al., 1998) for these simple gases—the NMDA subtype of the ionotropic glutamate receptor superfamily. The inhibition of the NMDA receptor by clinically relevant concentrations of nitrous oxide and xenon neatly accounts for some of the key pharmacological properties of these gases, notably the fact that they provide effective analgesia and that they cause a degree of excitement or euphoria. In addition, xenon and nitrous oxide have been shown to provide neuroprotection in a variety of in vivo and in vitro models (Jevtovic-Todorovic et al., 1998; Ma et al., 2002; Wilhelm et al., 2002). All of these are characteristic features of known NMDA receptor antagonists.

For the majority of general anesthetics, however, the most likely target is thought to be the $GABA_A$ receptor (Franks and Lieb, 1994). This inhibitory receptor channel is potentiated by virtually all anesthetics, although it has recently been shown that xenon is a marked exception to this rule, having little or no effect on $GABA_A$ receptors (Franks et al., 1998; de Sousa et al., 2000). Similarly, most (Jevtovic-Todorovic et al., 1998; Mennerick et al., 1998; Yamakura and Harris, 2000) but not all (Dzoljic and Van Duijn, 1998) reports show nitrous oxide to be relatively ineffective at potentiating $GABA_A$ receptors. Thus, these simple gases seem to belong to a specific class of general anesthetics that inhibit NMDA receptors but do not potentiate $GABA_A$ receptors. As a result of a recent study (Raines et al., 2001), other simple, apolar gaseous agents must also now be added to this class, including one that was used clinically for many years—cyclopropane.

The idea that these drugs act solely on the NMDA receptor to produce their myriad effects (loss of consciousness, analgesia, euphoria, neuroprotection, etc.) is open to doubt. This is partly because a number of other weak targets for these gases have been described previously (Yamakura et al., 2001) but also because supposedly selective NMDA receptor antagonists, such as ketamine, cause a distinct state of immobility

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; HEK, human embryonic kidney; MAC, minimum alveolar concentration.

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(sometimes referred to as "dissociative" anesthesia) that can be distinguished from the state of sedation that is induced by "conventional" general anesthetics.

In this article, we report a discovery that certain members of the two-pore-domain superfamily of background potassium channels can be activated by the anesthetic gases xenon, nitrous oxide, and cyclopropane. It has long been thought that the activation of potassium channels might be important in the actions of general anesthetics (Nicoll and Madison, 1982), and it was proposed that potassium channels selectively activated by volatile anesthetics may an important target (Franks and Lieb, 1988). These channels were first characterized (Franks and Lieb, 1988) in identified neurons of the pond snail *Lynmaea stagnalis*, and their mammalian counterparts subsequently cloned, and found to represent a special class of so-called two-pore-domain K^+ channels, because two pore-forming consensus regions could be identified in their primary sequences (Goldstein et al., 2001; Patel and Honore, 2001b; Lesage, 2003). Certain members of this superfamily (TREK and TASK) have been shown to be activated by volatile general anesthetics, with halothane being particularly effective (Patel and Honore, 2001a). Herein we show that the anesthetic gases xenon, nitrous oxide, and cyclopropane are as effective as halothane in activating TREK channels but have no effect at all on TASK channels. We also show that the cytoplasmic C terminus, which has been shown to be essential for the activating actions of halothane (Patel et al., 1999), plays a modulatory role in the actions of the anesthetic gases but is unlikely to contain primary binding sites. Finally, we identify Glu306 as an amino acid that is critical for the anesthetic activation. We conclude that the TREK family of two-pore-domain K^+ channels may represent an important target for the anesthetic actions of xenon, nitrous oxide, and cyclopropane.

Materials and Methods

Tissue Culture. Modified HEK-293 cells (tsA 201) were maintained in 5% CO_2/95% O_2 in a humidified incubator at 37°C in growth media [89% Dulbecco's modified Eagle's medium, 10% heat-inactivated fetal bovine serum, 1% penicillin (10,000 units/ml), and streptomycin (10 mg/ml)]. When the tsA cells were 80% confluent, they were split and plated for transfection onto glass coverslips coated with poly-D-lysine (1 mg/ml) to ensure good cell adhesion.

The tsA 201 cells were transiently transfected using the calcium phosphate method. One microgram of cDNA encoding a two-pore-K^+ channel subunit was added to each 35-mm well, and 1 μg of a plasmid encoding the cDNA of green fluorescent protein was included to identify cells expressing two-pore-domain channels. After a 24-h incubation period at 3% CO_2, the cells were rinsed with saline and fresh growth medium was added to the wells. The cells were incubated at 37°C with 5% CO_2 for 12 to 72 h before electrophysiological measurements were made. A number of experiments were performed with mock-transfected cells; these were treated identically, except that the cDNA encoding the two-pore-K^+ channel subunit was replaced by water.

A cell-line of HEK 293 cells stably-transfected with human TREK-1 (also known as KCNK2) was maintained in 5% CO_2 in a humidified incubator at 37°C in growth media [89.3% Dulbecco's modified Eagle's medium, 8.9% heat-inactivated fetal bovine serum, and 1.8% G-418 (20 mg/ml)]. When the cells were 80% confluent, they were split and plated onto glass coverslips coated with poly-D-lysine (1 mg/ml) and stored in an incubator at 5% CO_2. The cells were used after 4 to 5 days for electrophysiological measurements.

HEK 293 cells stably expressing human TREK-1 two-pore-channels, and human TASK-3 (also known as KCNK9) (Chapman et al., 2000) and TREK-1 (Meadows et al., 2000) two-pore-channel clones in the pcDNA 3.1 vector were kindly provided by Dr. Helen Meadows (GlaxoSmithKline, Uxbridge, Middlesex, UK).

Mutations and Truncations. To generate mutations and truncations, point mutations were introduced into the K^+ channel clones using the QuikChange kit (Stratagene, Amsterdam, The Netherlands). A pair of short (39–45 bases) complementary oligonucleotide primers, incorporating the intended mutation, was synthesized (MWG-Biotech, Ebersberg, Germany). To aid identification of successful mutants, a diagnostic restriction site was included in the primer sequence. Mutant DNA constructs were sequenced (MWG-Biotech) to confirm the introduction of the correct mutated bases.

Solution Preparation. Solutions for the anesthetic gas experiments were prepared by bubbling pure gases (oxygen, nitrogen, xenon, nitrous oxide, or cyclopropane) through fine sintered-glass bubblers in 250-ml Dreschel bottles filled with extracellular saline (see *Electrophysiology*). During bubbling, the solutions were continuously stirred at room temperature. The solutions were bubbled at a rate of 30 ml/min for 20 min. Gas chromatography measurements showed that gases would saturate a solution bubbled in this way within 10 min. Mixing nitrogen- and oxygen-saturated solutions in a 4:1 ratio formed a control saline solution with the partial pressures of the dissolved gases equivalent to 0.8 atm nitrogen and 0.2 atm oxygen. The anesthetic gas test solution was prepared in a similar manner and contained variable proportions of anesthetic gas (i.e., xenon, nitrous oxide, or cyclopropane) and nitrogen, with the balance being a fixed percentage of oxygen corresponding to 0.2 atm. Xenon (99.9%; research grade) was obtained from Air Products (Basingstoke, UK); cyclopropane was obtained from Linde (Unterschleißheim, Germany); and nitrous oxide, nitrogen, and oxygen were obtained from BOC (Guildford, UK). All other chemicals were obtained from Sigma (Poole, Dorset, UK). Solutions containing the volatile anesthetic halothane were prepared as volume fractions of a saturated aqueous solution. The concentration of a saturated aqueous solution of halothane was taken to be 17.5 mM (Raventós, 1956). Halothane concentrations in the gas phase (which are referred to in this article) were calculated using the Bunsen solubility coefficient as described previously (Franks and Lieb, 1993). Anesthetic solutions were prepared in sealed volumetric flasks and transferred immediately after mixing to glass syringe barrels containing a polypropylene float. Gas chromatography measurements showed that losses from such devices are negligible over periods as long as 12 h. Polytetrafluoroethylene tubing and nylon valves were used throughout to minimize nonspecific losses of anesthetic. Anesthetics were applied for >60 s to allow equilibrium to occur.

Anesthetic Concentrations. The most commonly used endpoint for inhalational anesthetics is the minimum alveolar concentration (MAC), which is the concentration required to prevent a purposeful response to a painful stimulus, such as a surgical incision. For the anesthetics we have used, these concentrations are (for humans) 0.75% for halothane (Steward et al., 1973), 71% for xenon (Cullen et al., 1969), 104% for nitrous oxide (Hornbein et al., 1982), and 9.2% for cyclopropane (Saidman et al., 1967). It should be noted, however, that these concentrations were determined at 37°C, and MAC values decrease with decreasing temperature. This effect can be quite significant for the larger volatile anesthetics (Franks and Lieb, 1996) but is much less for the smaller agents such as nitrous oxide (Antognini et al., 1994). In this article, we give all anesthetic concentrations as percentages of an atmosphere at our experimental temperature of about 22°C.

Electrophysiology. The composition of the control extracellular solution was 145 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM D-Glucose, 10 mM HEPES, titrated to pH 7.4 with NaOH. Glass microelectrodes were pulled from thick-walled borosilicate glass capillaries (GC150F-10; Harvard Apparatus, Edenbridge, Kent, UK) using a two-stage vertical puller (PP-830; Narishige,

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Tokyo, Japan). Fire-polished pipettes were back-filled with 0.2 μm-filtered intracellular solution (120 mM KCH_3SO_4, 4 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM HEPES, 3 mM MgATP, and 0.3 mM Na_2GTP, titrated to pH 7.2 with KOH; total K^+ concentration was 155 mM). For acidic internal solution, HEPES was substituted with 2-morpholinoethanesulfonic acid, pH was adjusted to 5.0 by KOH and K^+ was added to a total concentration of 155 mM using KCH_3SO_4. The pipette resistance ranged from 2 to 7 MΩ. Series resistance was compensated by 75 to 90%. Voltage-clamp recordings were made using either outside-out patches or the whole-cell recording technique. Cells and patches were usually held at -80 mV, and 750-ms voltage-ramps from -120 to 0 mV were recorded every 10 to 30 s. For continuous recordings, cells or patches were clamped at a fixed potential (-50 mV, unless otherwise stated). Anesthetic sensitivity was found to be identical whether assessed using voltage ramps or continuous recordings. Only whole-cell recordings with a positive holding current at -80 mV (indicating a negligible ohmic leak conductance) and an access resistance <10 MΩ were included in the analysis. Current-voltage curves from outside-out patches were corrected for an ohmic leak using the equation $I_{corr} = I_{obs} - I_{rev}V_m/V_{rev}$, where I_{corr} is the corrected current, I_{obs} is the observed current at a membrane potential V_m and I_{rev} is the current measured at the K^+ reversal potential. The percentage anesthetic activation was calculated relative to the average of controls taken just before and just after the anesthetic application.

Recordings were usually digitized at 10 kHz (2 kHz for continuous recordings) and the output of the patch-clamp amplifier (Axopatch 200B; Axon Instruments, Union City, CA) was usually filtered at 5 kHz using the 4-pole Bessel filter of the patch-clamp amplifier. Continuous recordings were digitally filtered at 100 Hz (8-pole Bessel filter, -3dB, Axon Instruments, Union City, CA), unless otherwise stated, before analysis using Clampfit software (Axon Instruments, Union City, CA). All recordings were stored on a computer hard disk.

For the measurements with $GABA_A$ receptors, HEK 293 (tsA 201) cells were transiently transfected with human cDNA coding for α1, β2, and γ2s subunits (kindly supplied by Neil Harrison, Cornell University, New York, NY). The coding sequences were subcloned into pcDNA3.1 (Invitrogen, Paisley, UK), a vector designed for high expression in mammalian cells. All other details were essentially as described above, except that the control extracellular solution was 145 mM NaCl, 3.0 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 5.5 mM D-Glucose, and 10 mM HEPES, titrated to pH 7.4 with NaOH; the intracellular solution was 140 mM CsCl, 1 mM $MgCl_2$, 11 mM EGTA, and 10 mM HEPES, titrated to pH 7.2 with CsOH. The data were digitized at 200 Hz, filtered at 50 Hz (8-pole Bessel filter, -3dB; Frequency Devices, Lyons Instruments, Hertfordshire, UK), and anesthetic potentiations were measured at -60 mV.

All electrophysiological measurements were carried out at room temperature (21–23°C). Values are given throughout as mean ± S.E.M. Error bars are not shown where they are smaller than the size of the symbols. Statistical significance was assessed using the Student's t test.

Results

Mock-transfected HEK-293 cells had resting potentials around -35 mV and passed only a few picoamperes or less when held at a membrane potential of -50 mV under voltage clamp. In contrast, cells expressing human TREK-1 channels had resting potentials around -85 mV and, under voltage clamp, passed large outwardly rectifying currents, consistent with many previous reports (Goldstein et al., 2001; Patel and Honore, 2001b; Lesage, 2003). Because the currents in the transfected cells were on the order of 1000 times larger than those of the untransfected cells, we considered all of the current measured under voltage clamp to be passed by the TREK-1 K^+ channels. This current increased markedly in the presence of clinically relevant concentrations of the inhalational general anesthetics halothane, xenon, nitrous oxide, and cyclopropane. Figure 1A shows a continuous current recording from a cell held under voltage clamp at -50 mV (our standard test potential), which illustrates the rapid and reversible activation of the current. In the presence of anesthetic concentrations near the MAC values (see *Materials and Methods*), halothane (0.6%), nitrous oxide (80%), xenon (80%), and cyclopropane (10%) increased the TREK-1 channel current by a very similar extent, between 28 and 35% (Fig. 1B). When tested with outside-out patches (Fig. 1B, two rightmost columns), halothane (0.6%) and xenon (80%) activated the current to a comparable degree, showing that the anesthetic activation does not require the intact whole cell. Figure 1, C–F, shows current-voltage curves in the presence of increasing concentrations of the four anesthetics. At concentrations of anesthetics close to MAC values, the anesthetic-activated current was reasonably well-fitted (see Fig. 2) by a Goldman-Hodgkin-Katz equation (Hille, 2001), but with a deviation that is consistent with a small degree of voltage gating at more positive potentials (Goldstein et al., 2001; Patel and Honore, 2001a). The percentage activation of the current was essentially constant with voltage (Fig. 2, insets).

Concentration-response curves for the four inhalational anesthetics are shown in Fig. 3. They are qualitatively similar in that the percentage activation monotonically increases with no hint of saturation. Quantitatively, however, the four agents differ greatly in their potencies; the concentrations required to achieve an arbitrary value of 25% activation were 0.72% for halothane, 80% for nitrous oxide, 60% for xenon, and 12% for cyclopropane, in line with their relative anesthetic potencies in humans (Saidman et al., 1967; Cullen et al., 1969; Steward et al., 1973; Hornbein et al., 1982).

In contrast to the TREK-1 K^+ channel currents, cells expressing TASK-3 channels behaved very differently in response to general anesthetics. Although TASK-3 channels still show a robust response to halothane, they were completely insensitive to the anesthetic gases nitrous oxide, xenon, and cyclopropane (Fig. 4, A–E). In fact, TASK-3 currents were significantly more sensitive to halothane than were TREK-1 currents, with 0.6% halothane increasing the current by 84 ± 17%. On the other hand, even the highest concentration (80%) of cyclopropane produced no significant effect on TASK-3 currents (Fig. 4E).

Because of the likely importance of the $GABA_A$ receptor in general anesthesia, we determined the sensitivity of this inhibitory receptor channel to the four inhalational agents. We used receptors containing the combination of subunits (α1β2γ2s) that are most commonly expressed in the central nervous system. The pattern of activation (Fig. 4F) was remarkably similar to that shown by TASK-3 channels (Fig. 4E), with a strong potentiation by halothane, but little or no response to xenon, nitrous oxide, and cyclopropane (even up to 80%).

Because of the reported importance of the C-terminal cytoplasmic domain in the activation of TREK-1 by volatile anesthetics (Patel et al., 1999), we investigated the requirement of this region of the channel for the activation by the anesthetic gases. We did this in two stages. We first looked at the sensitivity of a "semitruncated" channel in which the last 49 amino acids had been deleted by introducing a stop codon

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into the DNA base sequence at amino acid position 363. This channel expressed well and gave currents that were comparable with the wild-type channel (Fig. 5A). This construct TREK-1 Δ49 was still strongly activated by the inhalational anesthetics, although the degree of activation at MAC was between 53 and 79% of that seen with the wild-type channel (Fig. 5B). Whereas this reduction in sensitivity seems to be real, because it occurred for all the anesthetics, the difference was only statistically significant in the case of xenon ($P < 0.05$).

We went on to investigate a construct in which most of the cytoplasmic C terminus had been removed (although in the absence of a high-resolution structure, the definition of exactly where this domain begins is obviously uncertain). This involved introducing a STOP codon at position 323 that resulted in the deletion of the last 89 amino acids. When HEK 293 cells were transiently transfected with cDNA encoding this TREK-1 Δ89 construct, the outward current at −50 mV was very small (a few picoamperes) and comparable with that measured with mock-transfected cells. However, when 80% cyclopropane was applied, the transfected cells behaved differently from mock-transfected cells, and a clear anes-

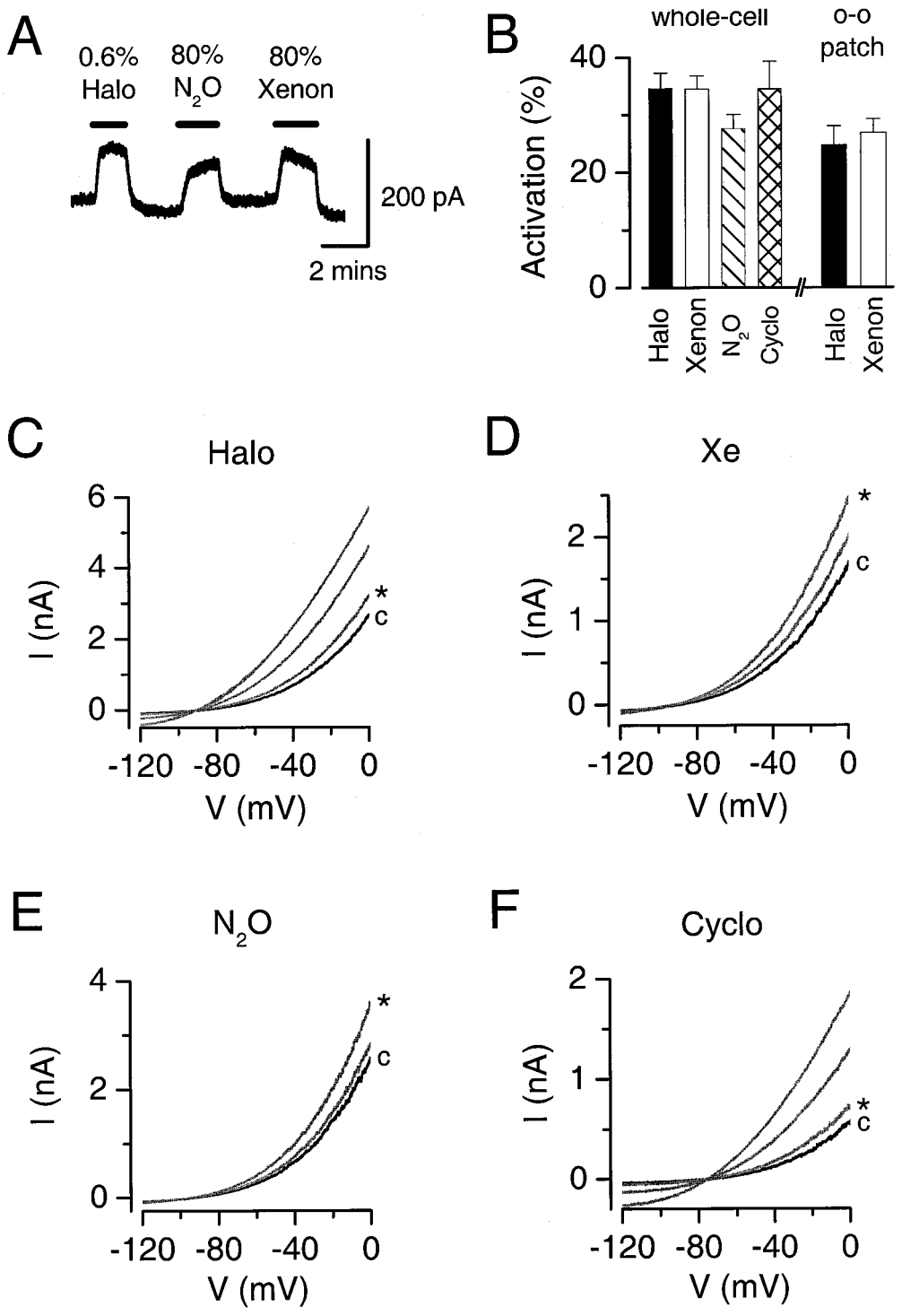

Fig. 1. Activation of human TREK-1 by halothane, xenon, nitrous oxide, and cyclopropane. A, halothane, xenon, and nitrous oxide activate human TREK-1 channels at clinically relevant concentrations. The activation has a fast onset, is maintained during the presence of the anesthetics, and is rapidly reversible. This example shows a continuous current recording for a cell voltage-clamped at −50 mV. B, summary of anesthetic effects on TREK-1 currents from whole-cell and outside-out patch recordings measured at −50 mV. Halothane (0.6%), xenon (80%), nitrous oxide (80%), and cyclopropane (10%) activate TREK-1 by 35 ± 3% (n = 28), 35 ± 2% (n = 21), 28 ± 2% (n = 25), and 35 ± 5% (n = 12), respectively. The columns on the right show the effect of 0.6% halothane (25 ± 3%; n = 5) and 80% xenon (27 ± 2%; n = 6) on TREK-1 in outside-out patches. C–F, recordings from voltage-ramps (−120 to 0 mV in 750 ms) showing TREK-1 activation by 0.6, 1.2, and 4.8% halothane, 40 and 80% xenon, 20 and 80% nitrous oxide, and 10, 40, and 80% cyclopropane. C, control traces; *, traces showing approximate MAC concentrations.

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thetic activation was observed (Fig. 6B). This anesthetic-activated current (Fig. 6B, inset) was very small in absolute terms but qualitatively similar to that observed with the wild-type receptor (Fig. 6A). This indicated that although only a small number of active TREK-1 Δ89 channels were present in the cells, they retained much of their sensitivity to cyclopropane. In subsequent experiments with outside-out patches, we found that, as previously reported (Maingret et al., 1999), lowering the internal pH greatly increased channel activity (Fig. 6C), although fluctuations in this basal activity made quantitative recordings difficult. This showed that the truncated channels were being expressed, but their opening probability at normal pH had been greatly reduced. When the channel activity was "rescued" by reducing the internal pH, we found that the TREK-1 Δ89 construct did indeed largely retain its sensitivity to the anesthetic gases (Fig. 6, C and D). Cyclopropane (80%) activated the current by 137 ± 40% (n = 10) and xenon (80%) activated the current by 16 ± 2% (n = 7). The extent of the activation, as with the semi-truncated construct, was approximately half that of the wild-type channel, indicating the C-terminal cytoplasmic domain must play some role in the modulation of the channel by anesthetic gases but could not contain the primary binding site(s).

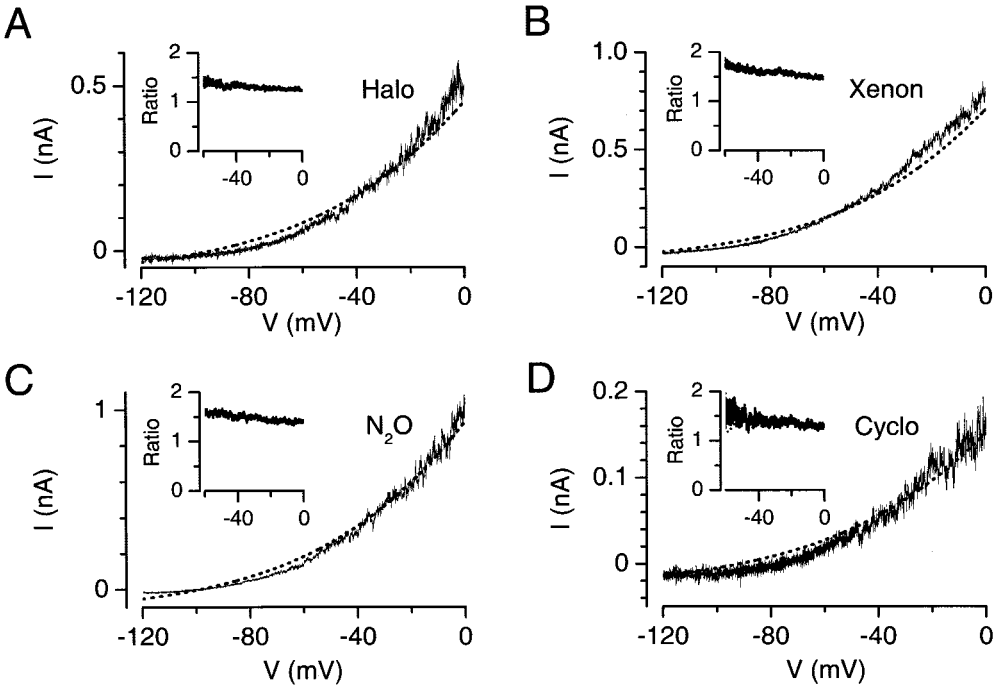

Fig. 2. Voltage-dependence of anesthetic-activated currents. Currents activated by halothane (0.6%) (A), xenon (80%) (B), nitrous oxide (80%) (C) and cyclopropane (10%) (D) at concentrations of anesthetics close to MAC values were well fitted by the Goldman-Hodgkin-Katz constant field equation (Hille, 2001) (dashed lines in figures). Each inset shows the ratio of the current in the presence of anesthetics to the control current; this ratio shows little or no voltage dependence.

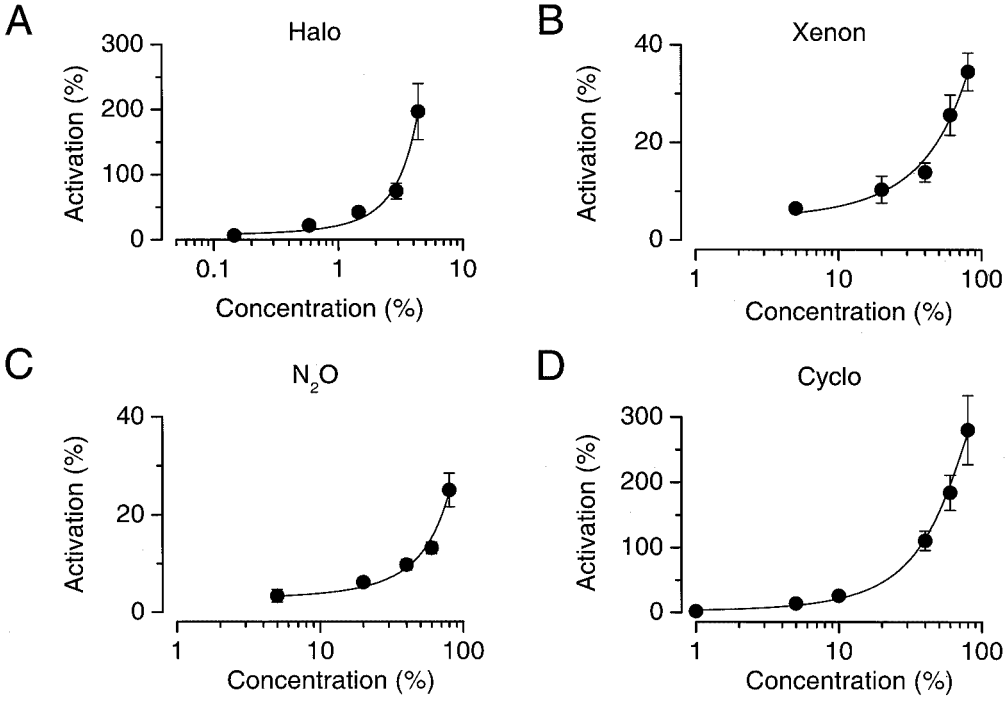

Fig. 3. Concentration-response curves for the activation of TREK-1 channels by halothane, xenon, nitrous oxide, and cyclopropane. Concentration-response curves for TREK-1 activation by halothane (A), xenon (B), nitrous oxide (C), and cyclopropane measured at −50 mV from ramps (−120 to 0 mV in 750 ms) (D). Data represent means ± S.E.M. from five to seven cells for halothane, seven to 14 cells for xenon, four to six cells for nitrous oxide, and four to seven cells for cyclopropane. The lines are drawn by eye and have no theoretical significance.

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To further explore the mechanism of anesthetic activation of the TREK-1 channels, we investigated the effect of a point mutation E306A that has been shown to eliminate the activation of TREK-1 by arachidonic acid, membrane stretch, and internal acidosis (Honore et al., 2002). We found that this mutation almost completely blocked the anesthetic activation of the channel (Fig. 7), with the activation being reduced by between 8- and 13-fold.

Discussion

General anesthetics are usually subdivided into intravenous and inhalational agents. Among the inhalational anes-

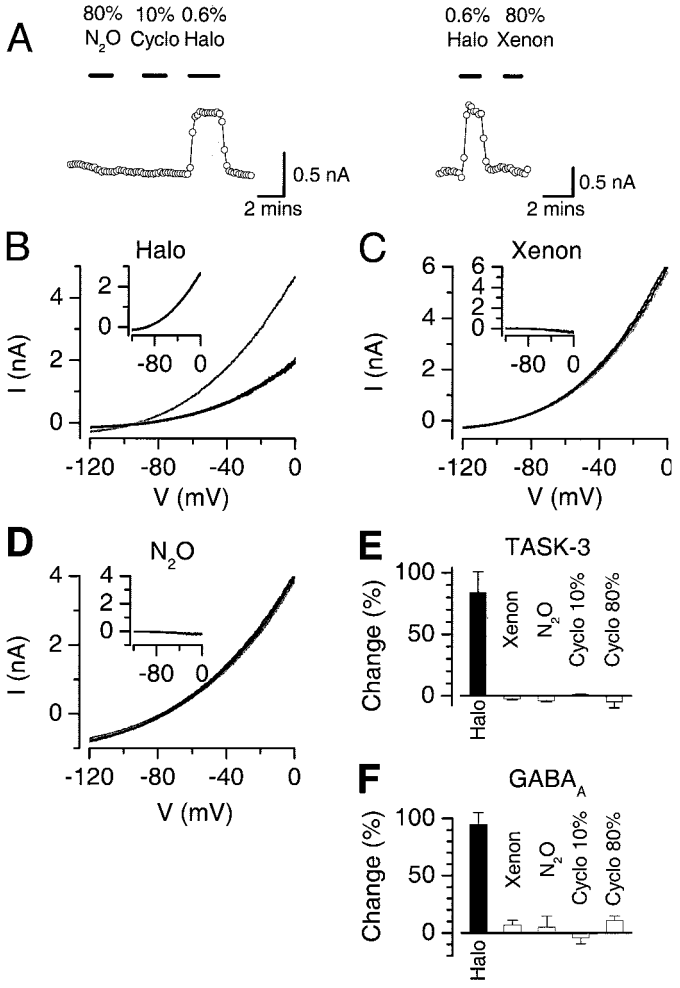

Fig. 4. Xenon, nitrous oxide, and cyclopropane do not affect human TASK-3 channels and human $\alpha1\beta2\gamma2s$ $GABA_A$ receptors. A, current recordings (at −50 mV from standard voltage-ramps) showing that 80% xenon, 80% nitrous oxide, and 10% cyclopropane have no effect on TASK-3 channels, in contrast to halothane, which causes a large activation. B–D, recordings from voltage-ramps (−120 to 0 mV; 750 ms) show the activation by 0.6% halothane and no significant effects for 80% xenon and 80% nitrous oxide. The insets show the difference between currents in anesthetic and control solution. E, summary of anesthetic effects on TASK-3 channel currents measured at −50 mV. Halothane (0.6%) activates TASK-3 channels by 84 ± 17% (n = 15) whereas xenon (80%), nitrous oxide (80%), and cyclopropane (10 and 80%) induced only small changes of −2.6 ± 0.6% (n = 9), −4.2 ± 0.6% (n = 9), 0.7 ± 0.8% (n = 10), and −5.0 ± 5.0% (n = 15), respectively. F, summary of anesthetic effects on $\alpha1\beta2\gamma2s$ $GABA_A$ receptors. Halothane (0.6%) potentiates currents evoked by 10 μM GABA by 95 ± 11% (n = 5), whereas xenon (80%), nitrous oxide (80%), and cyclopropane (10 and 80%) induced only small changes of 7 ± 4% (n = 19), 5 ± 10% (n = 5), −4 ± 5% (n = 5), and 11 ± 4% (n = 5), respectively.

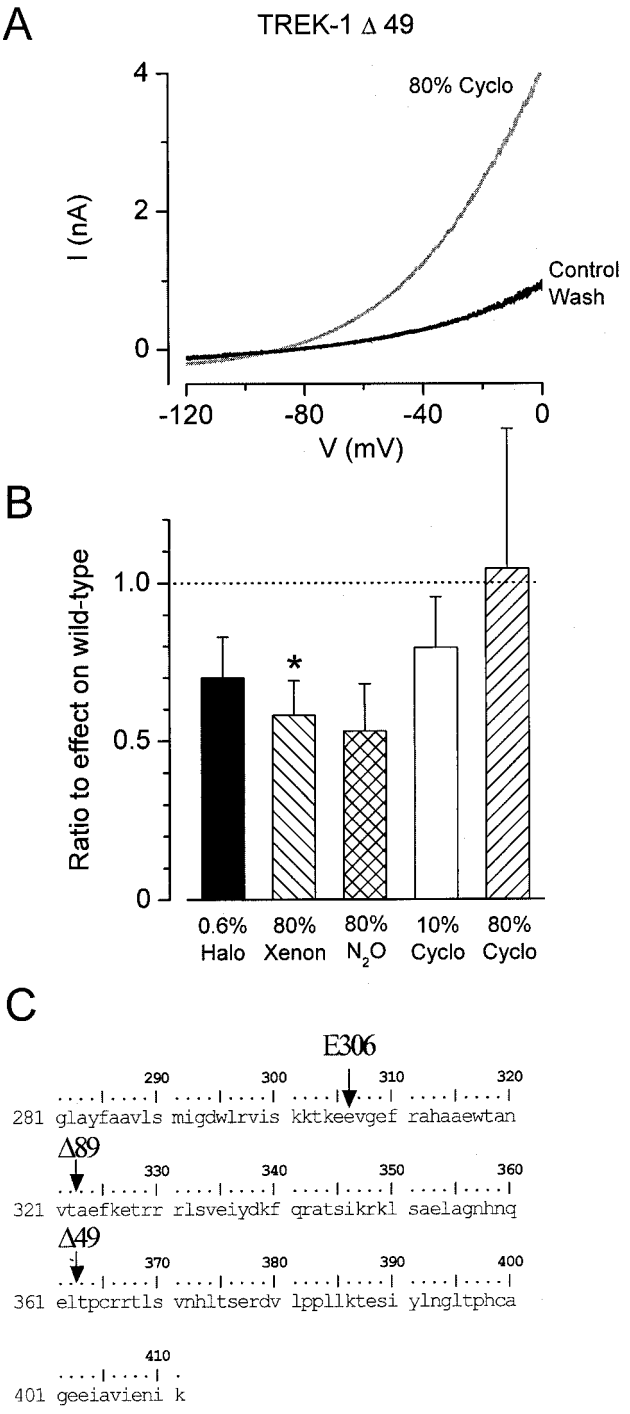

Fig. 5. The effects of a partial truncation of the carboxyl terminus of TREK-1 channels on anesthetic action. The activating effect of anesthetics is somewhat reduced when the last 49 amino acids of the carboxyl terminus are deleted (TREK-1 Δ49). A, original recordings showing the activation of TREK-1 Δ49 by 80% cyclopropane. B, summary showing the effects of the different anesthetics on TREK-1 Δ49 relative to wild-type TREK-1 controls, measured at −50 mV from standard voltage-ramps. The activations are expressed as a ratio of that seen with the wild-type channel, and were 0.70 ± 0.13 (n = 10) for halothane, 0.58 ± 0.11 (n = 8) for xenon, 0.53 ± 0.15 (n = 7) for nitrous oxide, 0.79 ± 0.16 (n = 8) for 10% cyclopropane, and 1.04 ± 0.44 (n = 12) for 80% cyclopropane. Only in the case of xenon was the activation of the TREK-1 Δ49 construct significantly less than that of the wild-type ($P < 0.05$). C, the amino acid sequence of the TREK-1 C terminus (the terminal cytoplasmic domain is thought to begin at approximately 295). The truncation positions for the two truncated channels Δ49 and Δ89 and the position of the point mutation Glu306 are indicated by arrows.

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thetics, however, a further subdivision can usefully be made between the volatile agents (such as halothane and isoflurane) that are liquids at the standard temperature and pressure (20°C and 1 atm.) and those that are gases (such as xenon and nitrous oxide).[2] This subdivision is useful because of the apparently different pharmacological profiles and molecular mechanisms involved. The anesthetic gases nitrous oxide and xenon are notable for their potent analgesic action, their minimal effects on muscle tone and contractibility, as well as their ability to cause euphoria, properties that are not generally associated with the volatile liquid anesthetics. In addition, over the last few years, clear differences have emerged from studies on ion channels between those inhalational anesthetics (such as halothane and isoflurane) that cause significant potentiations of the $GABA_A$ receptor (Franks and Lieb, 1994) and those (the anesthetic gases) that have little or no effect (Mennerick et al., 1998; de Sousa et al., 2000; Raines et al., 2001). Another difference seems to be the fact that the gases are more effective than the volatiles at inhibiting the NMDA receptor subtype of the glutamate receptor when their effects are compared at equianesthetic concentrations (e.g., at MAC), and, presumably as a direct consequence of their ability to inhibit NMDA receptors, the gases show greater potencies as neuroprotectants (Jevtovic-Todorovic et al., 1998; Ma et al., 2002; Wilhelm et al., 2002).

Except for the NMDA receptor, no other plausible target has been described that is likely to be important in the anesthetizing actions of xenon and nitrous oxide (Yamakura et al., 2001). The results presented here show that certain members of the two-pore-domain potassium channel superfamily may represent important new targets for anesthetic gases. This class of channels is responsible for the background, or leak, potassium currents that play an important role in modulating neuronal excitability (Goldstein et al., 2001; Patel and Honore, 2001b; Lesage, 2003). In humans, the superfamily has 15 members that have a complex and varied anatomical distribution and differ largely in the ways in which they can be modulated and regulated. They can be affected by changes in internal and external pH, membrane stretch, temperature, arachidonic acid, as well as by a variety of internal second messenger systems. Of particular relevance to the present discussion is the fact that some members have been shown to be activated by volatile general anesthetics (Gray et al., 1998; Franks and Lieb, 1999; Patel et al., 1999; Patel and Honore, 2001a). This has been shown for both TASK and TREK members of the superfamily, although most work in this area has focused on the TASK channels, TASK-1 and TASK-3. TASK channels have been shown to be activated by halothane, isoflurane, and other volatiles in recombinant expression systems (Patel et al.,

[2] Some gases below their critical temperatures are referred to as "vapors" because they can be condensed into liquids at high enough pressures, although this distinction is not important here.

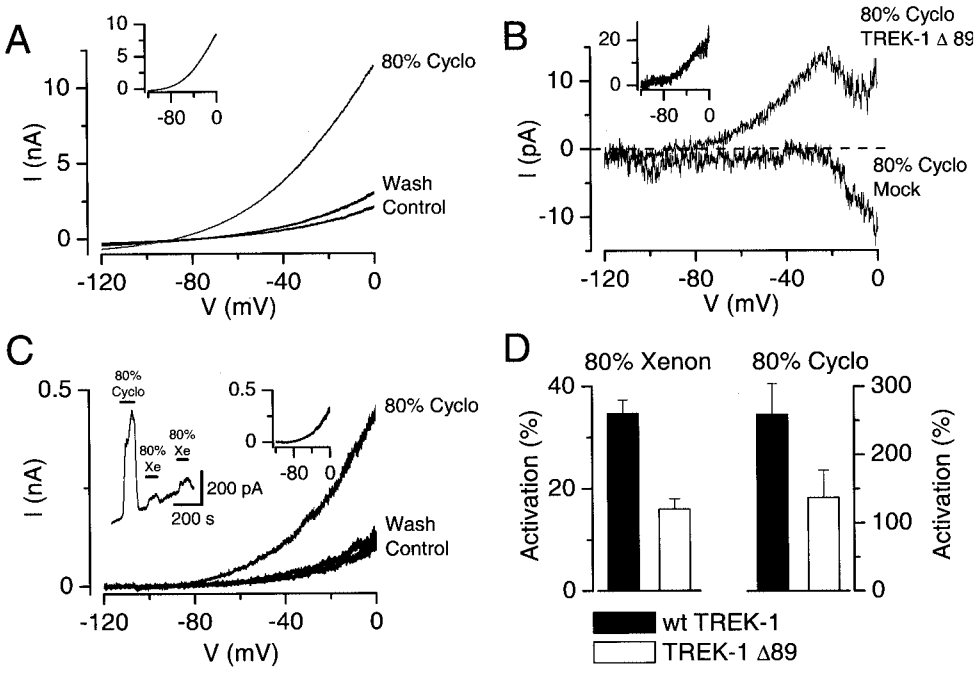

Fig. 6. Anesthetic gases still activate TREK-1 channels when most of the cytoplasmic carboxyl terminus is removed. Anesthetic gases cyclopropane and xenon activate a truncated TREK-1 channel in which 89 amino acids have been removed at the carboxyl terminus (TREK-1 Δ89). A, current-voltage curve for wild-type TREK-1 showing the large activating effect of 80% cyclopropane. The inset shows the difference current between 80% cyclopropane and control. B, effects of 80% cyclopropane on TREK Δ89 and mock-transfected tsA-cells. Because of the very small currents observed with this truncated channel (see text for details), data from several whole-cell experiments (n = 11 for TREK Δ89, n = 4 for mock-transfected tsA-cells) were averaged. The inset shows the difference between the mean currents and shows the activation of a current that obeys the Goldman-Hodgkin-Katz equation. C, the effect of 80% cyclopropane on TREK Δ89 in an outside-out patch. Cyclopropane caused a large activation (150 ± 31%, n = 10, measured from standard voltage-ramps at −50 mV). The pH of the internal solution was lowered to 5.0 to stimulate the basal current (Maingret et al., 1999). Right inset, difference between 80% cyclopropane and control; left inset, continuous current recording (+40 mV; filtered at 1 Hz) showing the activation of 80% cyclopropane and 80% xenon on TREK Δ89 in an outside-out patch. D, the percentage activation of TREK-1 currents by 80% xenon (16 ± 2% activation; n = 7) and 80% cyclopropane (137 ± 40% activation; n = 7) are significantly reduced when most of the cytoplasmic C terminus is removed by truncation, indicating that this domain plays a role in modulating the anesthetic effect. The TREK Δ89 data were obtained using outside-out patches with an internal pH of 5.0.

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1999; Talley and Bayliss, 2002), and TASK channels have been shown to underlie the principal effects of volatile anesthetics (specifically halothane) on rat somatic motoneurons and locus ceruleus cells (Sirois et al., 2000) and on thalamocortical neurons (Ries and Puil, 1999). Thus, a good case can be made for TASK channels playing some role in the actions of volatile agents, particularly halothane.

Less work has been done with anesthetics and TREK channels, although both TREK-1 (Patel et al., 1999) and TREK-2 (also known as KCNK10) (Lesage et al., 2000) channels have been shown to be sensitive to volatile agents. Although the distribution of TREK channels in the central nervous system is known (Talley et al., 2001) and has been shown to be rather different from that of TASK channels, in contrast to TASK channels (Millar et al., 2000), no specific neuronal populations have been identified in which TREK channels predominate. A great deal is known, however, about how TREK channels can be modulated. In addition to being sensitive to volatile anesthetics, TREK channels are mechanosensitive, are activated by unsaturated fatty acids and the neuroprotective drug riluzole, can be strongly modulated by internal acidosis, and can be regulated by G-protein-mediated neurotransmitters (Goldstein et al., 2001; Patel and Honore, 2001b; Lesage, 2003). Our finding (Fig. 1) that TREK channels can be activated by anesthetic gases shows that they may play a role in the anesthetic actions of this particular group of agents. In cells in which TREK channels are capable of modulating the membrane potential, the 30 to 40% activation of these K^+ channels that we observe (Fig. 1B) at MAC concentrations could obviously result in physiologically important changes in neuronal excitability, provided the channel is expressed at sufficiently high levels. However, the relevance of channel activation to the induction of general anesthesia will also depend upon the anatomical distribution of the channel in the central nervous system and how these anatomical sites are related to the neuronal pathways involved in the production of the anesthetic state. TREK channels are expressed throughout the central nervous system with a complex distribution that may be species dependent (Meadows et al., 2000; Talley et al., 2001) but with levels of expression in many regions that could be relevant for general anesthesia (e.g., thalamus and spinal cord). The fact that the anesthetic gases, in contrast to the volatile agents, have no measurable effect on TASK-3 channels or $GABA_A$ receptors (Fig. 4) may be part of the reason these two groups of inhalational anesthetics have such different pharmacological profiles. Information on the likely relevance of two-pore-domain channels to general anesthesia may come from genetically modified animals in which certain members of the superfamily are either modified or excluded. A recent study (Gerstin et al., 2003) on such a knock-out animal has used this approach and concluded that TASK-2 (KCNK5) channels are unlikely to be important for general anesthesia. Because the anatomical distribution and anesthetic sensitivities of two-pore-domain channels vary so greatly, the lack of importance of one member of the family cannot easily be extrapolated to other members, and the role of the channels we have studied (TREK-1 and TASK-3) in general anesthesia remains unknown.

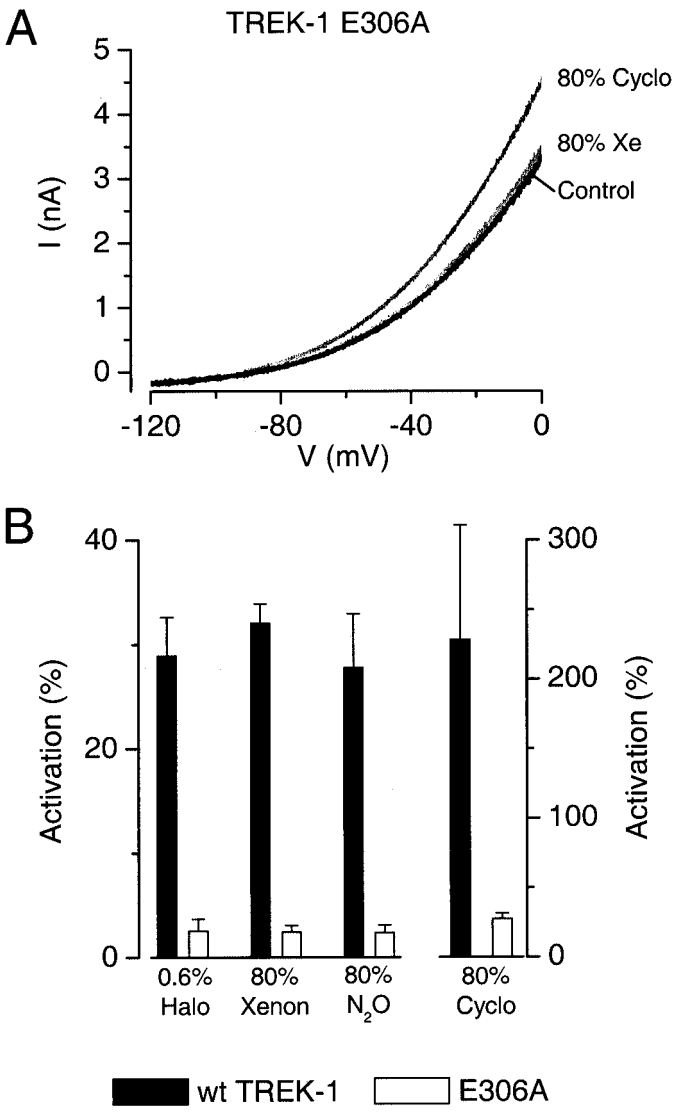

Fig. 7. Anesthetic activation of TREK-1 channels requires glutamic acid Glu306. The activating effect of anesthetics is greatly reduced in a mutated channel in which the glutamic acid at position 306 is replaced by an alanine (TREK-1 E306A). A, current-voltage curves showing that xenon (80%) has a negligible effect on TREK-1 E306A and that the effect of a high concentration of cyclopropane (80%) is greatly reduced compared with the wild-type channel (compare Figure 1F). B, summary showing the effects of different anesthetics on wild-type TREK-1 and TREK-1 E306A measured at −50 mV from standard voltage-ramps. Activation of TREK-1 E306A is reduced between 8- and 13-fold compared with wild-type to 2.5 ± 1.2% (n = 7) for halothane, 2.4 ± 0.6% (n = 7) for xenon, 2.4 ± 0.7% (n = 7) for nitrous oxide, and 27.0 ± 3.0% (n = 11) for 80% cyclopropane. Note the different ordinate scale for the data at 80% cyclopropane.

Interestingly, the neuroprotective agent riluzole can also activate TREK-1 channels (Duprat et al., 2000), although its actions are complex and biphasic, with the activation turning to inhibition during prolonged exposure. Nitrous oxide and xenon have also been shown to have neuroprotective properties; their main actions have been attributed to their ability to inhibit NMDA receptors (Mennerick et al., 1998; Ma et al., 2002; Wilhelm et al., 2002). It is possible that TREK activation by these anesthetic gases provides an additional neuroprotective pathway.

The concentration-response curves of Fig. 3 for anesthetics activating TREK-1 channels imply relatively weak binding because there is no evidence of saturation over the range of concentrations studied. The location of these binding sites, however, is uncertain. Previous work with TREK (Patel et

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al., 1999) and TASK (Talley and Bayliss, 2002) channels has shown that the C terminus is critical to the activation of these channels by halothane, and the progressive deletion of the C terminus results in a gradual reduction in anesthetic sensitivity. Whether this occurs because of the disruption of the halothane binding site(s) or because of an effect on channel gating is, at this stage, impossible to say. In the case of TREK-1, the deletion of most of the cytoplasmic C terminus (with the last amino acid of the construct being Thr322) resulted in the channel's almost complete insensitivity to halothane and chloroform (Patel et al., 1999). With the anesthetic gases, the semitruncated construct TREK-1 Δ49 had a somewhat reduced anesthetic sensitivity (Fig. 5). When most of the cytoplasmic C terminus was removed (TREK-1 Δ89 construct), the very low level of basal activity in the intact cells (Fig. 6B) showed the crucial role this domain plays in modulating the activity of the channel. However, it was clear that cyclopropane was still having a sizeable effect that is, in percentage terms, roughly comparable with that seen with the wild-type channel (Fig. 6A). This was clear in outside-out patches in which the basal activity of the TREK-1 channels had been enhanced by reducing the intracellular pH to 5.0. Under these conditions, cyclopropane and xenon activated the TREK-1 channels to an extent comparable to that seen with the semitruncated construct TREK-1 Δ49 and was approximately 50% of that found with the wild-type channel (Fig. 6, C and D). Thus it seems that whereas the C terminus is clearly involved in modulating the anesthetic sensitivity of the channel, the principal binding sites must lie elsewhere.

We also investigated the possible role of an amino acid (Glu306) that has been shown to be important in modulation of TREK-1 channels. It has previously been shown that mutating this glutamic acid to an alanine results in TREK-1 channels that are insensitive to activation by arachidonic acid, membrane stretch, and internal pH (Honore et al., 2002). The data in Fig. 7 show that this amino acid is also critical in regulating the sensitivity of the channel to inhalational anesthetics. The mutation of E306A resulted in an almost complete ablation of anesthetic effects. Because most if not all of the factors that modulate TREK-1 seem to be critically dependent on Glu306, this implies that activation of one pathway might influence the modulation of another pathway. Thus, neurons expressing TREK-1 channels could have a varied response to inhalational anesthetics, depending upon the channel state.

In summary, we have shown that the gaseous general anesthetics xenon, nitrous oxide, and cyclopropane can activate TREK-1 channels. The substantial activation of the channels over a clinically relevant range of concentrations suggests that TREK channels may play a significant role in the production and maintenance of the general anesthetic state and the neuroprotective properties of anesthetic gases.

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