Sites of alcohol and volatile anaesthetic action on GABA \(_A\) and glycine receptors

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Volatile anaesthetics have historically been considered to act in a nonspecific manner on the central nervous system. More recent studies, however, have revealed that the receptors for inhibitory neurotransmitters such as \(\gamma\)-aminobutyric acid (GABA) and glycine are sensitive to clinically relevant concentrations of inhaled anaesthetics. The function of GABA \(_A\) and glycine receptors is enhanced by a number of anaesthetics and alcohol, whereas activity of the related GABA \(_\pi\) receptor is reduced. We have used this difference in pharmacology to investigate the molecular basis for modulation of these receptors by anaesthetics and alcohol. By using chimeric receptor constructs, we have identified a region in the \(\gamma\)-aminobutyric acid receptor (GABA \(_A\)) that is modulated by alcohol and volatile anaesthetics. These observations support the idea that anaesthetics exert a specific effect on these ion-channel proteins, and allow for the future testing of specific hypotheses of the action of anaesthetics.

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We then individually mutated 12 residues within this general region of the Gly-receptor α1 subunit to the equivalent amino acid found in the GABA-receptor ρ1 sequence. Each mutant receptor was tested for its sensitivity to ethanol and compared with the wild-type Gly-receptor α1, which is sensitive to 10–200 mM ethanol. Of the 12 mutants tested, the substitution of serine with isoleucine at amino acid 267 (S267I) was the only mutation to abolish the potentiating effects of 200 mM ethanol when tested in either Xenopus oocytes (Fig. 3a) or HEK 293 cells (data not shown). All other mutants tested in oocytes retained the normal enhancement by ethanol of glycine currents, except for Gly-receptor α1 T264S, in which the effect was significantly reduced but not abolished (Fig. 3a). Wild-type and Gly-receptor α1 S267I mutant receptors expressed in oocytes had glycine half-maximal effector concentrations (EC50s) 50% lower than the wild-type receptor.

**Figure 1** Effects of ethanol and enflurane on wild-type glycine α1, GABA ρ1 and chimaeric receptors. Ethanol and enflurane potentiated the effects of glycine on Gly-receptor (Gly-R) α1 (white bars) and inhibited the effect of GABA-receptor ρ1 (black bars). The numbers on the right represent the percentage change of the control agonist response (Fig. 3a) or HEK 293 cells (data not shown). All other mutants tested in oocytes retained the normal enhancement by ethanol of glycine currents, except for Gly-receptor α1 T264S, in which the effect was significantly reduced but not abolished (Fig. 3a). Wild-type and Gly-receptor α1 S267I mutant receptors expressed in oocytes had glycine half-maximal effector concentrations (EC50s) 50% lower than the wild-type receptor.

**Figure 2** Sequence alignment of the TM2 and TM3 domains that are important for the action of ethanol and enflurane. An alignment of the Gly-receptor α1, GABA-receptor α1, α2, β1, β3 and ρ1 polypeptide amino-acid sequences within this general region highlighted 14 amino acids (indicated by asterisks) that are identical in Gly-receptor α1 and GABA-receptor ρ1, but different in β1. Two other amino acids (indicated by hash signs), which differ between the glycine α1 and ρ1 subunits, were also considered to be potentially relevant for alcohol and anaesthetic effects.

**Figure 3** Identification of amino-acid residues in glycine and GABAα receptors critical for the actions of ethanol. a. Mutation of 12 individual amino-acid residues of the Gly-receptor α1 subunit (highlighted in Fig. 2) to their ρ1 counterparts identified Ser 267 as being critical for the modulatory actions of ethanol. In the wild-type receptor, 20–200 mM ethanol increased glycine-receptor currents. All mutants were tested with 200 mM ethanol. The D247R mutant failed to express; A288W (asterisk) was unusual in producing channels that were open in the action of ethanol and enflurane. An alignment of the Gly-receptor α1, GABA-receptor α1, α2, β1, β3 and ρ1 polypeptide amino-acid sequences within this general region highlighted 14 amino acids (indicated by asterisks) that are identical in Gly-receptor α1 and GABA-receptor ρ1, but different in β1. Two other amino acids (indicated by hash signs), which differ between the glycine α1 and ρ1 subunits, were also considered to be potentially relevant for alcohol and anaesthetic effects.
of 240 ± 80 and 155 ± 17 μM, respectively (n = 6). The S267I mutation did not affect the reversal potential for glycine-activated currents, and the half-maximal inhibitory concentration (IC50) of the Gly-receptor antagonist strychnine was 26.9 ± 3.9 nM (n = 9) in the wild-type Gly-receptor and 86.9 ± 15.9 nM (n = 10) in the S267I mutant.

Studies on anaesthetic binding sites in proteins of known structure lead us to hypothesize that an alcohol-binding pocket in glycine receptors could be formed between two or more transmembrane domains, and so we examined TM3 for residues that might also be important. The Gly-receptor α1 subunit has an alanine residue at position 288 in TM3, whereas the corresponding residue in GABA receptor α1 is tryptophan. Because of the non-conservative nature of this substitution, we constructed homomeric glycine α1 A288W mutant receptors (Fig. 3a) to test for ethanol potentiation. These receptors were tonically active in the absence of glycine, precluding tests of ethanol modulation of glycinergetic function. Application of the chloride-channel blocker picrotoxin induced an outward current, consistent with closure of tonically open channels. Ethanol and enflurane, applied in the absence of glycine, were also found to inhibit the tonic activity of this mutant receptor (data not shown).

To determine whether residues homologous with Ser 267 and Ala288 in Gly-receptor α1 were essential for the action of ethanol in GABAα receptors, we next made mutants at the equivalent positions in GABAα receptor α and β subunits. GABAα receptors consisting of wild-type α1β1 subunits show modest potentiation by ethanol (Fig. 3b). Coexpression of wild-type β1 with the α1 (S270I) mutant produced receptors resistant to ethanol enhancement (Fig. 3b), and similar results were obtained using α2(S270I)β1 receptors (data not shown). Coexpression of wild-type α1 with β1(S265I) subunits reduced ethanol enhancement of the resulting receptors to a lesser degree (Fig. 3b), and similar results were obtained with α1β3(N265I) receptors. GABAα receptors comprising both α1 (S270I) and β1 (S265I) subunits exhibited inhibition by ethanol of the effects of GABA.

We reasoned that the Gly-receptor α1 (A288W) mutant exhibits unusual behavior perhaps because it contains one additional bulky tryptophan residue in each of the five TM3 domains, and that mutant receptors containing fewer Trp residues would show normal ligand gating. The A288W Gly-receptor contains ten Trp residues in TM3, whereas the wild-type GABA α1 receptor contains only five, perhaps explaining why the latter channel is not tonically open. We mutated the homologous residues in TM3 of the GABA-receptor α and β subunits to Trp and found that coexpression of GABAαα receptor α2 (A291W) with wild-type β1 produced a receptor with normal ligand gating; indeed, this mutant had increased sensitivity to GABA. These receptors were completely insensitive to ethanol (Fig. 3b). Coexpression of GABAαα receptor β1 (M286W) with wild-type α2 produced receptors in which ethanol still had some effects (Fig. 3b). Coexpression of the GABAαα receptor α (A291W) and β (M286W) mutant subunits produced a receptor with unusual gating properties, similar to those of Gly-receptor α1 (A288W). The current associated with the resting state of this receptor was blocked by 200 mM ethanol in the absence of GABA.

Modulation by enflurane was associated with the same 45 amino-acid domain as the action of ethanol (Fig. 1). All of the original Gly-receptor mutants tested retained normal or increased sensitivity to enflurane (Fig. 4a). Although the S267I mutation rendered the glycine α1 receptor resistant to the potentiating effects of ethanol, it did not markedly affect potentiation by enflurane. However, substitution of Ser 267 with tyrosine, an even larger amino-acid residue than isoleucine, produced a mutant glycine receptor (S267Y) that

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**Figure 4** Amino-acid residues in glycine and GABAα receptors critical for the action of the volatile anaesthetic enflurane. **a**, Mutation of Ser 267 to tyrosine, but not to isoleucine, abolished the effect of enflurane on Gly-receptor α1. All other mutants tested responded normally or supranormally to 1 mM enflurane. **b**, Expression of either GABAαα receptor α1 (S270I) or β1 (S265I) TM2 mutants significantly decreased (P < 0.001) the enhancement by 1 mM enflurane. Expression of either GABAαα receptor α1 (A291W) or β1 (M286W) TM3 mutants also abolished (P < 0.001) the enhancement of GABA responses by 1 mM enflurane, but with no difference between the effects of mutating either the α or β mutant. Mutant subunits are depicted by an asterisk. **c**, Recordings from HEK 293 cells (n = 4) coexpressing wild-type GABAαα α2β1 receptors or mutant α2(S270I)β1 or α2(A291W)β1 receptors. Wild-type α2β1 receptors (top) show significant potentiation by 0.23 and 0.46 mM enflurane, unlike α2(S270I)β1 (middle) and α2(A291W)β1 (bottom) receptors. Horizontal bars indicate the period of drug application. An EC50 concentration of GABA for each receptor was used in these experiments.
was virtually insensitive to 1 mM enflurane (Fig. 4a), suggesting that Ser 267 is also important for the actions of enflurane on Gly-receptors. The M287I mutation exhibited significantly greater enhancement by enflurane than the wild-type Gly-receptor (Fig. 4a), but this was not observed for ethanol (Fig. 3a).

We then investigated the effects of mutations in TM2 and TM3 of GABA<sub>A</sub>-receptor α and β subunits on sensitivity to enflurane. Mutation of Ser 270 in TM2 of GABA<sub>A</sub>-receptor α1 or α2 to isoleucine completely abolished 1 mM potentiation by enflurane: the same mutation in GABA<sub>A</sub>-receptor β1 had a much weaker effect (Fig. 4b). Coexpression of these two TM2 mutants produced a receptor with normal GABA gating but that exhibited inhibition by enflurane (Fig. 4b). Mutation of Ala 291 in TM3 of GABA<sub>A</sub>-receptor α2 or Met 286 in TM3 of GABA<sub>A</sub>-receptor β1 to tryptophan completely abolished enflurane potentiation (Fig. 4b, c). Similar results were obtained in each case with another volatile anesthetic, isoflurane (data not shown).

Finally, we investigated whether these mutations in GABA<sub>A</sub> receptors were specific to ethanol or enflurane, or whether they caused general insensitivity to multiple anesthetic modulators. The intravenous anaesthetic propofol, like the volatile anaesthetics, enhances GABA<sub>A</sub> receptor function in a manner that does not depend on the presence of the γ subunit in the receptor<sup>1,16</sup>. The two point mutations GABA<sub>A</sub>-receptor α2 (S270I) and GABA<sub>A</sub>-receptor α2 (A291W), which abolish regulation of the receptor by isoleucine and enflurane (Fig. 4c), did not alter the potentiation of GABA by 1 μM propofol (wild-type α2β1, 124 ± 46%, n = 7; α2(S270I)β1, 88 ± 29%, n = 7; α2(A291W)β1, 106 ± 9%, n = 6).

Studies of the action of anesthetics and alcohol on related receptors, such as the serotonin-3 (5-HT<sub>3</sub>) and nicotinic acetylcholine receptors (nAChR), have yielded conflicting results concerning their site of action. Experiments on a single chimera between the nAChR α7 and 5-HT<sub>3</sub> receptor subunits suggest that the N-terminal domain of nAChR α7 is responsible for alcohol<sup>17</sup> and anaesthetic<sup>18</sup> inhibition, but mutations in TM2 increase receptor sensitivity to anesthetics and alcohols in studies of mouse muscle nAChR subunits<sup>19</sup>. In contrast, our data clearly implicate TM2 and TM3. In particular, our result with chimera C6 shows that this region is not only necessary but also sufficient for the enhancement of receptor function by enflurane and ethanol. Studies using the cysteine substitution mutagenesis method have identified residues in TM2 of the GABA<sub>A</sub>-receptor α1 subunit that are accessible from within the aqueous pore. Ser 270 is not among these, and is believed to face directly away from the channel lumen into the hydrophobic interior of the protein<sup>20</sup>. One possibility suggested, although not proven, by our findings is that Ser 270 and Ala 291 in GABA<sub>A</sub>-receptor α1 are part of a binding pocket formed between at least two transmembrane domains. Support for such an idea is provided by the observation that the GABA<sub>A</sub> receptor mutations specifically abolished modulation by ethanol and enflurane but did not alter modulation by propofol. Considerable data support the existence within various proteins of sequestered hydrophobic binding sites for small anaesthetic molecules. The existence of such small binding pockets is suggested by the observation of "cut-off" phenomena in anaesthetic activity as molecular volume is increased<sup>10,21</sup>. Another possibility is that the anesthetics bind elsewhere on the glycine and GABA<sub>A</sub> receptors and that the residues identified here play some other crucial role in transducing their effects. Physical methods will ultimately be required to resolve this issue beyond reasonable doubt, but high-resolution structures are not yet available for the receptors studied here. Techniques such as X-ray crystallography have already been applied to other proteins of known structure, demonstrating the existence of specific anaesthetic-binding sites between adjacent α-helices in both myoglobin<sup>22,23</sup> and adenylate kinase<sup>24</sup>. The creation of receptor mutants such as Gly-receptor α1 (S267I) and GABA<sub>A</sub>-receptor α2 (A291W), which lack alcohol or enflurane sensitivity but otherwise function normally, should help further investigations into the mechanisms of action of volatile anaesthetics and alcohol. The availability of mutants such as these, coupled with transgenic animal technology, now allows the examination of the involvement of specific receptor subunits in the behavioural actions of these compounds. If a mouse expressing a mutated ethanol- or anaesthetic-insensitive receptor were to display altered behavioural sensitivity to those drugs when compared with a wild-type animal, this would provide strong supporting evidence for the role of that receptor in the actions of alcohols or volatile anaesthetics in vivo. It would thereby help in distinguishing between nonspecific and specific hypotheses of anaesthetic action.

Methods

Construction of chimaeras and mutants. The C1 (N-Gly-ALLE/YAAV-p-c in TM3), C2 (N-p-SVLE/YAAV-Gly-C in TM3), C4 (N-Gly-AAPA/RVPL-p-c in TM1/TM2 loop) and C5 (N-p-AVPA/RVGL-Gly-C in TM1/TM2 loop) chimaeras were constructed by the introduction of restriction sites into homologous regions of the cDNAs encoding the human Gly-receptor α1 and GABA-receptor β1 subunits. XbaI sites were engineered into the nucleotide sequences of Gly-receptor α1 and GABA-receptor β1 corresponding to the region close to the end of TM1 (Fig. 1), and BstII sites were incorporated into the cDNA sequences encoding the loops between TM1 and TM2 (Fig. 1). Chimaeras were then constructed by standard techniques<sup>25</sup>. The C3 chimaera (N-p-TLMV/ILSW-Gly-C in TM1) was constructed using the random chimaeragenesis technique<sup>26</sup>. Gly-receptor α1 and GABA-receptor β1 cDNAs were subcloned into the pBK-CMV vector (Stratagene) that had been modified by the removal of the lac promoter and the lacZ ATG. The GABA-receptor α1 and Gly-receptor α1 cDNAs were inserted into this vector in tandem in the SpeI and ClalKpnI sites, respectively. The resulting tandem-containing plasmid was linearized with SalI and XbaI; unique restriction sites for these two enzymes were both located between the two parental cDNAs. The linearized DNA was gel purified (QIAEX Resin, Qiagen, Chatsworth, CA) and 200 ng of DNA was used to transform Escherichia coli DH5α (ME; Gibco/BRL). Resultant colonies were screened for the occurrence of homologous recombination events and the presence of GABA-receptor β1/Gly-receptor α1 chimaeras. Finally, chimera C6 was produced by introducing BstIII sites into the cDNA sequences of chimaeras C1 and C2, and then exchanging fragments in the usual manner. All chimaeras were sequenced (Sequenase version 2.0, U.S. Biochemical) to identify the precise location of the chimaera junctions. Point mutations in GABA-receptor α1, α2, β1, β3, β1 and Gly-receptor α1 subunits were performed on cDNAs subcloned into pcIS2 or pBK-CMV vectors (Stratagene) using either the QuikChange site-directed mutagenesis kit (Stratagene) or the USE kit (Pharmacia Biotech, Piscataway, NJ). All point mutations were verified by double-stranded DNA sequencing.

Electrophysiological recording. Xenopus oocytes were isolated and injected<sup>27</sup> with human cDNAs (1.5 ng per 30 nl) subcloned into pcIS2 or a modified pBK-CMV vector. Electrophysiological recording was performed as described<sup>27</sup>. All drugs were applied for 30–180 s and the maximum peaks obtained were measured. To determine the concentration of GABA or glycine producing 10% of the maximal effect (EC<sub>10</sub>), 1 mM GABA or 10 mM glycine was used to produce a maximal current against which all other concentrations of ligand were compared. The bath concentration of enflurane was determined by gas chromatography<sup>28</sup>. HEK 293 cells (ATCC) were grown on glass coverslips and transfected with cDNAs<sup>29</sup>. Recordings were made in whole-cell mode at −60 mV using patch pipette solutions based on 145 mM N-methyl-D-glucamine HCl and extracellular saline based on 145 mM NaCl as described<sup>8</sup>. All drugs were applied locally to the cell using a motor-driven solution exchange device<sup>25</sup>. Unless otherwise stated, all data in Figs 1, 3 and 4 were obtained in Xenopus oocytes using a concentration of agonist (EC<sub>50</sub>) appropriate for the receptor tested, and are expressed as mean ± s.e.m. of between 4 and 14 oocytes.

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Single cyclic nucleotide-gated channels locked in different ligand-bound states

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Cyclic nucleotide-gated (CNG) channels are directly activated by the binding of several ligands\textsuperscript{1-4}. For these channels as well as for other allosteric proteins, the functional effects of each ligand-binding event have been difficult to assess because ligands continuously bind and unbind at each site. Furthermore, in retinal rod photoreceptors the low cytoplasmic concentration of cyclic GMP means that channels exist primarily in partially liganded states, so it is important to determine how such channels behave.


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Figure 1 Covalent attachment of ligands persistently activates channels. a. Top, structure of APT-cGMP; bottom, representation of APT-cGMP binding and, upon photolysis, covalently attaching cGMP moieties to channel subunits. b. Traces from a single channel recording at +50 mV before and after covalent attachment of ligand. Dashed lines indicate closed (C) levels; open, O. Top, maximal activation with 1 mM cGMP; middle, after application of APT-cGMP and exposure to UV light, the patch was washed for 20 min to remove all unthereted ligand. Maximal activation persisted in the absence of free cGMP. Bottom, 1 mM Mg\textsuperscript{2+} blocked the persistent current.

Previous studies of single channels have suggested that they occasionally open to subconducting states at low cGMP\textsuperscript{2,3,8–10} but the persistent currents and these how and they arise is poorly understood. Here we combine the high resolution of single-channel recording with the use of a photoaffinity analogue of cGMP\textsuperscript{11,12} that tethers cGMP moieties covalently to their binding sites to show single retinal CNG channels can be effectively locked in four distinct ligand-bound states. Our results indicate that channels open more than they would spontaneously when two ligands are bound (~1% of the maximum current), significantly more with three ligands bound (~33%), and open maximally with four ligands bound. In each ligand-bound state, channels opened to two or three different conductance states. These findings place strong constraints on the activation mechanism of CNG channels.

Channels formed from the a-subunit of the retinal rod CNG channel\textsuperscript{13} were expressed in Xenopus laevis oocytes and studied in excised membrane patches. This produced homomultimeric channels with four cGMP-binding sites\textsuperscript{14,15}. Patches containing a single channel (see Methods) were exposed to the photoaffinity analogue 8-p-azidophenacylthio-cGMP (APT-cGMP\textsuperscript{12}; Fig. 1a) and illuminated with long-wavelength ultraviolet light. Previous work on multichannel patches showed that this procedure resulted in persistent currents that were not removed by extensive perfusion with cGMP-free solution. The persistent currents had several macroscopic properties that precisely matched the behaviour of currents activated by cGMP, indicating that cGMP moieties were covalently tethered to the binding sites\textsuperscript{13,12}. Our goal was to lock single channels into each possible ligand-bound state (Fig. 1a, bottom). Figure 1b compares the behaviour of a single channel fully activated by either cGMP, or by covalently tethered cGMP moieties: in saturating...