Modulation of TASK-1 (Kcnk3) and TASK-3 (Kcnk9) Potassium Channels

VOLATILE ANESTHETICS AND NEUROTRANSMITTERS SHARE A MOLECULAR SITE OF ACTION*

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TASK-1 and TASK-3, members of the two-pore-domain channel family, are widely expressed leak potassium channels responsible for maintenance of cell membrane potential and input resistance. They are sites of action for a variety of modulatory agents, including volatile anesthetics and neurotransmitters/hormones, the latter acting via mechanisms that have remained elusive. To clarify these mechanisms, we generated mutant channels and found that alterations disrupting anesthetic (halothane) activation of these channels also disrupted transmitter (thyrotropin-releasing hormone, TRH) inhibition and did so to a similar degree. For both TASK-1 and TASK-3, mutations (substitutions with corresponding residues from TREK-1) in a six-residue sequence at the beginning of the cytoplasmic C terminus virtually abolished both anesthetic activation and transmitter inhibition. The only sequence motif identified with a classical signaling mechanism in this region is a potential phosphorylation site; however, mutation of this site failed to disrupt modulation. TASK-1 and TASK-3 differed insofar as a large portion of the C terminus was necessary for the full effects of halothane and TRH on TASK-3 but not on TASK-1. Finally, tandem-linked TASK-1/TASK-3 heterodimeric channels were fully modulated by anesthetic and transmitter, and introduction of the identified mutations either into the TASK-1 or the TASK-3 portion of the channel was sufficient to disrupt both effects. Thus, both anesthetic activation and transmitter inhibition of these channels require a region at the interface between the final transmembrane domain and the cytoplasmic C terminus that has not been associated previously with receptor signal transduction. Our results also indicate a close molecular relationship between these two forms of modulation, one endogenous and the other clinically applied.

TASK1 channels are members of the two-pore-domain family of potassium channels, whose presumptive structure consists of two pore-forming regions flanked by four membrane-spanning domains (1, 2). Their name (an acronym for “TWIK-related acid sensitive K+ channels”) is derived from their structural similarity to the first mammalian member of the two-pore domain family to be characterized (TWIK-1), and from the fact that the activity of these channels is sensitive to changes in extracellular pH in the physiological range (3). Like other two-pore domain family members, these channels show little time or voltage dependence. Thus they have characteristics of leak K+ channels, generating background currents that contribute to membrane potential and the shaping of cell excitability.

The first TASK channel to be cloned, TASK-1 (designated KCNK3 by the Human Genome Organization), has been established as a site of modulation by a variety of agents, including hydrogen ions, oxygen, volatile anesthetics, and hormones/neurotransmitters (4). This modulation appears to be important in a number of physiological contexts, including the regulation of breathing (5, 6), control of aldosterone secretion (7), and transmitter and anesthetic regulation of neuronal activity (8–10).

In addition to TASK-1, four other two-pore domain channel genes have also been given the name TASK; two of these (TASK-2 (KCNK5) and TASK-4/TALK-2 (KCNK17)) are only distantly related to TASK-1 (11–13), and one (TASK-5 (KCNK15)) has not produced functional channels in heterologous expression systems (14–17). The fourth, TASK-3 (KCNK9), is >50% identical to TASK-1 at the amino acid level, and in whole-cell recordings the two channels have similar physiological properties but different pH sensitivities (3, 18–21). They are co-expressed in a number of different neuronal populations (16, 22, 23) and can form heterodimers when expressed in Xenopus oocytes (24).

For both TASK-1 and TASK-3, a molecular basis has been established for modulation by extracellular protons, as pH sensitivity is essentially abolished by replacing a histidine residue adjacent to the presumed selectivity filter region of the ion-conducting pore (18, 21, 25). Progress also has been made in identifying channel regions required for the actions of volatile anesthetics on human TASK-1 (26) (see below). However, the mechanisms underlying effects of neurotransmitters on these channels are poorly understood. For TASK-1 (and recently TASK-3) (24), inhibition via G protein-coupled receptors has been demonstrated for the cloned channels and for channels with similar properties in native systems (7, 8, 10). However, experiments have failed to implicate downstream effectors commonly associated with these receptors (3, 19, 27, 28). Here, we report the results of experiments in which we used mutagenesis of TASK-1 and its homologue TASK-3 to investigate the molecular bases for neurotransmitter inhibition of these channels in HEK 293 cells stably expressing the TRH-R1 receptor. We found that mutations disrupting anesthetic activation of these channels also disrupt transmitter inhibition; both of these effects require a region of the channel that does not contain motifs suggestive of a traditional signaling pathway.
EXPERIMENTAL PROCEDURES

cDNA encoding rat TASK-1 (GenBank™ accession number AF031384) and rat TASK-3 (GenBank™ accession number AF391084) were obtained from Andrew Gray (University of California, San Francisco) (27) and Donghee Kim (the Chicago Medical School) (18). Both cDNAs were subcloned into a mammalian expression vector (pcDNA3, Invitrogen). Portions of mouse TREK-1 (29) (GenBank™ accession number U73488.2), obtained by PCR from mouse cerebellar RNA (22) and mouse TRAAK (30) (GenBank™ accession number AF056492, obtained from Andrew Gray) were used to make chimeric constructs. Site-specific mutations and chimeras were generated by PCR using the overlap-extension method (31). A TASK-1/TASK-3 tandem dimer was also produced using PCR. The resulting channel contains a three-residue (glycine-serine-alanine) linker region, placed after the C-terminal amino acid of TASK-1 and fused to the start methionine of TASK-3. Mutations were introduced into this dimeric construct by ligation of fragments excised from cDNAs coding for the relevant monomeric mutant channels.

For both TASK-1 and TASK-3, a hemagglutinin epitope tag was added to the wild-type channel and to a number of different mutant constructs. These included the four TASK/TREK chimeric channels (i.e. TASK-1 and TASK-3 containing either a portion of the TREK-1 C-terminus or six residues substituted from TREK-1, see “Results”). The sequence encoding the epitope was excised from pKH3 (32) (obtained from Ian Macara, University of Virginia). Each construct was modified by PCR to allow for insertion of the epitope upstream of the start methionine, with an intervening three-residue (glycine-serine-alanine) linker. For all six of these epitope-tagged constructs, the responses to halothane and TRH were not different from the responses of constructs without the epitope, and therefore the data were pooled. All subsequent mutations, including the point mutations for TASK-1 and C-terminal deletions for TASK-3, were made in the context of the epitope-tagged channels. Plasmids were prepared by column elution (Endo-Free Plasmid Maxi Kit, Qiagen, Inc.) and further purified by phenol/chloroform extraction. All constructs were fully verified by sequence analysis.

HEK 293 cells stably expressing the TRH-R1 receptor (E2 cells (33), obtained from Graeme Milligan) were grown under standard conditions with 10% fetal bovine serum. HEK 293 cells stably expressing the TRH-R1 receptor were transfected with rat TASK-1 (A) or TASK-3 (B) and recorded by whole-cell voltage clamp. Cells were held at −60 mV and subjected to voltage ramps (0.2 V/s) at 5-s intervals. Conductance (calculated using linear fits to currents obtained between −130 and −60 mV) was plotted over time to reveal changes in response to variations in pH (7.3, 5.9, and 8.4), as well as to applications of halothane (0.3 mM) and TRH (0.2 μM). Insets show current traces from the indicated time points. TASK-1 and TASK-3 channels were inhibited at acidified pH (pH 5.9) and activated at alkalized pH (pH 8.4, less the conductance obtained when the channels were fully inhibited, pH 5.9). Data are presented as means ± S.E. Statistical analyses of anesthetic and transmitter effects on wild-type and mutant channels were performed by one-way analysis of variance (ANOVA) using a statistics software package (SigmaStat, Jandel Scientific). Pairwise comparisons were made using the Student-Newman-Keuls method, with p < 0.05 as criteria for acceptance as statistically significant. Linear regression analysis was performed using the same software.

TRH (American Peptide) was used at 0.2 μM. Halothane was bubbled into the perfusate via a vaporizer (Ohmeda, Austell, GA) using a room air/gas mixture (21% O2/balance N2). Aqueous concentrations were determined by gas chromatographic analysis of solutions taken from the recording chamber (34).

RESULTS

TASK Channels: Regulation by Extracellular pH, Volatile Anesthetics, and Neurotransmitters—TASK-1 and TASK-3 are subject to modulation by multiple agents; examples of this modulation are shown in Fig. 1. HEK 293 cells stably expressing the TRH-R1 receptor were transiently transfected with rat TASK-1 or TASK-3 and tested for responsiveness to changes in pH, as well as to applications of the volatile anesthetic halothane and the neuropeptide TRH. Cells were subjected to vol-

FIG. 1. Opposing regulation of pH-sensitive K+ currents by halothane and TRH in cells expressing TASK channels. HEK 293 cells stably expressing the TRH-R1 receptor (E2 cells (33), obtained from Graeme Milligan) were grown under standard conditions with 10% fetal bovine serum. HEK 293 cells stably expressing the TRH-R1 receptor were transfected with rat TASK-1 (A) or TASK-3 (B) and recorded by whole-cell voltage clamp. Cells were held at −60 mV and subjected to voltage ramps (0.2 V/s) at 5-s intervals. Conductance (calculated using linear fits to currents obtained between −130 and −60 mV) was plotted over time to reveal changes in response to variations in pH (7.3, 5.9, and 8.4), as well as to applications of halothane (0.3 mM) and TRH (0.2 μM). Insets show current traces from the indicated time points. TASK-1 and TASK-3 channels were inhibited at acidified pH (pH 5.9) and activated at alkalized pH (pH 8.4, less the conductance obtained when the channels were fully inhibited, pH 5.9). Data are presented as means ± S.E. Statistical analyses of anesthetic and transmitter effects on wild-type and mutant channels were performed by one-way analysis of variance (ANOVA) using a statistics software package (SigmaStat, Jandel Scientific). Pairwise comparisons were made using the Student-Newman-Keuls method, with p < 0.05 as criteria for acceptance as statistically significant. Linear regression analysis was performed using the same software.

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age ramps at 5-s intervals (see “Experimental Procedures”) to assess changes in whole-cell conductance. As expected, decreasing extracellular pH from 7.3 to 5.9 resulted in a complete inhibition of both TASK-1 (Fig. 1A) and TASK-3 (Fig. 1B). Switching to an alkalized extracellular solution (pH 8.4) restores the currents. In the case of TASK-1, alkalization induced an increase in conductance relative to physiological pH (pH 7.3), whereas for TASK-3 there was little change. These distinct responses to bath alkalization reflect a difference in the pH sensitivities of the two channels (pK \(-7.2\)–7.5 for TASK-1 versus pK \(-6.0\)–6.7 for TASK-3) (3, 10, 18–21).

After bath pH was raised, a clinically relevant dose of halothane (0.3 mM) activated the channels further. For TASK-1 the activation represented an \(\sim 50\%\) increase over the pH-sensitive conductance (52.5 \(\pm\) 3.6%; \(n = 5\)), with an even larger activation of TASK-3 (134.6 \(\pm\) 20.0%; \(n = 6\)). This value for rat TASK-1 is consistent with data for the human channel (26), but the rat TASK-3 response was somewhat greater than that reported for the corresponding human orthologue (20). For both channels, application of TRH in the continued presence of halothane resulted in a robust inhibition of the total (i.e. halothane- and pH-sensitive) current (TASK-1, 94.2 \(\pm\) 1.5\% inhibition, \(n = 6\); TASK-3, 86.4 \(\pm\) 1.6\% inhibition, \(n = 3\)). TASK channel inhibition by TRH is consistent with our previous work (10) and with results showing inhibition of these channels by a number of other G\(_\alpha\)q/11-coupled receptors (8, 24, 28).

**Removal of the C Terminus of TASK-1 Fails to Disrupt Modulation by Halothane and TRH**—To identify regions of the channel necessary for receptor-mediated inhibition of TASK-1, intracellular domains of the channel were targeted for mutation. The presumed intracellular domains of TASK-1 include a minimal (seven-residue) N terminus, a 30–40-residue linker between transmembrane domains two and three (the M2-M3 linker), and a C terminus of \(\sim 160\) residues. The first domain targeted for mutation was the C terminus, which was removed by introducing a stop codon after the fourth transmembrane domain (after residue 248). This mutation left a core channel with currents that were indistinguishable from those of wild-type TASK-1 (Fig. 2B). As expected from previous studies of the human orthologue (26), this core channel was fully responsive to changes in pH and to halothane (Fig. 2B). In addition, we found that this mutant was fully inhibited by bath application of TRH (Fig. 2B). Thus the C terminus is not necessary for either anesthetic activation or transmitter inhibition, even though this region contains all of the identified consensus motifs for phosphorylation by protein kinase C and protein kinase A, as well as for tyrosine phosphorylation (3, 19, 27, 35).

We also tested whether mutations in the M2-M3 cytoplasmic linker region could perturb receptor modulation. Chimeras were generated in which a 20-residue section of rat TASK-1 (amino acids 132–151) was replaced with a corresponding section of mouse TREK-1 (residues 181–203) or of mouse TRAAK (residues 143–165), but these mutant constructs failed to make functional channels (data not shown). Mutations also were introduced in the M2-M3 linker domain by substituting alanine residues for a threonine at position 134 and for arginines at positions 137, 142, 145, and 150. For each of these point mutants, the responses to changes in pH, halothane, and TRH were indistinguishable from wild-type TASK-1 (data not shown).

**A Six-residue Segment at the Beginning of the Cytoplasmic C Terminus of TASK-1 Is Necessary for Both Halothane Activation and TRH Inhibition of the Channel**—To probe further into the transmembrane-spanning regions of the channel, we took advantage of earlier mutagenesis of human TASK-1 (26). Results from this previous work indicated that further deletion of the C terminus (by introducing a stop codon after residue 242, *i.e.* six residues upstream of our C-terminal deletion) resulted in a loss of channel function. However, replacing the C terminus of human TASK-1 (starting from residue 243) with the corresponding region from TREK-1 resulted in a construct generating functional channels that were not activated by volatile anesthetics (26). We made a similar construct using rat TASK-1, but we used a truncated portion of the C terminus from TREK-1 (residues 293–327) lacking regions critical for modulation of the channel by phosphorylation (36, 37). As shown in Fig 2C, this TASK-1/TREK-1 chimeric construct produced currents that were pH-sensitive but not activated by halothane. In fact, halothane caused an inhibition of these currents (see below). The same chimeric channel was essentially unaffected by application of TRH (Fig. 2C). These results suggested that the six-amino acid stretch of TASK-1 (residues 243–248; VLRFMT) that was contained in the C-terminal deletion mutant but not in the TASK-1/TREK-1 chimera is critical for both halothane activation and transmitter inhibition of the channel. To test this possibility, the corresponding six amino acids from TREK-1 (residues 293–298, GDWLRV, without the rest of the TREK-1 C-terminal region) were substituted into the full-length TASK-1. The resulting construct generated pH-sensitive currents, but once again the channels were not activated by halothane and were only minimally inhibited by TRH (15.6 \(\pm\) 2.2\%).

As noted above, introduction of residues from TREK-1 into the proximal C terminus of TASK-1 created a channel that was inhibited, rather than activated, by halothane. For the construct containing a six-residue TREK-1 substitution, the inhibition was substantial (30.6 \(\pm\) 0.2\% of the pH-sensitive conductance). It also was dose-dependent, being much greater at higher concentrations of halothane (reaching 85.2 \(\pm\) 1.3\% inhibition at 1 mM). One possible interpretation of this result is that there are multiple and competing sites for halothane effects on TASK-1. Consistent with this view, we found that halothane activation of the wild-type channel diminished with time (see Fig. 1A and Fig. 2A), particularly at higher concentrations of the anesthetic (data not shown). Analysis of the basis for the biphasic response to halothane will require further investigation. In any case, mean data showing peak activation by halothane (derived from the maximal conductance obtained during halothane treatment, Fig. 2E) demonstrated that channel activation was eliminated in these two TASK-1/TREK-1 chimeras. Likewise, TRH inhibition also was abolished for the two chimeric channels (Fig. 2F). Therefore, this six-residue segment of TASK-1 (243–248) is essential for both halothane activation and TRH inhibition of the channel.

The region we identified as essential for transmitter inhibition of TASK-1 was not expected, because initial descriptions did not identify consensus motifs for receptor signaling in this part of the channel (3, 19, 27, 35). However, the sequence VLRFMT does contain a threonine (residue 248) that could serve as a site of phosphorylation by calcium-calmodulin-dependent protein kinase (38). Nonetheless, substitution of the corresponding valine from TREK-1 failed to affect modulation by halothane or TRH (Fig. 3, A, C, and D), indicating that phosphorylation of this residue does not participate in anesthetic activation or transmitter inhibition of TASK-1. Thus, there are no known motifs in this region that would suggest a modulatory mechanism underlying anesthetic and transmitter effects.

We also targeted an arginine at position 245 for mutation, because this positively charged residue could be important for interaction with the head groups of phospholipids implicated in receptor signaling, such as phosphoinositides. As shown in a
FIG. 2. C-terminal mutations abolish halothane activation and TRH inhibition of TASK-1. A, responses of a cell expressing wild-type TASK-1 to changes in pH, as well as to applications of halothane and TRH. Whole-cell conductance was inhibited following bath acidification (from pH 7.3 to pH 5.9) and activated following alkalinization (to pH 8.4). Halothane caused a further increase in conductance, whereas TRH (applied after washing halothane) induced a robust channel inhibition. B, deletion of 163 residues (249–411) from the C terminus failed to affect the response to halothane or TRH. C and D, substitution of a section from the C terminus of TREK-1, including a replacement of an additional six TASK-1 residues (243–248) closer to the presumed transmembrane domain, resulted in a channel that was neither activated by halothane nor inhibited by TRH. Furthermore, replacing these six residues in the full-length TASK-1 (D) also resulted in a channel that was not activated by halothane and had very little response to TRH. Both of these TASK-1/TREK-1 chimeric channels actually were inhibited by halothane; this inhibition may occur via a separate mechanism (see “Results”). E and F, mean data demonstrate that both halothane activation and TRH inhibition require residues 243–248 of TASK-1. Peak halothane activation (derived from the maximal conductance obtained during halothane treatment (E)) and TRH inhibition (F) are plotted as a percentage of the pH-sensitive conductance (n ≥ 5 for each data point). Statistical significance was established using one-way ANOVA; asterisks indicate difference from wild-type (WT) TASK-1 (p < 0.05, pairwise comparison using the Student-Newman-Keuls test).
The protein sequences of rat TASK-1 and TASK-3 differ substantially in the C terminus (18), but the six-residue sequence necessary for halothane activation and TRH inhibition in TASK-1 also is contained in TASK-3 with only one minor difference: TASK-3 contains a leucine instead of methionine at position 247, resulting in a sequence of VLRFLT (residues 243–248). To test if this region is required for halothane activation and TRH inhibition of TASK-3, the same mutations used for TASK-1 were introduced into TASK-3, with slightly different results (Fig. 4). As with TASK-1, removal of the C terminus from TASK-3 did not abolish halothane activation or TRH inhibition (Fig. 4B), although both of these effects were diminished (Fig. 4, E and F, see below). Substitution with residues from TREK-1, either replacing the C terminus or only replacing residues 243–248, resulted in a loss of halothane activation (to less than 15% of the wild-type levels) and TRH inhibition (to less than 10% of wild-type levels), indicating that this six-residue stretch is essential for effects of these compounds on both TASK-1 and TASK-3.

Both Halothane Activation and TRH Inhibition Are Increasingly Attenuated by Removal of Successive Portions of the TASK-3 C Terminus—Removal of the C terminus of TASK-3 partially attenuated halothane activation and TRH inhibition, indicating that a portion of this domain is necessary for the full effects of these agents. We investigated this issue in more detail using two more constructs, one in which the final 11 residues were deleted (Δ386–396) and another in which 92 residues were removed (Δ305–396). These were compared with the initial C-terminal deletion construct (see Fig. 4), in which 148 residues had been eliminated (Δ249–396). As shown in Fig. 5, deletion of 11 residues (Δ386–396) failed to attenuate halothane activation and TRH inhibition; the effects on this mutant were not different from those on the wild-type channel. However, removal of a greater portion of the C terminus (Δ305–396) resulted in partial but significant decreases in both effects (to 68 and 76% of the wild-type channel). The actions of both compounds were nevertheless significantly larger than those on the Δ249–396 construct, which were diminished to 43% (halothane activation) and 59% (TRH inhibition) of wild-type levels. Thus there were incremental decreases in the effects of halothane and TRH upon removal of 92 and 148 residues of the C terminus of TASK-3. Once again, the results for halothane were correlated with those for TRH. This is represented graphically in Fig. 5C, in which halothane activation is plotted against TRH inhibition for individual cells. Linear regression analysis of these values indicates that halothane activation and TRH inhibition were highly correlated ($R^2 = 0.669$, $p < 0.0005$).

Introduction of a Single Mutated Domain (Substitution of Six Residues from TREK-1) into a Tandem-linked Channel Is Sufficient to Disrupt the Effects of Halothane and TRH—As noted above, TASK-1 and TASK-3 are co-expressed in a number of different cell types, suggesting the possibility that they form heterodimeric channels (16, 22, 23). To test for neurotransmitter and anesthetic effects on such channels, we generated a construct in which the C terminus of TASK-1 was fused to the N terminus of TASK-3 (via a short linker region, see “Experimental Procedures”). As shown in Fig. 6, these heterodimeric channels were fully activated by halothane (150.8 ± 12.6%) and inhibited by TRH (82.4 ± 5.4%). As was found for TASK-3, the effect of halothane on the tandem construct was more potent than on TASK-1 (see Figs. 2 and 3). We also made constructs with substitutions of the six residues from TREK-1 (identified above) into either the TASK-1 or the TASK-3 portion of the channel. This created channels with only a single mutated domain, as opposed to un-linked channels, which (because they assemble as dimers (25)) contain two mutated domains per
FIG. 4. C-terminal mutations also attenuate halothane activation and TRH inhibition of TASK-3. A, like TASK-1, wild-type TASK-3 was activated by halothane and inhibited by TRH. B, deletion of 148 residues from the C terminus of TASK-3 (249–396) resulted in a construct with pH-sensitive currents that were still activated by halothane and inhibited by TRH, although both effects were partially attenuated. C and D, replacement of the TASK-3 C terminus with residues from TREK-1 (C), or replacement of six TASK-3 amino acids (243–248) proximal to the transmembrane region (D), resulted in channels with little response to halothane or TRH. E and F, averaged responses of wild-type TASK-3 and the three different mutant channels to halothane (peak activation, E) and TRH (percent inhibition, F) are plotted as a percentage of the pH-sensitive conductance (n ≥ 6 for each data point). Statistical significance was established using one-way ANOVA; asterisks indicate difference from wild-type TASK-3 (p < 0.05).
channel. One might predict that a single mutated domain would be only half as effective in attenuating the actions of halothane and TRH. Instead, we found that the effects of these compounds on the singly mutated tandem-linked channels were reduced to levels very similar to those seen for the mutated un-linked channels (Fig. 6, D and E). Thus a single substitution of six residues from TREK-1, into either the TASK-1 or the TASK-3 portion of the heterodimeric channel, was sufficient to disrupt the effects of halothane and TRH.

**DISCUSSION**

We generated mutations in TASK-1 and TASK-3 in order to identify regions critical for neurotransmitter inhibition of these channels, and we found that alterations disrupting anesthetic activation also interfered with neurotransmitter inhibition. For TASK-1, removal of the cytoplasmic C terminus failed to affect either form of regulation, even though this domain contains in its primary sequence a number of motifs associated with receptor signal transduction (3, 19, 27, 35). Also, mutations in the M2–M3 cytoplasmic linker region failed to disrupt modulation. Instead, a region not associated with known signal transduction-related motifs, at the interface between the presumed fourth transmembrane domain and the cytoplasmic C terminus, was critical for both halothane activation and TRH inhibition of TASK-1 currents. Substitution of six residues from TREK-1 into this region (in the context of full-length TASK-1 or in the replacement of the C terminus with a section from TREK-1) essentially abolished both effects. Mutagenesis of TASK-3 produced similar although not identical results. We used the same strategy (substitution of residues from TREK-1) and found that the corresponding region in TASK-3 was critical for modulation by halothane and TRH. However, a large portion of the C terminus of TASK-3 was necessary for the full effects of these agents.

**Identification of a Domain in TASK-1 and TASK-3 Critical for Transmitter Inhibition**—We identified a region in both TASK-1 and TASK-3 at the cytoplasmic face of the fourth transmembrane domain that is critical for channel inhibition by transmitters. Similar or identical sequence is present in the corresponding regions of presumed channels from *Caenorhabditis elegans* (GenBank™ accession number AF083652) and *Drosophila melanogaster* (GenBank™ accession numbers AAF54970 and AAF54374), indicating that this sequence is ancient and may have the same importance in regulating channel activity in those species as well. Also, it has been noted that an analogous region (immediately following the S6 transmembrane domain) is likely to be important for transmission of intracellular gating signals in voltage- and cyclic nucleotide-gated channels (39).

The importance of this region was unexpected, because the

**TASK-3 C terminus.** A and B, constructs were generated by removal of different lengths of the TASK-3 cytoplasmic C terminus. Deletion of this entire region (Δ249–396, see Fig. 4) resulted in partial loss of the effects of halothane and TRH. Removal of a smaller portion of the C terminus (92 residues, Δ305–396) resulted in a partial recovery of these effects, whereas removal of only 11 residues (Δ386–396) resulted in effects of halothane and TRH that were not different from those of the wild-type channel. For both halothane and TRH, there were significant differences in effects between each of the groups (one-way ANOVA, *n* ≥ 5 for each group). *, different from Δ386–396; #, different from Δ249–396. C, correlation of the magnitudes of halothane and TRH effects. Individual cells (open symbols) transfected with the three different TASK-3 C-terminal deletion mutants (squares, Δ249–396; circles, Δ305–396; and triangles, Δ386–396) are plotted with respect to halothane activation and TRH inhibition. Mean data (± S.E.) for each construct are represented as filled symbols. Linear regression analysis of the individual data points (solid line) indicated a significant correlation between the two effects (*p* < 0.0005).
Fig. 6. Introduction of a single mutated domain (substitution of six residues from TREK-1) into a tandem linked channel is sufficient to disrupt the effects of halothane and TRH. A, a tandem heterodimeric construct was generated by linking the C terminus of TASK-1 to the N terminus of TASK-3. This construct was fully activated by halothane and inhibited by TRH. B and C, the six residues identified as critical for halothane activation and TRH inhibition in the context of wild-type TASK-1 and TASK-3 were introduced into the tandem construct, either in the TASK-1 (B) or the TASK-3 (C) portion of the channel. D and E, mean data (± S.E.) show that either mutation was sufficient to reduce the effects of halothane and TRH to an extent similar to that seen for un-linked channels. Asterisks indicate significantly different from the non-mutated tandem construct.

relevant sequence is not identified with any known signal transduction motifs. However, this fact is consistent with previous negative findings regarding second messenger pathways responsible for TASK channel inhibition by neurotransmitters and hormones (3, 19, 27, 28). Inhibition of TASK-1 and TASK-3, or native counterparts, has been demonstrated for a number of different G protein-coupled receptors besides the TRH-R1 receptor, all of which specifically couple to G proteins of the \( \alpha_{q/11} \) family (7, 8, 10, 24). The downstream signaling pathway most commonly associated with activation of these receptors entails hydrolysis of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) by phospholipase C (PLC), resulting in production of inositol 3,4,5-trisphosphate and diacylglycerol. However, initial reports (3, 19, 27) and a more complete study (28) failed to implicate the products of this lipid hydrolysis, nor did they implicate downstream signaling events, including release of calcium from intracellular stores and activation of protein kinase C. Other signaling pathways initiated by \( \alpha_{q/11} \)-coupled receptors also do not appear to be involved, including tyrosine kinase activation (28), production of arachidonic acid via phospholipase A (36, 40), and activation of Rho kinase.2

Although most of the evidence has been negative, two proposed signaling mechanisms have received some positive support. One proposal suggests that the mechanism does not involve pathways downstream of PLC but instead results from PLC-mediated depletion of PI(4,5)P2 (28), as has been suggested for receptor-mediated inhibition of members of the inwardly rectifying K+ channel family (41–43). Supporting a role for PLC in receptor-mediated TASK-1 inhibition, the \( \alpha_{q/11} \)-coupled M2 muscarinic receptor was able to inhibit TASK-1 currents only upon overexpression of PLC\( \beta_{2} \), an isoform that can be activated by \( \alpha_{q/11} \)-coupled receptors. In addition, the PLC inhibitor U73122 reduced receptor effects on TASK-1 in Xenopus oocytes, and the phospholipid kinase inhibitor wortmannin (used at concentrations high enough to block phosphatidylinositol 4-kinases) slowed recovery from receptor inhibition, presumably as a result of a reduced ability of the cells to replenish PI(4,5)P2 levels (28). However, the pharmacological evidence has not been universally positive. In HEK 293 cells, U73122 (but not its inactive analogue U73343) partially blocked TRH inhibition, but another PLC blocker, D609, was ineffective.2 Also, treatment of cerebellar granule neurons with U73122 failed to prevent receptor-mediated inhibition of \( I_{K,SO} \), a native correlate of TASK channels (44). In any case, the possibility that PI(4,5)P2 depletion contributes to receptor inhibition of TASK-1 and TASK-3 awaits more experimentation, such as testing for channel activation by the compound itself.

A second signaling pathway proposed for receptor actions on TASK-1 involves the endocannabinoid anandamide, which inhibits human TASK-1 currents at sub-micromolar levels, although it is less potent at inhibiting TASK-3 (40). This compound can be released in neurons by activation of G\( \alpha \)-coupled receptors also do not appear to be involved, including tyrosine kinase activation (28), production of arachidonic acid via phospholipase A (36, 40), and activation of Rho kinase.2

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E. M. Talley and D. A. Bayliss, unpublished results.
activation and transmitter inhibition. Moreover, mutations resulting in partial blockade of one effect also produced partial obstruction of the other. These mutations included an arginine-to-tryptophan substitution in TASK-1, and TASK-3 constructs in which increasing portions of the C terminus were removed. The TASK-3 C-terminal deletion mutants were especially interesting in this regard, because halothane and TRH shared the same rank order of potency in their effects on these channels, with increasing lengths of the C terminus resulting in incrementally greater effects for both anesthetic and transmitter. The data clearly indicate that these agents share structural requirements at the channel and may act via a common mechanism, albeit with opposite effects.

It is not clear if the identified convergence occurs directly at the channel or involves interaction with some upstream intermediary signaling molecule(s). A direct anesthetic effect has been suggested, because halothane activated TASK-1 channels in excised patches (26). Therefore the effects of these compounds, if not direct, are at least likely to be closely associated with the channel. If the receptor mechanism involves depletion of an endogenous channel-activating compound (such as PI(4,5)P2) (28), then it might be supposed that volatile anesthetics substitute for this compound, acting as agonists at the same site on the channels. If this were true, then it would be expected that depletion of the endogenous compound would be compensated by anesthetics and that receptor-mediated channel inhibition therefore would be offset in their presence. However, our data indicate that TRH inhibition is fully effective during the continued application of halothane (see Fig. 1) and therefore do not support this model.

Modulation of TASK-1/TASK-3 Heterodimers—As noted in the Introduction, mRNAs encoding TASK-1 and TASK-3 are co-expressed in a number of different neuronal populations (16, 22), including cells known to express functional TASK-like currents (8–10, 23). Recently, heterodimerization of these two channel subunits was demonstrated in Xenopus oocytes (24). Whether these two genes form heterodimers in vivo remains to be established, but it is possible that such heterodimeric channels have properties that are emergent, as opposed to having properties common to those of TASK-1 and TASK-3. Here we show that heterodimers composed of TASK-1 and TASK-3 (in a forced tandem-linked conformation) are regulated by TRH and halothane. This regulation apparently occurs via the same mechanism as in un-linked homodimeric channels, because substitution of individual domains into either the TASK-1 or the TASK-3 portion of the channel resulted in nearly complete disruption of the effects of halothane and TRH. The magnitude of the TRH effect was not different between the un-linked channels and the tandem heterodimer. However, halothane activated TASK-3 and the tandem construct much more potently than TASK-1, with a nearly 3-fold difference in the degree of activation.

The difference in magnitude of the halothane effect raises the question of how these values compare with those from native cells in which both TASK-1 and TASK-3 are expressed, such as motoneurons (10, 15, 16, 22). In previous recordings of hypoglossal motoneurons (HMs) from our laboratory, a pH- and neurotransmitter-sensitive leak K+ current was identified as resulting from expression of TASK-1 (10), because the pH sensitivity (pK~7.4) of the current was inconsistent with any other known leak K+ channel (including TASK-3). This motoneuronal leak current also was activated by volatile anesthetics (9), once again consistent with the expression of TASK-1. However, in the present study we found that TASK-1 was only activated by ~50% using 0.3 mm halothane; in HMs, the same concentration of halothane produced an activation that was ~100% relative to the pH-sensitive current (9). Thus, although the motoneuronal experiments were performed somewhat differently from those of the present report, the potency of the anesthetic effect appears to have been larger than would be expected solely from the expression of TASK-1. Therefore it is possible that the magnitude of the anesthetic effect in HMs results from the activation of some combination of TASK-1, TASK-3, and/or TASK-1/TASK-3 heterodimers. The existence of heterodimers in these neurons would appear to be even more likely if the heterodimeric channels had a pH sensitivity close to that of TASK-1 (and not TASK-3), given the TASK-1-like pH sensitivity of the currents observed in HMs.

In any case, here we demonstrate that a domain contained in both TASK-1 and TASK-3 is necessary for the effects of transmitters and anesthetics. This domain is not associated with any previously identified signaling motifs, consistent with the fact that the mechanism for transmitter regulation of these channels has not been elucidated. Because the same domain is required for transmitter and anesthetic modulation of TASK-1/TASK-3 heterodimers, the present results are equally relevant to potential heterodimeric channels existing in vivo.

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Modulation of TASK-1 (Kcnk3) and TASK-3 (Kcnk9) Potassium Channels:
VOLATILE ANESTHETICS AND NEUROTRANSMITTERS SHARE A
MOLECULAR SITE OF ACTION
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