General Anesthetic Action at an Internal Protein Site Involving the S4-S5 Cytoplasmic Loop of a Neuronal K⁺ Channel*

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The structural bases of general anesthetic action on a neuronal K⁺ channel were investigated using the series of homologous 1-alkanols, electrophysiology, and mutational analysis. Domain swapping between dShaw2 (alkanol-sensitive) and hKv3.4 (alkanol-resistant) and site-directed mutagenesis demonstrated that a 13-amino acid cytoplasmic loop (S4-S5) determines the selective inhibition of native dShaw2 channels by 1-alkanols. The S4-S5 loop may contribute to a receptor for both 1-alkanols and the inactivation particle, because the enhanced 1-alkanol sensitivity of hKv3.4 channels hosting S4-S5 mutations correlates directly with disrupted channel inactivation. Evidence of a discrete protein site was also obtained from the analysis of the relationship between potency and alkyl chain length, which begins to level off after 1-hexanol. Rapid application to the cytoplasmic side of inside-out membrane patches shows that the interaction between dShaw2 channels and 1-alkanols equilibrates in <200 ms. By contrast, the equilibrium time is >1000-fold slower when the drug is applied externally to outside-out membrane patches. The data strongly favor a mechanism of inhibition involving a discrete internal site for 1-alkanols in dShaw2 K⁺ channels. A new working hypothesis proposes that 1-alkanols lock dShaw2 channels in their closed conformation by a direct interaction at a crevice formed by the S4-S5 loop.

General anesthetics (including 1-alkanols) at clinically relevant concentrations mainly alter the function of neuronal ion channels by a direct action (1–5). Site-directed mutagenesis has provided strong evidence favoring the presence of critical protein sites necessary for the interaction between 1-alkanols and the ion channel protein (e.g. Refs. 6–12). Furthermore, these studies suggest that diverse sites but similar molecular mechanisms may underlie general anesthesia and alcohol intoxication. The application of mutational analysis and electrophysiology to the investigation of model ion channels that directly interact with 1-alkanols can provide detailed information about the general characteristics of the 1-alkanol sites in membrane proteins and the molecular interactions that underlie the effects of general anesthetics and 1-alkanols on more complex neuronal ion channels.

We have investigated the molecular properties of the putative 1-alkanol site in Drosophila dShaw2 K⁺ channels. These channels are expressed in Drosophila neurons and exhibit low voltage sensitivity, low open probability, and no inactivation (13–16). dShaw2 channels are selectively inhibited by anesthetic concentrations of ethanol (25–100 mM) and homologous 1-alkanols in a manner that agrees with a drug-receptor interaction (6, 17). This inhibition is due to the stabilization of the closed state(s) of the channel by preferentially reducing the probability of entering a long duration open state (with no effect on the mean open time) (6). The earlier work also showed that dShaw2 subunits confer 1-alkanol sensitivity to hybrid channels and suggested that a dShaw2-specific isoleucine in the S4-S5 cytoplasmic loop of the channel subunit contributes to a hydrophobic site of action (6).

The cytoplasmic S4-S5 loop (13 amino acids) is also associated in various ways with the gating of voltage-gated K⁺ channels. Point mutations or chemical modification in the S4-S5 loop alter the development of rapid inactivation and destabilize the inactivated state of the channel (18–20). Thus, it has been suggested that the S4-S5 loop is a component of the receptor for the inactivation particle of the channel. Other studies have also shown that the S4-S5 loop contributes to voltage-dependent gating and the structural stability of K⁺ channels (21, 22). Based on this information and on structural criteria, current models of voltage-gated K⁺ channels depict the S4-S5 loop as a short α-helical segment that together with the cytoplasmic sections of S5 and S6 form the inner mouth of the pore (23, 24). Critical conformational changes that control the gating of the channel may take place here (19, 25–28).

In this study, mutational analysis identifies the cytoplasmic S4-S5 loop of dShaw2 channels as a necessary molecular determinant of 1-alkanol action and suggests that the structure of this region is critical to maintain both the 1-alkanol site and the receptor site of the inactivation particle. Further evidence of a discrete site is obtained by examining long chain 1-alkanols, and kinetic analysis confirms that the 1-alkanol site is most likely located at the cytoplasmic side of the channel. The data are consistent with a new working model where 1-alkanols interact with discrete protein crevices near the putative internal gate (which is influenced by the S4-S5 loop) and thereby lock the pore in its closed conformation.

EXPERIMENTAL PROCEDURES

Reagents—Ethanol and 1-butanol were purchased from Fisher. 1-Hexanol was from Sigma, and 1-heptanol, 1-octanol, 1-decanol, and 1-undecanol were from Acros Organics (Geel, Belgium). All 1-alkanols were mixed in the external or internal bath solutions. For adequate mixing, solutions containing long-chain 1-alkanols (1-octanol, 1-decanol, and 1-undecanol) were sonicated (bath sonicator, 15–30 min). In some cases, long-chain 1-alkanols were also predissolved in dimethyl sulfoxide (maintaining the final concentration of dimethyl sulfoxide below 0.02%). The concentration of the long-chain 1-alkanols was verified by gas chromatography in the perfusion reservoir (see below), the recording chamber, and the perfusate. For all of the 1-alkanols, no losses were detected in the perfusion system.

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Direct Interaction of 1-Alkanols with a K+ Channel

Gas Chromatography—Two to four ml of the buffer from the perfusion reservoir, the recording chamber, or the perfusate were extracted with 0.2 ml of hexane and dried briefly over anhydrous sodium sulfate. Completeness of the extraction was assessed by extracting and analyzing known amounts of standard 1-alkanols added to the buffer. For an analysis differing by one atom from the sample, the sample was added to the buffer as an internal standard. The 1-alkanols were separated by capillary gas liquid chromatography on a Hewlett-Packard 5890A gas-liquid chromatograph with a fused silica highly polar stabilized bisacyanopropylglycol polysiloxane phase SP-2380 column (30 m × 0.32 mm internal diameter, 0.2-μm film thickness) (Supelco, Bellefont, PA). The column temperature was linearly programmed from 120 to 140 °C at 5 °C/min with an initial time of 5 min and a final time of 1 min, and the injector and detector temperatures were 250 °C. The 1-alkanols were identified by comparison with retention times of standard 1-alkanols and were analyzed using a Hewlett-Packard 3393A computing integrator to calculate the area percentage of each 1-alkanol.

Molecular Biology and Site-directed Mutagenesis—Wild-type cDNAs encoding dShaw2 and hKv3.4 were maintained as described previously (6). Quick Change (Stratagene, La Jolla, CA) was used to make the mutations according to the manufacturer specifications and as described before (29). The objective of the mutagenesis was to convert all seven cytochrome P450-dependent hydroxylases between the S4–S5 loop of the F座谈会上k3.4 (including a total of 13 amino acids). Table I summarizes the main mutations investigated in this study. Multiple dShaw2 mutations were created sequentially as follows: wild-type dShaw2 → I319G → I319G/F322L → S3 → S5 → S6 → SK chimera (complete conversion). The S4–S5 loop of the SK chimera was identical to that of hKv3.4. I319G and F322L were used to make I319G/A326T and F322L/A326T, respectively. Introducing G371I in the SK chimeras created a reversal mutant. S5 was also made by cassette mutagenesis (named S5′). Briefly, plasmids hosting wild-type dShaw2 and the mutant dShaw2 were digested with two restriction endonucleases (HpaI and PstI) at the 5′- and 3′-ends, respectively. These enzymes excised the mutant fragment of interest (residues 314–328) and created compatible restriction endonuclease sites in the wild-type dShaw2 vector for subcloning (following digestion, the vector was also treated with alkaline phosphatase to prevent self-ligation). The fragment and the wild-type host vector were then gel-purified and ligated (10:1, vector:insert) using commercially available techniques and standard procedures (Qiagen Inc., Valencia, CA). Multiple hKv3.4 mutations were created sequentially as follows: Wild-type hKv3.4 → G371I → G371I/L374F → K3 → K5 → K6 and KS chimera (two possible combinations of the mutations). For the S5′ loop for Asn797, the S4–S5 loop of the KS chimera is identical to that of dShaw2. The mutation N379K creates K7. The S4–S5 loop of K7 is identical to that of dShaw2 (Table I). G371I and L374F were used to make G371I/T378A and L374F/T378A, respectively. K5 was made from the KS chimera by introducing F374L. The mutants K5, K6, and K7 did not express or expressed very poorly (<300 nA). The K7 mutant allow- ed accurate analysis of energy contributions, which accounts for the stability of the α-helix in solution (31, 33). The energy contributions include the intrinsic tendency of amino acids to be in helical dihedral angles, short range interactions, and long range electrostatics. AGADIR has accurately predicted the α-helical content of more 1000 peptides (including more than 200 protein fragments).

RESULTS

The Cytoplasmic S4–S5 Loop of a K+ Channel, a Candidate Site of 1-Alkanol Action—Fig. 1 illustrates the sequence alignment and putative α-helical wheel projections of the S4–S5 loops in dShaw2 and hKv3.4 subunits (both members of the Shaw family). Although no direct information about the secondary structure of the S4–S5 loop is available yet, the results of other studies are consistent with such a model (18, 19, 23). Between the dShaw2 and related Kv3 subunits there are seven amino acid differences in the S4–S5 loop (Fig. 1), and, compared with dShaw2, hKv3.4 and rKv3.1 K+ channels, expressed in HEK293 cells, express the S4–S5 loop of the Shaw family. The Shaw family (6, 17). Note, however, that the upper right-hand quadrant of the wheel is nearly identical between the two and that most of the differences are in the lower quadrants including various hydrophobic substitutions. In particular, isoleucine occupies position 319 in dShaw2 and glycine occupies the equivalent position in hKv3.4 and the vast major-
ity of voltage-gated K⁺ channels. The mutation G371I in hKv3.4 confers enhanced 1-alkanol sensitivity (6). Thus, a unique hydrophobic residue in the S4-S5 loop of dShaw2 appears to contribute to the site of 1-alkanol action. Here, we report a systematic analysis to test the contribution of the S4-S5 loop to the putative 1-alkanol site in dShaw2 and examine the mechanism of action.

K⁺ Channel Chimeras with Altered Sensitivity to 1-Alkanols—We created the chimeras depicted in Fig. 2 to test whether the S4-S5 loop is indeed a critical determinant of the 1-alkanol binding site in dShaw2 K⁺ channels. The loops were effectively swapped to obtain the KS chimera (hKv3.4 is the host, and dShaw2 is the donor) and the SK chimera (dShaw2 is the host and hKv3.4 is the donor). Fig. 3A (top row) shows wild-type hKv3.4 and dShaw2 outward currents (elicited by a pulse to +40 mV) in the absence and presence of 11 mM 1-butanol. While dShaw2 currents are inhibited 39% in a reversible manner, hKv3.4 currents are nearly unaffected. The apparent equilibrium constants (Kᵢ) estimated from the 1-butanol equilibrium dose-inhibition analysis are 17 and 70 mM for dShaw2 and hKv3.4, respectively (Fig. 3B). Relative to the host channel, the chimeras produce nearly opposite results that closely match the donor channel. Under the same conditions described above, the SK chimera is inhibited only 8.6%, whereas the KS chimera is inhibited 50% (Fig. 3A). The Kᵢ values were 50 and 10 mM for the SK and KS chimeras, respectively (Fig. 3B). The Hill coefficients revealed another interesting difference. While the values for the 1-alkanol-sensitive channels are 1.2 and 1.4 (dShaw2 and the KS chimera, respectively), those for the 1-alkanol-resistant channels were 2 and 2.8 (hKv3.4 and the SK chimera, respectively). This result suggests that the latter channels may exhibit multiple interacting sites.

hKv3.4 channels exhibit rapid inactivation that is mediated by the first 28 amino acids of the channel protein (34–36). The 1-alkanol sensitivity of the NH₂-terminal-deleted hKv3.4 (Δ1–28) was examined to rule out the influence of the inactivation domain on the inhibition by 1-alkanols. As expected, the deletion mutant exhibits no rapid inactivation, and as for the wild-type hKv3.4 and the SK chimera, the inhibition by 11 mM 1-butanol is also very small (4.8%; Fig. 3A). In fact, the dose-inhibition curves from hKv3.4 and Δ1–28 were nearly identical (Fig. 3B). Thus, the presence of the inactivation domain does not determine the low 1-alkanol sensitivity of hKv3.4. It is clear, however, that swapping the S4-S5 loop between hKv3.4 and dShaw2 is sufficient to enhance or reduce 1-alkanol action.

The Inhibition of K⁺ Channels by 1-Alkanols Is Dictated by the Structure of the S4-S5 Loop—Using site-directed mutagenesis, the S4-S5 loop of dShaw2 was progressively converted into that of hKv3.4 and vice versa to examine the contribution of added mutations to the inhibitory action of 1-alkanols (see Table I for nomenclature; Fig. 4). Their effects were assessed by a protocol described above. In dShaw2 (Fig. 4A), a minimum of six mutations was necessary to reduce the inhibitory effect of 1-butanol by 2-fold, and a complete conversion (seven substitutions) produced nearly an additional 2-fold reduction. Interestingly, the F322L and I319G/F322L mutations abolish functional expression, but when combined with other mutations they enhance inhibition by 1-alkanols (F322L/A326T and S3). This effect is reversed by two additional mutations K316R/I328D (see “Experimental Procedures”).

In hKv3.4 (Fig. 4B), a previously characterized single mutation (G371I) was sufficient to enhance inhibition by ~4-fold, and other double and triple mutations (L374F/T378A; G371I/T378A; and K3) also enhanced the inhibition considerably (~3–8-fold).
5-fold). Notice, however, that two of these mutants already include the G371I mutation, and the mutation L374F in two mutants (G371I/L374F and K3) appears to partly reverse the level of inhibition established by G371I. As with F322L in dShaw2, the reverse mutation L374F at the equivalent site in hKv3.4 destroyed functional expression. Also, a hKv3.4 mutant with six substitutions exhibits enhanced inhibition above the level expected in wild-type dShaw2 channels (this mutant is characterized as the KS chimera in Fig. 3). The mutant K5 had already reached the level of inhibition observed with dShaw2 channels (equivalent to that observed after complete conversion with K7). Thus, F374 appears to enhance the inhibition by 1-butanol in the KS chimera (relative to K5), and K379 appears to suppress it (to a level equivalent to that of K5). Although the effects of the mutations in the S4-S5 loops of dShaw2 and hKv3.4 are complex, it is clear that a progressive structural conversion allows an eventual progressive exchange of 1-alkanol sensitivities between dShaw2 and hKv3.4 as the differences in the S4-S5 loops are eliminated. This apparent complexity may reflect equivalent contributions of the amino acid differences and structural changes.

Because the point mutation G371I in hKv3.4 conferred considerably enhanced inhibition by 1-butanol (Fig. 4B), we asked whether the same mutation in the SK chimera (G319I) would be sufficient to reverse and restore the sensitivity to 1-butanol. Answering this question can help to determine how the background of the S4-S5 loop contributes to the 1-butanol sensitivity of the SK chimera. In this chimera (with a low sensitivity to 1-butanol that mimics that of wild-type hKv3.4), the host is dShaw2, and the donor of the S4-S5 loop is hKv3.4 (dShaw2 and hKv3.4 share ~50% amino acid identity in the core of the protein). The G319I mutation in the SK chimera also enhances the inhibition by ~3-fold (Fig. 4A). This result shows that independently of the host or background (wild-type hKv3.4 or dShaw2 in the SK chimera) a specific hydrophobic substitution in the S4-S5 loop is sufficient to confer enhanced inhibition by 1-alkanols. By contrast, other single substitutions that helped to convert the 1-alcohol sensitivity of dShaw2 or hKv3.4 (Fig. 4) had little or no effect on the inhibition by 1-butanol when tested alone. The mutations H372Q (in hKv3.4) and Q320H (in dShaw2) exhibit wild-type sensitivities to 1-butanol, and K327N (in dShaw2) increased the inhibition by ~20% only (data not shown). Altogether, the results from the site-directed mutants demonstrate that the structure of the S4-S5 loop is probably the main factor that dictates a favorable interaction between 1-alkanols and the dShaw2 channel. In particular, the highly conserved glycine in the S4-S5 loop of hKv3.4 appears to create a significant structural constraint to lower the sensitivity to 1-alkanols. Also, the structure of the S4-S5 loop seems critical for proper protein folding, subunit assembly, or channel gating, because some mutations (see above) prevented functional expression. These mutations could have disrupted a putative “leucine zipper” motif that contributes to channel gating and/or maintains a proper tertiary folding between the S4-S5 loop and the S5 region (22, 23). L374 (in hKv3.4) and F322 (in dShaw2) are part of the heptad repeat that forms the putative “leucine zipper.” Presently, it is not clear whether the absence of functional expression of some S4-S5 mutants is the result of no surface expression or the inability of the channels to open.

**Defective Inactivation Gating and Enhanced Inhibition by 1-Alkanols Are Correlated in hKv3.4 Channels**—In rapidly inactivating K⁺ channels, the S4-S5 loop is an important component of a receptor site for the cytoplasmic inactivation particle (18–20). Thus, we hypothesized that mutations that alter the inhibition by 1-alkanols as the result of structural changes in the S4-S5 loop can also disrupt inactivation gating (from closed and open channels) (20, 37). Examining premature inactivation of wild-type and mutant channels helped us to investigate the
presence of that relation. Prepulse inactivation determines the equilibrium probability that resting or activated channels do not inactivate after a prolonged depolarization, even before the magnitude of the depolarizing pulse is sufficient to open a significant fraction of the available channels. This analysis, therefore, helps to determine the relative stability of the activated and inactivated states before channels open. Fig. 5A shows hKv3.4 outward currents elicited by a test pulse to 140 mV after an increasingly depolarized prepulse (10 s). While the wild-type currents (filled symbols) inactivate rapidly in 100 ms, the mutant currents (open symbols) inactivate at a much slower rate. Also, both wild-type and mutant currents decrease as the prepulse depolarization increases (Fig. 5B), but the mutant channels require significantly stronger depolarizations to obtain inactivation equivalent to that of the wild-type channels. This is the result of a 26.5-mV rightward shift of the midpoint of prepulse inactivation. The mutant prepulse inactivation curve also exhibits an apparently reduced slope and an enhanced level of noninactivating current (Fig. 5B). In the chimeric channel (also characterized in Fig. 3), the slow development of inactivation, the rightward shift of the midpoint of prepulse inactivation, and an enhanced noninactivating current demonstrate that inactivation gating is unfavorable. Supporting the idea of a structural change in the S4-S5 loop that simultaneously alters inactivation gating and the inhibition by 1-alkanols, a clear correlation between the inhibition by 1-butanol and the midpoint of prepulse inactivation was clearly observed (Fig. 5C). More depolarized midpoints correspond to the mutants exhibiting greater inhibition by 1-butanol. Thus, the S4-S5 loop helps to control inactivation gating and, as previously suggested (Figs. 3 and 4), probably plays a direct role in the interaction between dShaw2 and 1-alkanols. The dual role of the S4-S5 loop is also apparent from the effects of 1-butanol on the KS chimera (Fig. 3A). There, 1-butanol inhibits the current by ~50% and simultaneously slows inactivation mediated by the N-terminal inactivation particle.

**A Physically Delimited 1-Alkanol Site in dShaw2 K\textsuperscript{\textdagger} Channels—**The inhibition of dShaw2 K\textsuperscript{\textdagger} channels by 1-alkanols involves a saturable site (6). This is clearly demonstrated in Fig. 6A, which shows saturation of the equilibrium dose-inhibition curve for 1-butanol. The best fit of the Hill equation to this curve produced a $K_{1/2}$ of 17 mM and a Hill coefficient of 1.2. In a previous study, we showed that between ethanol and 1-hexanol the $K_{1/2}$ decreases as a function of chain length according to the solubility of the 1-alkanols in organic solvents (the Meyer-Overton rule) (6). This relation reveals that each methylene group contributes an equivalent free energy change to the binding of the 1-alkanol ($\approx 3 \text{kJ/mol}$) and suggests the presence of a hydrophobic site at the water/protein interface. To test whether this ideal behavior is maintained for long-chain 1-alkanols, we estimated the $K_{1/2}$ for 1-heptanol, 1-octanol, 1-decanol, and 1-undecanol (for the last two, the IC\textsubscript{50} values were used as an estimate of the $K_{1/2}$). An apparent leveling off in the $K_{1/2}$-chain length relation can further support the presence of a discrete 1-alkanol site (38). Clearly, for 1-alkanols longer than 1-hexanol the relation between the observed potency and chain length deviates from the line predicted by an ideal behavior and tends to level off (Fig. 6B). Thus, the free energy change

### Table I

**S4-S5 loop mutations examined in this study**

The 13 residues of the loop are labeled as single digit numbers. The actual positions can be obtained from Fig. 1. The results are reported in Figs. 3 and 4. For each mutant, amino acids that remained unchanged are highlighted.

| Channel | S4-S5 Loop Amino Acid Sequence |
|---------|-------------------------------|
| dShaw2 (WT) | GLX1LITTPRASS |
| dShaw2 (A326T) | GLX1LITTPRASS |
| dShaw2 (F322L) | GLX1LITTPRASS |
| dShaw2 (F322L/A326T) | GLX1LITTPRASS |
| dShaw2 (I319G) | GLX1LITTPRASS |
| dShaw2 (I319G/F322L) | GLX1LITTPRASS |
| dShaw2 (I319G/A326T) | GLX1LITTPRASS |
| dShaw2 (S3) | GLX1LITTPRASS |
| dShaw2 (S5) | GLX1LITTPRASS |
| dShaw2 (S6) | GLX1LITTPRASS |
| SK chimera | GLX1LITTPRASS |
| SK chimera (G319I) | GLX1LITTPRASS |

**hKv3.4**

| Channel | S4-S5 Loop Amino Acid Sequence |
|---------|-------------------------------|
| hKv3.4 (WT) | GLX1LITTPRASS |
| hKv3.4 (L374F) | GLX1LITTPRASS |
| hKv3.4 (L374F/T378A) | GLX1LITTPRASS |
| hKv3.4 (G371I) | GLX1LITTPRASS |
| hKv3.4 (G371I/L374F) | GLX1LITTPRASS |
| hKv3.4 (K3) | GLX1LITTPRASS |
| hKv3.4 (K5) | GLX1LITTPRASS |
| hKv3.4 (K5') | GLX1LITTPRASS |
| hKv3.4 (K6) | GLX1LITTPRASS |
| KS chimera | GLX1LITTPRASS |
| KS chimera (K7) | GLX1LITTPRASS |

**Fig. 4. The structure of S4-S5 loop determines the inhibition of K\textsuperscript{\textdagger} channels by 1-butanol.** A and B, the 1-butanol sensitivity of various S4-S5 mutants form dShaw2 and hKv3.4 channels, respectively. The black and white bars represent the data from wild-type channels and the S4-S5 chimeras (Figs. 2 and 3), respectively. Gray bars represent the data from various mutants named according to the nomenclature given in Table I. The numbers in parenthesis indicate the number of oocytes studied. N.E., mutants that exhibited no functional expression. 

- **Fig. 3A** shows the structure of S4-S5 loop determining the inhibition of K\textsuperscript{\textdagger} channels by 1-butanol. A and B, the 1-butanol sensitivity of various S4-S5 mutants form dShaw2 and hKv3.4 channels, respectively. The black and white bars represent the data from wild-type channels and the S4-S5 chimeras (Figs. 2 and 3), respectively. Gray bars represent the data from various mutants named according to the nomenclature given in Table I. The numbers in parenthesis indicate the number of oocytes studied. N.E., mutants that exhibited no functional expression. 

- **Fig. 6A** shows saturation of the equilibrium dose-inhibition curve for 1-butanol. The best fit of the Hill equation to this curve produced a $K_{1/2}$ of 17 mM and a Hill coefficient of 1.2. In a previous study, we showed that between ethanol and 1-hexanol the $K_{1/2}$ decreases as a function of chain length according to the solubility of the 1-alkanols in organic solvents (the Meyer-Overton rule) (6). This relation reveals that each methylene group contributes an equivalent free energy change to the binding of the 1-alkanol ($\approx 3 \text{kJ/mol}$) and suggests the presence of a hydrophobic site at the water/protein interface. To test whether this ideal behavior is maintained for long-chain 1-alkanols, we estimated the $K_{1/2}$ for 1-heptanol, 1-octanol, 1-decanol, and 1-undecanol (for the last two, the IC\textsubscript{50} values were used as an estimate of the $K_{1/2}$). An apparent leveling off in the $K_{1/2}$-chain length relation can further support the presence of a discrete 1-alkanol site (38). Clearly, for 1-alkanols longer than 1-hexanol the relation between the observed potency and chain length deviates from the line predicted by an ideal behavior and tends to level off (Fig. 6B). Thus, the free energy change
per additional methylene group of the long-chain 1-alkanols becomes less significant and approaches 0 (i.e., additional methylenes tend to remain in water, contributing less to the interaction with the putative hydrophobic site). This result is unlikely to be simply the result of a reduced water solubility of the long-chain 1-alkanols, because the maximum aqueous solubilities (C sat) of 1-heptanol, 1-octanol, and 1-decanol were clearly above the estimated K 1⁄2 (Fig. 6B). For 1-undecanol, however, the C sat value lies below the estimated potency. Therefore, the potency of 1-undecanol could not be measured.

Fig. 5. A relationship between inactivation of Kv3.4 K + channels and their inhibition by 1-butanol. A, whole-oocyte currents elicited by a test pulse in prepulse inactivation experiments for the wild-type Kv3.4 and KS chimeric channels (Fig. 1 and corresponding legend). Peak currents were 16 and 1.4 μA from the hKv3.4, and the KS chimera, respectively. The pulse sequence (inset) consisted of two consecutive pulses: a 10-s prepulse to a variable holding potential (from −100 to 0 or +40 mV in 5-mV increments) and a constant 250-ms test pulse to +40 mV (the gap in the lines is a reminder of the much longer duration of the prepulse compared with the test pulse). To allow recovery from inactivation, in the interepisode interval the membrane was held at −100 mV for at least 10 s. B, the relation between prepulse voltage and the fractional current that remains after the prepulse. The symbols and bars represent the mean and S.D. of six independent measurements. The solid lines are the best fit Boltzmann distributions with parameters indicated in the graph (midpoint voltage (V 0.5), slope (s), and noninactivating fraction of the current (I NI)). C, the correlation between the midpoints of prepulse inactivation and the inhibition of the wild-type and mutant hKv3.4 currents by 11 mM 1-butanol. The symbols and bars represent the mean and S.D. of 2–6 independent measurements. S.D. bars are not visible when the radii of symbols exceed their length. No S.D. bars are shown when n < 3.

Fig. 6. The relation between K 1⁄2 and alkyl chain length deviates from the Meyer-Overton rule. A, 1-butanol equilibrium dose-inhibition curve of the dShaw2 K + channels expressed in Xenopus oocytes. These data were well described by the Hill equation (solid line) with the following best fit parameters: K 1⁄2 = 16.6 mM and n H = 1.2. The inset shows representative dShaw2 currents in the absence and presence of 16 mM 1-butanol (currents elicited as described in the Fig. 2 legend). Symbols represent the means of 2–6 independent measurements (S.D. bars are shown for means of three or more determinations). B, the relation between the K 1⁄2 or C sat and the number of carbon atoms in the hydrocarbon chain of the 1-alkanols. The data for ethanol, 1-propanol, 1-pentanol, and 1-hexanol are replotted from an earlier study (6). The K 1⁄2 value for 1-butanol is new and essentially identical to that obtained earlier (6). The graph also shows the potencies of 1-heptanol, 1-octanol, 1-decanol, and 1-undecanol. Symbols and bars are the means and S.D. of 3–6 independent measurements. S.D. bars are not visible when the radii of symbols exceed their length. For 1-decanol and 1-undecanol, single estimations of an IC 50 were made from pooled data including 2–5 measurements at concentrations that ranged between 38 and 200 μM. The maximum possible aqueous concentrations (solubilities; C sat) of a series of 1-alkanols are shown as a reference (1-pentanol, 1-hexanol, 1-heptanol, 1-octanol, 1-nonanol, 1-decanol, 1-undecanol, and 1-dodecanol; open symbols) (43). Note that, except for 1-undecanol, the K 1⁄2 or IC 50 values are below the corresponding C sat values. Solid lines are the best straight lines fitted through the data points included between ethanol and 1-hexanol (K 1⁄2) or between 1-pentanol and 1-dodecanol (C sat). The dashed line represents a second-order polynomial fitted to the K 1⁄2 data points included between 1-hexanol and 1-decanol.
reliably (38). These observations are similar to those reported in a previous study that examined the effect of long-chain 1-alkanols on dShaw2 channels (39). The nonideal behavior of long-chain 1-alkanols was also confirmed in inside-out patches expressing dShaw2 currents (data not shown). Because the interaction between dShaw2 channels and the homologous series of 1-alkanols does not seem to obey the Meyer-Overton rule, it can be concluded that these channels are likely to exhibit a size-delimited pocket for these agents (Refs. 2 and 38; see “Discussion”).

**Kinetic Analysis of the Interaction between dShaw2 and 1-Alkanols Confirms the Presence of an Internal Site**—The analysis of the chimeras demonstrated that the cytoplasmic S4-S5 loop could represent a component of the 1-alkanol site in dShaw2 channels. This implies that the primary site of 1-alkanol action in these channels is internal. To test this hypothesis more directly, we examined the kinetics of the interaction upon application of 1-alkanols to inside-out or outside-out patches expressing dShaw2 currents (internal and external application, respectively). To ensure proper temporal resolution, we implemented a rapid solution switching system with an exchange time of ~2 ms (see “Experimental Procedures”, Fig. 7). Because dShaw2 channels do not inactivate, a sustained membrane depolarization to +50 mV maintained the dShaw2 K⁺ current at steady state, and the time-dependent current inhibition and recovery were monitored upon rapid internal application of ethanol (200 mM) or 1-butanol (40 mM) and upon rapid wash-out, respectively (Figs. 7, A and B). In both instances, the results revealed two clear rapid relaxations, which are well described, assuming exponential time dependences. The development of the inhibition and the recovery are nearly complete in <250 ms (Table II). These relaxations may correspond to the binding and unbinding of the 1-alkanols, because the time constant of the on phase is concentration-dependent, whereas that of the off phase is not (data not shown). In sharp contrast, rapid application of 1-butanol to outside-out patches appears to cause no inhibition (Fig. 7C). After a delay (~1 min), however, time-dependent inhibition in outside-out patches occurs at a much slower rate that is at least 1000-fold slower and reaches the expected steady-state level estimated from equilibrium experiments (Fig. 7D). The slow development of the inhibition in outside-out patches is similar to that observed in whole-oocyte experiments. Clearly, the 1-alkanol site is more readily accessible from the cytoplasmic side of the membrane. Therefore, it is more likely that 1-alkanols interact at an internal site in dShaw2 channels.

**DISCUSSION**

We have examined the interaction between 1-alkanols and a neuronal K⁺ channel encoded by the *Drosophila* dShaw2 gene, as well as various mutant K⁺ channels derived from dShaw2 and hKv3.4 (a human homologue). Earlier studies showed that while clinical concentrations of 1-alkanols selectively inhibited dShaw2 channels, they had little or no effect on rat and human Kv3 channels (6, 17). It is therefore unlikely that Kv3 homologues are targets of 1-alkanols in humans and that these channels mediate general anesthesia. Interestingly, however, the inhibition of dShaw2 channels by 1-alkanols could be explained as a direct interaction involving a saturable site in the channel protein (17). Furthermore, the initial analyses also suggested that the S4-S5 loop could play a role in that interaction and that 1-alkanols mainly stabilize the closed state of the channel. From these earlier observations it became apparent that the differential 1-alkanol sensitivity of dShaw2 and hKv3.4 was probably due to specific structural differences. Thus, although the mammalian homologues were resistant to 1-alkanols, the selective sensitivity of the dShaw2 channel to 1-alkanols and other important properties described here have rendered this protein as a unique model system to learn about the structural bases and mechanisms of general anesthesia and alcohol intoxication.

**A Direct Interaction between dShaw2 Channels and 1-Alkanols at a Discrete Internal Site**—The data presented here demonstrate that a short cytoplasmic segment of 13 amino acids in dShaw2 channels is a necessary determinant of the inhibition of these channels by 1-alkanols (Fig. 3). Further support for this notion is found from the effects of single and

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**Table II**

| 1-Alkanol | ON (ms) | OFF (ms) |
|-----------|---------|----------|
| Ethanol (200 mM) | 31.4 ± 6.4 (n = 3) | 58.9 ± 20.6 (n = 3) |
| 1-Butanol (40 mM) | 28.5 ± 8.8 (n = 4) | 75.5 ± 11 (n = 4) |

1 M. Shahidullah and M. Covarrubias, manuscript in preparation.
combined mutations in that region. Even a single mutation could significantly enhance the inhibition of the channels by 1-alkanols, independently of more global differences in the channel’s primary structure (G371I in hKv3.4, and G319I in the dShaw2 chimera; Fig. 4). Therefore, the simplest explanation for these results is that the S4-S5 loop directly contributes to a discrete 1-alkanol binding site in the channel. However, given the relative complexity of the proteins under study and the methods used here, more complex mechanisms involving allosteric interactions cannot be completely ruled out. In such a case, the mutations in the S4-S5 loop could influence how the 1-alkanols interact with the channel at distant sites (e.g., through effects of the mutations on channel gating).

How could the S4-S5 loop contribute directly to the 1-alkanol site? A possible answer could be obtained from the mutational analysis showing that most S4-S5 mutations do not act reciprocally in dShaw2 and hKv3.4 and that their effects were not unidirectional (i.e., reduced or enhanced inhibition) in either channel (Fig. 4). Therefore, instead of specific amino acid determinants, the 1-alkanol site in dShaw2 channels might rather be determined by the secondary structure of the S4-S5 loop. Structural modeling suggests that the S4-S5 loop may adopt an α-helical structure, which could be broken near its center at a highly conserved glycine corresponding to Gly371 in hKv3.4 (Fig. 1) (23). Uniquely, isoleucine occupies the equivalent position in dShaw2. Thus, changes in the primary sequence could shift the α-helix/random-coil equilibrium. To explore this possibility, the α-helix/coil transition theory (see “Experimental Procedures”) was used to compute the theoretical free energy change (ΔG_H) associated with the α-helix/coil equilibrium in protein fragments corresponding to the S4-S5 loops from wild-type and mutant channels. The ΔG_H values for dShaw2 and hKv3.4 are 8.5 and 10.7 kcal/mol, respectively (the lower the value, the greater the stability of the α-helix segment). For the mutants, on the other hand, ΔG_H range between 7.4 and 11.7 kcal/mol, and for some mutants (e.g., G371I in hKv3.4 and G319I in the SK chimera) there is a good correlation between the inhibition and the theoretical stability of the α-helix. However, the fact that this correlation is not always found suggests more complex interactions involving other regions (see below). Nevertheless, given that the S4-S5 loop appears to be the main determinant of 1-alkanol action and the substantial difference in the 1-alkanol sensitivities of dShaw2 and hKv3.4 channels, it is likely that structural differences between the S4-S5 loops of these channels could be of critical importance. For instance, the α-helix stability of the S4-S5 loop could influence the packing of an amphipathic protein-protein interface, which constitutes a narrow crevice capable of accommodating short-chain 1-alkanols. Adjacent domains forming the interface may include the internal sections of S5 and S6 (Fig. 8).

The analysis of equilibrium and kinetic experiments provided additional evidence in favor of a discrete internal 1-alkanol site in the dShaw2 protein. Equilibrium dose-inhibition curves exhibit clear saturation and can be described assuming a simple bimolecular interaction (Fig. 6). Furthermore, the 1-alkanol site appears to be a pocket of circumscribed dimensions because the K153ρ-chain length relation begins to level off after 1-hexanol (Fig. 6), and the location of the 1-alkanol site in dShaw2 channels is more likely to be internal because the inhibition rates observed upon internal application are more than 3 orders of magnitude faster than those observed upon external application (Fig. 7). The side dependence of the inhibition argues strongly in favor of a protein site and is consistent with the putative location of the S4-S5 loop, the molecular determinant of 1-alkanol action in dShaw2 channels.

Related Studies—In the past 5 years, studies from several other laboratories have utilized genetic manipulations to demonstrate the presence of 1-alkanol and volatile anesthetic sites in ion channel proteins (40). These studies have concentrated on homologous neurotransmitter-gated ion channels and have shown that domain swapping and point mutations affecting various subunits can influence the interaction with 1-alkanols and volatile anesthetics. Blockade of the muscle nicotinic acetylcholine receptor by 1-alkanols involves an interaction with putative pore residues in the M2 segment (7, 9, 41), whereas the NH2-terminal domain of the neuronal acetylcholine receptor appears to contribute to the inhibition of this receptor by 1-alkanols (8, 10). In the first instance, 1-alkanols may directly occlude the open pore. In the second, however, interactions with the agonist binding site or an allosteric modulation of agonist binding or gating are also likely. Potentiation of the γ-amino-butyric acid and glycine receptors by 1-alkanols, on the other hand, is determined by residues in the M2 and M3 segments of the pore-forming subunits (11, 12). However, it is not yet known how such a potentiation is linked to the putative interaction of 1-alkanols with those residues. Altogether, there is clear support for a direct interaction between ion channel proteins and general anesthetics, but the molecular mechanisms that explain how 1-alkanols and other general anesthetics interfere with channel gating remain to be elucidated. Here, we attempted to close that gap by unambiguously identifying the region of the dShaw2 K+ channel that confers 1-alkanol action and providing further molecular and functional information to develop a feasible structural working hypothesis of how 1-alkanols directly interfere with channel gating.

A Structural Model of General Anesthetic Action in dShaw2

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2 M. Covarrubias and T. Harris, unpublished observations.
**Direct Interaction of 1-Alkanols with a K+ Channel**

*K+ Channels*—dShaw2 K+ channels exhibit at least two open states, and 1-alkanols do not affect the lifetime of these states. Instead, they inhibit these channels by preferentially reducing the probability of entering the longer duration open state (6). This observation indicates that 1-alkanols reduce the opening rate governing the transition into the long duration open state and thereby stabilize the closed state(s) of the channel. The main results demonstrate that the S4-S5 loop might be a key component of the putative site of 1-alkanol action in dShaw2 channels. Therefore, in a plausible molecular mechanism, it could be assumed that this internal loop directly participates in channel gating by coupling the voltage-dependent movement of the voltage sensor (activation) with a concerted conformational change that opens an internal gate (26). Others have proposed that the internal segment of S6 constitutes such a gate (25, 28). The S4-S5 loop could be seen as the latch that controls the gate. Thus, 1-alkanols may stabilize the closed state by directly interfering with this latch and effectively locking the channel in a closed conformation. Based on limited direct structural information (24, 28), modeling (23), and the results of this study, Fig. 8 depicts a structural working model of the internal mouth for a tetrameric K+ channel in the closed, open, and locked conformations. According to this model, when the channel activates, the S4-S5 loops move “out of the way” to allow a concerted shutter-like movement of the S6 segments that results in the opening of the central pore. 1-Alkanols could jam the voltage sensor (activation) with a concerted conformational change that opens an internal gate (26). Others have proposed that the internal segment of S6 constitutes such a gate (25, 28).

**Conclusion**—Mutational and biophysical analyses of two K+ channels have provided compelling evidence of a direct interaction between 1-alkanols and the cytoplasmic S4-S5 loop, which constitutes a key element of the channel’s gating apparatus. A structural working hypothesis based on this evidence explains how the S4-S5 loop may act as a latch that couples activation and channel opening and how 1-alkanols exert their anesthetic action by jamming the latch in its closed conformation. The results of this study demonstrate a solid model system to further investigate the structural bases of general anesthesia and alcohol intoxication.

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General Anesthetic Action at an Internal Protein Site Involving the S4-S5 Cytoplasmic Loop of a Neuronal K⁺ Channel
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