Molecular Modeling of a Tandem Two Pore Domain Potassium Channel Reveals a Putative Binding Site for General Anesthetics

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ABSTRACT: Anesthetics are thought to mediate a portion of their activity via binding to and modulation of potassium channels. In particular, tandem pore potassium channels (K2P) are transmembrane ion channels whose current is modulated by the presence of general anesthetics and whose genetic absence has been shown to confer a level of anesthetic resistance. While the exact molecular structure of all K2P forms remains unknown, significant progress has been made toward understanding their structure and interactions with anesthetics via the methods of molecular modeling, coupled with the recently released higher resolution structures of homologous potassium channels to act as templates. Such models reveal the convergence of amino acid regions that are known to modulate anesthetic activity onto a common three-dimensional cavity that forms a putative anesthetic binding site. The model successfully predicts additional important residues that are also involved in the putative binding site as validated by the results of suggested experimental mutations. Such a model can now be used to further predict other amino acid residues that may be intimately involved in the target-based structure–activity relationships that are necessary for anesthetic binding.

KEYWORDS: Tandem pore potassium channel, anesthesia, homology modeling

Volatile general anesthetics are thought to act, at least in part, by binding to and modulating two pore domain potassium channels (K2P). These K2P potassium channels are transmembrane ion channels whose current is modulated by the presence of a wide range of volatile and gaseous general anesthetics and whose genetic knock out, either globally or locally, has been shown to confer a level of anesthetic resistance. What is not known is where anesthetics bind within the channels, and how this binding translates into increased channel opening. For different K2P channels, using a combination of chimeric constructs and site-directed mutagenesis, a number of amino acids have been identified as key anesthetic determinants. However, whether these determinants function as parts of anesthetic binding sites or are involved in transduction mechanisms that convert binding into channel gating is unclear. The covalent modification work by Conway and Cotton suggests that a region around L159 forms a putative anesthetic binding site. Another approach to this problem is to use the structures of homologous potassium channels as templates to construct models of an anesthetic-sensitive channel to investigate the three-dimensional disposition of these anesthetic determinants. Here we describe how the construction of such models reveals the convergence of amino acid regions that are known to affect anesthetic sensitivity into a common three-dimensional locus that could serve as an anesthetic binding site. We test our prediction that particular amino acids form part of an anesthetic binding site using patch-clamp electrophysiology on wild-type and mutant K2P channels.

A novel anesthetic-activated potassium current was first characterized in a single molluscan neuron by Franks and Lieb. Following this, anesthetic activated mammalian K2P channels were identified and knockout mice lacking K2P channels have demonstrated decreased sensitivities to volatile anesthetics. Within the tandem pore potassium channel family, TRENK-1 is sensitive to the anesthetic gases Xe, N₂O, and cyclopropane, while TASK-3 is insensitive to these gases while retaining sensitivity to the volatile anesthetics. Additional studies producing single point mutations in TRENK-1 conferred relative resistance to certain volatile anesthetics. Through the generation of TASK-3 knockout mice, Pang et al. showed a significant role for the TASK-3 potassium channel in the theta oscillations of the cortical EEG that are associated with both sleep and anesthetized states. In particular, TASK-3 knockout animals show marked alterations in both anesthetic sensitivity and natural sleep behavior. This is particularly tantalizing, since certain electrophysiologic aspects of deep (non-REM) sleep have similarities to the anesthetized state.

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Molecular Modeling. We chose to model the TASK potassium channel from *Lymnaea stagnalis* (LyTASK), because this has the greatest sensitivity to volatile anesthetics of any known K2P channel. The BLAST-derived scores suggest a close homology between LyTASK and two K2P channels whose structures have been determined to high resolution. The first is the anesthetic-sensitive human TWIK-1 channel, 3UKM (51% sequence coverage, 33% maximum amino acid identity, and BLAST expectation value of $4 \times 10^{-29}$), and the second is the human anesthetic-insensitive TRAAK channel, whose structure has been described with two different chain connectivities 3UM7 and 4I9W (67% sequence coverage, 31% maximum amino acid identity, and BLAST expectation value of $3 \times 10^{-31}$). Subsequent CLUSTALW alignment of the sequence from LyTASK to the sequence profile (created from the sequences of the two templates 3UKM and 3UM7) also demonstrates reasonable sequence similarity (Figure 1).

Models of the anesthetic-sensitive LyTASK based on the overlapped TWIK-1 and TRAAK template structures show a dimer with symmetry about a central ion pore. Amino acid regions notable for modulating anesthetic action on this channel (L159 and the sequence ILRFLT) converged on a common pocket in three-dimensional space (Figure 2). This was the case even in a model with the alternative backbone connectivities based on 4I9W, though the latter was not used for further analysis. The different connectivity only affected regions that are at the opposite end of the channel to the putative anesthetic binding site and therefore is unlikely to impact on our results or conclusions. Three-dimensional visualization of our model suggested that L241, L242, and S155 were adjacent to the critical residues (L159 and the sequence ILRFLT) that have previously been demonstrated as having large effects on anesthetic modulation. Additionally, the model suggests that the L159 might form part of an intercalated hydrophobic side chain interaction with L241 and L242 (see Figures 3 and 4) as well as demonstrating residue proximities that could allow a possible polar interaction between S155 and R246 (Figure 3). The disruption of such interactions between alpha helical secondary structure units could lead to changes in the tertiary structure as well as the large scale motions of the protein.

Electrophysiology on Wild-Type and Mutant LyTASK Channels. Cells transfected with wild-type LyTASK cDNA exhibited robust outwardly rectifying currents that reversed close to the calculated potassium reversal potential (Figure 5A, blue line; Figure 5B−F, solid lines). Halothane (3%) resulted in activation of both wild-type and mutant LyTASK currents.
the wild-type LyTASK currents by 412 ± 54% (Figure 6C). The LyTASK L241A and the L242A mutants showed a significantly reduced activation by 3% halothane compared to the wild-type channel, with 190 ± 11% and 194 ± 14% activation, respectively (Figure 6C). To test a hypothesized polar interaction between S155 and R246, we mutated LyTASK S155 to an alanine. The effect of 3% halothane on this S155A mutant showed no significant difference compared to wild-type (393 ± 62% activation). We also mutated S155 to a bulky aromatic residue, tryptophan, to see if this might mimic the presence of an anesthetic in our putative binding pocket. The S155W mutant showed a marked increase in activation by 3% halothane, with the 1146 ± 20% activation being ∼2.8 times that of the effect of halothane on the wild-type channel (Figure 6C and Table 1).

**DISCUSSION**

General anesthetics have been employed for over 165 years, and their use is indispensable in a variety of invasive surgical procedures and an ever increasing body of screening and preventive medicine maneuvers (colonoscopy, bronchoscopy, etc.). The state of general anesthesia is characterized by profound lack of awareness, plus amnesia, analgesia, and immobility. Each of these desirable physiological responses is likely to be a consequence of anesthetic effects on different parts of the central nervous system, and considerable progress has been made toward identifying these sites.20–22 At the cellular level, it appears that anesthetics act predominantly at a relatively small number of molecular targets, in particular, GABA_A receptors, two-pore domain potassium channels, and NMDA receptors.21 In order to further pinpoint molecular sites of anesthetic action, a large number of in vitro site-directed mutagenesis studies have been performed, identifying particular amino acids and motifs that are required for the effects of both volatile and intravenous general anesthetics on a wide variety of ion channels.20–22 Effects have been catalogued across different ion channel proteins without convergence on a single site of action. Another approach to identify relevant receptors has involved the genetic modification of whole organisms in an effort to induce resistance to particular anesthetics and to test the importance of a putative target. To date, two key molecular targets have emerged from this combination of in vitro and in vivo approaches: the GABA_A receptor and K2P potassium channels. The N265 M knock-in mutation in the mouse β3 GABA_A receptor subunit23 conferred increased resistance to both propofol and etomidate for both loss of righting reflex (a rodent surrogate for loss of consciousness in humans) and loss of response to a painful stimulus. While a great deal of work has focused on the modeling of anesthetic binding sites within the GABA_A receptor,24,25 several studies have shown that this class of proteins is unlikely to mediate all of the effects of anesthetics, particularly for the volatile agents. It is also likely that anesthetics act by binding to and modulating K2P potassium channels.21 The knockout of two different tandem pore potassium channels4–7 conferred enhanced resistance to volatile general anesthetics.

In this Article, we now show how molecular modeling can be used to shed light on molecular mechanisms of anesthetic action as well as more efficiently suggest in vitro mutations for testing of molecular models. In this case, molecular modeling allows one to leverage the knowledge that is gleaned from the recently available high-resolution crystallographic coordinates for K2P potassium channels that are highly homologous to one...
that has been extensively studied by mutagenesis and electrophysiologic analyses. Mutations at L241 and L242 may blunt activation by anesthetics possibly through a disruption of an intercalated hydrophobic side chain interactions (Figure 4). However, the mutations at S155 seem to only have an effect of potentiating anesthetic activation when the substituted side chains are large enough, possibly simulating the presence of ligand. Initial predictions on the importance of a partial polar interaction between S155 and R246 seem disproven by the fact that there is no effect on anesthetic activation when the polar to nonpolar S155A mutation is introduced. The greater potentiation by the S155W mutation may indicate that a larger than normal side chain protruding into an anesthetic binding site accentuates an effect resulting from anesthetic binding, especially with the tryptophan side chain being significantly larger than a single halothane molecule. One could postulate that such an effect may indicate that the anesthetic binding site in LyTASK could accommodate two halothane molecules, as has been previously shown on anesthetic binding to cholesterol oxidase and firefly luciferase.26,27,28

Figure 5. Typical electrophysiology current−voltage relations for the WT and mutant LyTASK potassium channels. (A) Schematic diagram showing current−voltage relation for an untransfected cell (green line), LyTASK transfected cells exhibit a large outwardly rectifying potassium current (blue line) reversing close to −90 mV (R). In the presence of halothane (red line), the LyTASK current is increased. LyTASK currents are quantified by measuring the value of the current at a membrane potential of −50 mV, marked on the diagram is the control LyTASK current “L” and the halothane-activated LyTASK current, “H”. (B) Wild-type LyTASK. (C) LyTASK S155A mutant. (D) LyTASK L242A mutant. (E) LyTASK S155W mutant. (F) LyTASK L241A mutant. Solid lines are LyTASK currents in the absence of halothane, and dashed lines are the LyTASK currents in the presence of 3% halothane. Data were sampled at 20 kHz, and each trace contains 3000 data points. Lines shown are means of 10 individual voltage ramps for a given cell in each condition.

This causes Modeler to build the same loop in chain B of the LyTASK model based solely on TWIK, which is more collapsed in the direction of any binding site, causing slightly different pocket formations in this region versus that composed of template chains A. Also, the alignment of the positions in the templates that are homologous to L159 in LyTASK show reasonable homology (alanines instead of leucine). However, there is rather poor homology in the regions of the templates associated with the ILRFLT motif within LyTASK. This leaves some potential for variability in the alignments. Such alignments would require experimental validation as has been presented here.

Furthermore, at this stage, our model does not explain the differential anesthetic sensitivities of some K2P channels over others. Our unpublished preparatory work to the current study did not demonstrate any notable differences in pocket sizes between TRAAK and TWIK in these areas that would account for differential anesthetic sensitivities. However, the point of this work is to demonstrate the convergence of relevant residues on a common 3D locale that has pocket-like character allowing quite variable size accessibility from the extracellular space. Such accessibility is clearly present in both the TRAAK and TWIK templates. The differential sensitivity of these proteins is most likely due to different amino acid side chains being present in the binding pocket, as well as different large
Although most work on anesthetic binding to soluble proteins shows little effect on protein structure when anesthetics bind, such changes may occur in integral membrane proteins. Either way, the location of the binding pocket at the surface of K2P channels, in addition to its amphiphilic character, may lend itself to a considerable amount of ligand size promiscuity, as exemplified by the differential effects of ligands from Xe to isoflurane.

Finally, the localization of residues important for anesthetic binding may only infer a putative binding site. Another interpretation could be that such residues merely form a region which is critical for allosteric modulation of the anesthetic-mediated effect. Greater evidence for actual anesthetic binding could come from experiments involving the covalent localization of anesthetic-like ligand to the putative anesthetic binding site. Additionally, a concern could be that the open state probability of residue-241 and -242 mutants may be near 100%. We cannot exclude the possibility that the mutations affect channel gating, but the fact that we can activate all of the mutant channels with halothane indicates that the open probability is less than 100% in all cases. In addition, Conway and Cotten have shown that LyTASK L159C and TASK3M159C, both anesthetic resistant, tolerate further activation by alkylation, implying the channels are not locked open by loss of leucine or methionine in this region and that such changes may occur in integral membrane proteins. Either way, the location of the binding pocket at the surface of K2P channels, in addition to its amphiphilic character, may lend itself to a considerable amount of ligand size promiscuity, as exemplified by the differential effects of ligands from Xe to isoflurane.

**CONCLUSION**

Homology modeling produced a model of the K2P channel that revealed a putative anesthetic-binding pocket identified by the convergence of amino acid residues known to modulate anesthetic activity. The anesthetic binding pocket model was validated by the successful prediction of other amino acid residues also found to alter anesthetic modulation of the channel due to their spatial proximity to the putative binding site. Such a model can now be used to further predict other amino acid residues that may be intimately involved in the anesthetic activity. The anesthetic binding pocket model was validated by the successful prediction of other amino acid residues also found to alter anesthetic modulation of the channel due to their spatial proximity to the putative binding site. Such a model can now be used to further predict other amino acid residues that may be intimately involved in the anesthetic activity.

**METHODS**

**Molecular Modeling.** All protein construction calculations were performed in the Discovery Studio 3.5 software suite (Accelrys, San Diego, CA). The amino acid sequence of the K2P channel from the snail, *Lymnaea stagnalis* (LyTASK), was obtained from the National Institute of Standards and Technology (NIH). The K2P channel from the snail, *Lymnaea stagnalis* (LyTASK), was obtained from the National Institute of Standards and Technology (NIH). The K2P channel from the snail, *Lymnaea stagnalis* (LyTASK), was obtained from the National Institute of Standards and Technology (NIH). The K2P channel from the snail, *Lymnaea stagnalis* (LyTASK), was obtained from the National Institute of Standards and Technology (NIH). The K2P channel from the snail, *Lymnaea stagnalis* (LyTASK), was obtained from the National Institute of Standards and Technology (NIH). 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either minimal or not present). All experiments were carried out at 22 °C. Percentage activation was calculated from the ratio of the halothane activated current, \( H \), and the control LyTask current, \( L \), using the equation: activation = \( \frac{H}{L} - 1 \) × 100%.

### ASSOCIATED CONTENT

#### Supporting Information

Three-dimensional coordinates of the LyTask model in PDB format. This material is available free of charge via the Internet at http://pubs.acs.org/.

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#### Author Contributions

Bertacini and Trudell performed the molecular modeling. Dickinson and Franks performed the in vitro mutations and electrophysiology. All authors contributed to the approach to the work, as well as its analyses, interpretations, and actual manuscript composition.

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### Notes

The authors declare no competing financial interest.

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