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Divergence of Volatile Anesthetic Effects in Inhibitory Neurotransmitter Receptors

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Background: **The mechanism of volatile anesthetic (VA) action is unknown. Inhibitory receptors for the neurotransmitters γ-aminobutyric acid (GABA) or glycine are typically positively modulated by VAs and may be important targets for their action. The existence of a GABA receptor subtype (ρ), which is uniquely inhibited by VAs, suggested a chimeric receptor approach to identify portions of these proteins that may be necessary for anesthetic effects.**

Methods: **A silent mutation resulting in the addition of a unique restriction enzyme recognition site was introduced in GABA receptor type A α_2, glycine α_1, and ρ subunit cDNAs. Chimeras were constructed by rejoining restriction digest fragments and were expressed in *Xenopus* oocytes. Modulation of submaximal agonist-evoked peak currents by the VAs chloroform, enflurane, halothane, or isoflurane was measured using two-electrode voltage clamp.**

Results: **Four chimeras were constructed and designated glyrho, rhogly, $\alpha_2$rho, and rhoα_2. Glyrho formed glycine-gated receptors with currents that were enhanced by chloroform or halothane but were inhibited by enflurane or isoflurane. Chimeras rhogly and rhoα_2 each formed GABA-gated receptors with currents that were inhibited by chloroform or halothane but enhanced by enflurane or isoflurane.**

Conclusions: **These data show, for the first time, functional divergence of VA action on a single protein target. The VAs in this study fall into two distinct groups with respect to their effects on these receptors. This grouping parallels the chemistry of these compounds. Our results support the involvement of multiple protein domains in the mechanism of VA modulation of GABA and glycine receptors.**

THE molecular mechanism of volatile anesthetic (VA) action in the central nervous system remains controversial. Neurotransmitter receptor proteins are critical to the regulation of central nervous system excitability and are likely targets of VA action. VA effects may be explained, in part, by the ability to potentiate neuronal inhibition mediated by the neurotransmitters γ-aminobutyric acid (GABA) and glycine at GABA and glycine receptors, respectively.[1]

γ-Aminobutyric acid and glycine receptors are members of a superfamily of ligand-gated ion channels, which also include the nicotinic acetylcholine as well as serotonin type 3 receptors.[2] GABA and glycine receptors are members of a subfamily of these channel proteins that are selective for chloride ion. They are believed to exist as pentameric complexes formed by protein subunits; each subunit contains four putative transmembrane domains (M1–M4).[3] Within this subfamily are subgroups that show different sensitivities to VAs. Although most GABA type A ($GABA_A$) receptors[4] and glycine receptors[5] are positively modulated at submaximal agonist concentrations, the ρ subtype of GABA receptors (sometimes referred to as $GABA_C$) is insensitive or negatively modulated by these agents.[6]

Native $GABA_A$ receptors exist in the mammalian central nervous system largely as heteromers of α, β, and γ subunits[7] with stoichiometry $\alpha\alpha\beta\beta\gamma$,[8,9] but receptors expressed *in vitro* using only α and β retain anesthetic modulation.[10] The native strychnine-sensitive glycine receptor is composed of α and β subunits,[11] but α subunits readily form homomeric receptors that retain agonist and antagonist[12] as well as anesthetic sensitivity.[13] GABA ρ receptors are believed to exist as homomers, and expression of the ρ subunit reconstitutes the pharmacology of the native receptor.[14] Since the primary amino acid structures of these receptors are known, their differences in VA sensitivity prompt the question whether structural motifs shared by glycine and $GABA_A$ as opposed to GABA ρ receptors, confer the pattern of VA modulation. To gain information about the molecular basis of these interactions, chimeric receptors were constructed by combining segments of positively modulated ($GABA_A$ or glycine) receptors with those from negatively modulated (GABA ρ_1) receptors. Such a chimeric approach has been successfully applied to the characterization of other cell surface receptor proteins. Two-electrode voltage clamp was used to record currents from *Xenopus* oocytes expressing chimeric receptors in the absence and presence of the VAs chloroform, enflurane, halothane, or isoflurane. It was hypothesized that the presence of specific portions of the native receptor proteins would be permissive for their respective anesthetic pharmacology. The pattern of modulation (*i.e.*, whether VAs enhanced or inhibited responses) in chimeras would identify specific protein domains involved in the interaction of VAs with native receptors.

Materials and Methods

Preparation of Chimeras

The $GABA_A$ α_2, glycine α_1, and GABA ρ_1 receptor complementary DNA (cDNA) subunits were subcloned into the plasmid expression vector pRK7. A potential

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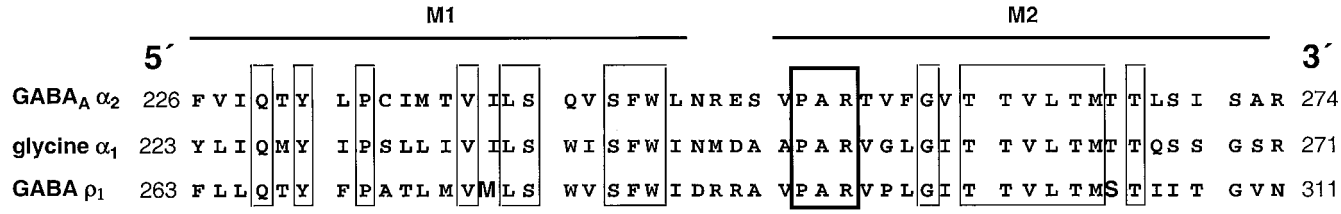

Fig. 1. Partial sequence amino acid alignment of wild-type GABA$_A$ α_2, glycine α_1, and GABA ρ_1 subunits. 5' and 3' indicate orientation toward to N-terminus and C-terminus, respectively. M1 and M2 regions are indicated by dark overline. Sequence identity among subunits is indicated by "boxed" residues. A *BssH* II site was inserted in the M2 "PAR" box.

chimeric splice site, containing the invariant amino acid triplet proline alanine arginine, was identified at the 5' end of the second transmembrane (M2) domain in all subunits (fig. 1). A silent mutation—a nucleotide substitution that does not alter amino acid sequence—was introduced into subunit cDNA clones to create a unique recognition site for the DNA restriction enzyme *BssH* II. Site insertion was performed using a commercially available kit. Briefly, thermostable, high-fidelity, proofreading *Pfu* DNA polymerase was used in the polymerase chain reaction to generate full-length nicked DNA products using primer oligonucleotides with the desired mutation and appropriate templates (*Bam H* I-linearized constructs of each receptor cDNA in plasmid pRK7). Primers were designed and obtained for each subunit as follows: glycine α_1 5′TGCTGCACCTGCGCGCGTGGGCCTAGGCA3′ (sense, nucleotides 825–853) and 5′TGCCTAGGCCCACGCGCGCAGGTGCAGCA3′ (antisense, nucleotides 825–853); GABA ρ_1 5′CAGAGCCGTGCCTGCGCGCGTCCCCTTAGGTATC3′ (sense, nucleotides 921–954) and 5′GATACCTAAGGGGACGCGCGCAGGCACGGCTCTG3′ (antisense, nucleotides 921–954); GABA$_A$ α_2 5′GAATCTGTGCCTGCGCGCACTGTGTTTGGAG3′ (sense, nucleotides 829–859) and 5′CTCCAAACACAGTGCGCGCAGGCACAGATTC3′ (antisense, nucleotides 829–859).

Polymerase chain reaction products were treated with *Dpn* I endonuclease, which is specific for *dam*-methylated DNA. Because DNA (including plasmid preparations) from virtually all *Escherichia coli* strains is *dam*-methylated and susceptible to *Dpn* I digestion, this step removes any parental template DNA, selecting for mutation-containing newly synthesized DNA. *Dpn* I–digested polymerase chain reaction products were used to transform *E. coli* XL1-Blue competent cells to repair and replicate the mutant plasmid. Plasmid DNA was then prepared by large-scale culture of an appropriately antibiotic-resistant colony and purified by alkali lysis and CsCl gradient centrifugation. All mutants were partial-length DNA sequenced to verify splice regions. Dideoxy sequencing of double-stranded DNA and rescued single-stranded DNA was performed according to established protocols with minor variations.[15]

Mutant cDNA–plasmid constructs (fig. 2A) were digested with *Nde* I and *BssH* II (New England Biolabs, Inc., Beverly, MA) to obtain two restriction digest fragments (fig. 2B). The first fragment (1.6–1.9 kb) contained a 0.7-kb portion of the plasmid from its unique *Nde* I site, extending up to and 0.9 kb into each subunit cDNA and ended at the newly introduced *BssH* II site. The second, larger (4.8–5.2 kb) *BssH* II–*Nde* I fragment consisted of residual plasmid and insert. Fragments were isolated by gel electrophoresis, exchanged between pairs, and ligated to generate chimeric cDNAs (fig. 2C). All manipulations of DNA, including preparation of restriction digests and ligations, were performed accord-

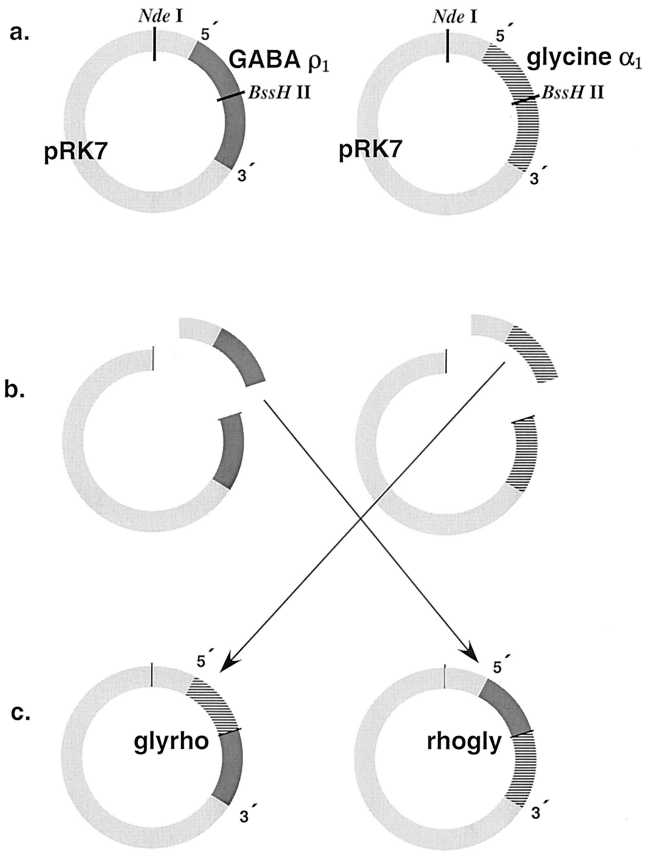

Fig. 2. Schematic for production of chimeric cDNAs. (*A*) Wild-type receptor cDNAs (GABA ρ_1, dark gray; glycine α_1, striped) in plasmid (pRK7, light gray). Note restriction enzyme recognition sites for *Nde* I and *BssH* II. 5' and 3' as defined in figure 1. (*B*) Fragments formed after digest with *Nde* I and *BssH* II. (*C*) Exchange of smaller fragments and ligation to generate two chimeric cDNAs.

ing to the protocols of the manufacturer or according to commonly accepted procedures.[16]

Expression of Chimeras

Chimera complementary RNAs (cRNAs) were generated *in vitro*. Phage polymerase SP6 was used to make full-length capped RNA transcripts from *BamH* I–linearized chimera template DNA using a commercial kit according to the manufacturer's protocol.

Adult female *Xenopus laevis* frogs were purchased from Nasco (Fort Atkinson, WI). Oocytes were obtained from frogs according to a protocol approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania and consistent with National Institutes of Health and American Physiological Society guidelines. Frogs were anesthetized by immersion in 0.2% iced 3-aminobenzoic acid ethyl ester methanesulfonate salt. An incision was made in the abdominal wall, and a small piece of ovary was excised and manually dissected to free oocytes from surrounding tissue. After removal of the oocyte follicular layer by collagenase incubation in OR2 solution (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5), oocytes were washed and placed in ND96 medium (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, 5 mM pyruvate, pH 7.5). Stage V–VI oocytes[17] were injected in the vegetal pole with cRNA (50 nl; 10–25 ng of cRNA transcript suspended in diethyl pyrocarbonate–treated water) using a digital microdispenser. Given that the wild-type $GABA_A$ α_2 subunit does not form functional homomeric receptors,[18,19] a subset of the oocytes injected with chimeras containing sequences from $GABA_A$ α_2 were co-injected with wild-type $GABA_A$ β_1 subunit cRNAs to facilitate heteromeric receptor expression. Oocytes were maintained at 18°C in ND96 solution with antibiotic (50 μg/ml gentamicin) for 2–6 days before use in experiments.

Electrophysiologic Recording

Agonist-evoked currents were measured using a two-electrode voltage clamp amplifier. For recording, oocytes were positioned in a small chamber (approximately 100-μL volume) and continuously superfused (5 ml/min) with ND96 buffer solution. Oocytes were impaled with borosilicate glass microelectrodes filled with 3 M KCl (typical resistance, 0.5–3 MΩ). Currents were low-pass filtered and digitized using an A/D interface with chart recording software and stored on a computer hard disk.

Chimera-injected oocytes were screened for agonist (GABA or glycine) specificity. Dose–response studies (GABA, 0.1 μM to 10 mM; or glycine, 1 μM to 100 mM) established agonist sensitivity of each construct. Peak oocyte currents evoked by EC_{10} (that which evoked 10% of the maximal current response) for the appropriate agonist were measured in the absence and presence of 1.2 mM chloroform, 0.75 mM enflurane, 0.3 mM halothane, or 0.4 mM isoflurane at 25°C. These anesthetic concentrations each approximate 1.5 times the minimum alveolar concentration (MAC) for humans,[20] with the exception of chloroform, for which the concentration was estimated from published MAC values for dogs.[21] Small agonist doses were used to increase sensitivity for detecting VA modulation, since VA effects are most prominent in this agonist range.[1]

All drugs were dissolved in buffer and applied by gravity-fed superfusion. VAs were applied to the preparation *via* the extracellular medium. Each VA was preapplied 5 min to assure equilibration. Anesthetic solutions were prepared by addition of liquid phase anesthetic to medium in airtight containers (plastic intravenous solution bags) that were vortexed (1 min) and stirred to allow equilibration (30 min). Appropriate volumes of liquid anesthetic were calculated using published MAC values, solubility coefficients, and temperature coefficients of solubility.[20] Experiments were repeated on at least four individual oocytes. Anesthetic concentrations in the assay chamber were verified by gas chromatography of aliquots removed during experiments; gas-tight Hamilton syringes were used, and aliquots were immediately deposited in airtight septum-capped vials. VA concentrations sampled from the oocyte bath remained at or above 90% of the applied drug level.

Halothane was obtained from Halocarbon Laboratories (River Edge, NJ), and isoflurane was obtained from Anaquest (Madison, WI). All other chemicals were obtained from Sigma (St. Louis, MO).

Statistical Analysis

Data were normalized for each oocyte to eliminate variation in control currents caused by receptor expression. Mean peak currents were analyzed with curve-fitting software to establish EC_{10}, EC_{50}, and Hill coefficients for each chimera.

Data from anesthetic experiments were expressed as mean $\pm$ SD percent change from control current and were analyzed by unpaired Student *t* test. $P < 0.05$ was considered significant.

Results

Chimera Nomenclature

Chimeric constructs (fig. 3) were designated as follows. The chimera with glycine α_1 sequence extending from its N-terminus to the M2 splice site, and the remaining sequence from the splice to C-terminus contributed by GABA ρ_1, was designated glyrho. The chimera with sequence from N-terminus to splice contributed by GABA ρ_1, and the remainder from glycine α_1, was designated rhogly. The chimera with $GABA_A$ α_2 sequence

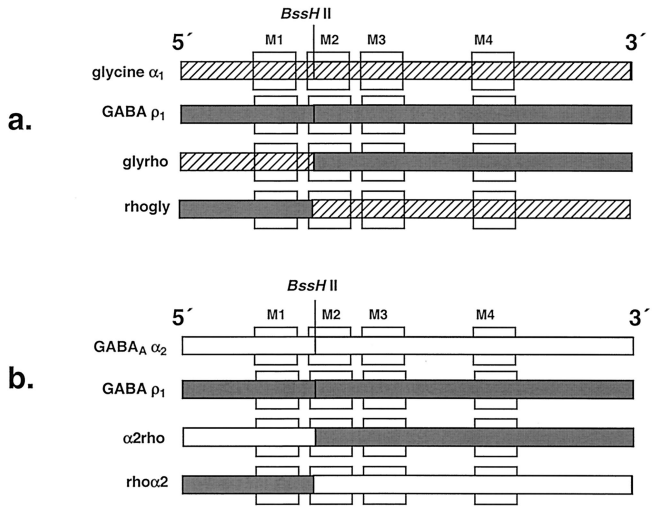

Fig. 3. Chimeric receptor constructs indicating relative contributions from wild-type receptor subunits. (*A*) glyrho and rhogly; (*B*) $\alpha_2$rho and rhoα_2. M1–M4 indicate transmembrane domains. Note *BssH* II restriction enzyme recognition site in M2, which forms splice site. 5' and 3' as defined in figure 1.

from N-terminus to the splice and the remainder from GABA ρ_1 was designated $\alpha_2$rho. The chimera with GABA ρ_1 sequence from N-terminus to splice, and the remainder from $GABA_A$ α_2, was designated rhoα_2.

Responses of Chimeric Receptors to Agonists

The agonist pharmacology of chimeras expressed in *Xenopus* oocytes is summarized in table 1. The glyrho construct showed specificity for glycine, while the rhogly and rhoα_2 constructs each showed specificity for GABA. Co-injection of wild-type $GABA_A$ β_1 did not appear to alter rhoα_2 responses. Oocytes injected with $\alpha_2$rho (irrespective of whether wild-type $GABA_A$ β_1 was co-injected) showed no response at GABA up to 1×10^{-2}. Dose–response curves for expressed chimeras are shown in figures 4A–C.

Volatile Anesthetic Modulation of Chimeric Receptors

Coapplication of VAs altered control responses to agonists. Representative oocyte recordings are shown in figures 5A–C. Effects of VAs are depicted graphically in figure 5D. Glycine-evoked currents in glyrho receptors were inhibited by enflurane ($-10 \pm 2.0\%$; n = 4) or isoflurane ($-17.7 \pm 4.4\%$; n = 6) but were enhanced by chloroform ($12 \pm 9.6\%$; n = 5) or halothane ($15 \pm 4.2\%$; n = 5). GABA-evoked currents in rhogly receptors were enhanced by enflurane ($11.8 \pm 5.8\%$; n = 4) or isoflurane ($16 \pm 6.6\%$; n = 4) but were inhibited by chloroform ($-11 \pm 7.6\%$; n = 4) or halothane ($-14 \pm 6.0\%$; n = 5). Currents in oocytes expressing rhoα_2 were inhibited by chloroform ($-15 \pm 10.5\%$; n = 5) or halothane ($-20 \pm 6.8\%$; n = 4) but were enhanced by enflurane ($24 \pm 11.6\%$; n = 4) or isoflurane ($30 \pm 11.6\%$; n = 6). All VA effects were significant *versus* control ($P < 0.05$).

To explore the dependence of these effects on VA concentration, a set of additional experiments were performed with the chimera glyrho. Three concentrations of VA approximating 1, 2, and 3 MAC (0.2 0.4, and 0.6 mM halothane or 0.3, 0.6, and 1.0 mM isoflurane, respectively) were co-applied with GABA EC_{10}. Isoflurane inhibited currents in oocytes expressing this chimera at all concentrations except the highest, whereas halothane enhancement of currents was observed only at 1.5 or 2 MAC. These results are summarized in table 2.

Discussion

The agonist selectivity of our chimeras, formed from native receptors with dissimilar agonists, were consistent with existing data that agonist binding domains for GABA and glycine receptors are found in the large 5' extracellular regions.[22,23] Yet our chimeras showed variance in agonist sensitivity when compared with wild-type receptors. The sensitivity of chimera rhogly to GABA is approximately 10-fold greater than wild-type ρ_1 (ρ_1 EC_{50} is 1.5 μM GABA in *Xenopus* oocytes; data not shown). The chimera rhoα_2 showed approximately 100-fold less GABA sensitivity than ρ_1. The chimera glyrho was approximately 100-fold less sensitive to glycine compared with wild-type glycine α_1, but within the range of apparent agonist affinity observed among all known glycine α subunit isoforms.[24] Whether such changes reflect alterations in the agonist binding equi-

Table 1. Agonist Pharmacology of Chimeras Expressed in *Xenopus* Oocytes

Injected cRNA	Glycine EC_{50} (M)	GABA EC_{50} (M)	Hill Coefficient	n
glyrho	$1.2 \pm 0.04 \times 10^{-3}$	*	2.2	5
rhogly	*	$1.4 \pm 0.09 \times 10^{-7}$	2.1	4
rhoα2	*	$9.8 \pm 1.2 \times 10^{-6}$	0.9	4
rhoα2 + GABA β_1	*	$9.7 \pm 1.1 \times 10^{-6}$	0.8	4
α2rho	*	*	NA	8
α2rho + GABA β_1	*	*	NA	8

Mean ± SEM. See text for chimera nomenclature.

cRNA = complementary RNA; EC_{50} = agonist concentration eliciting a peak current that is 50% of the maximal response; GABA = γ-aminobutyric acid; n = sample size (number of oocytes assayed).

* No detectable response.

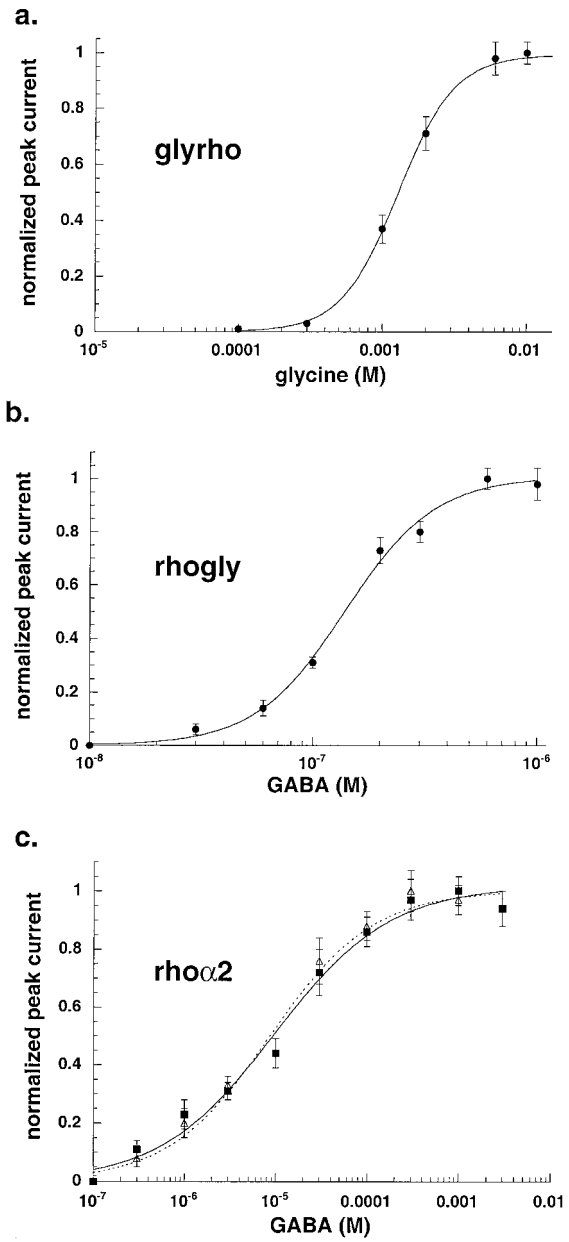

Fig. 4. Agonist dose–response curves for chimeras expressed in *Xenopus* oocytes. Data are normalized mean ± SEM of peak currents. (*A*) glyrho; (*B*) rhogly; (*C*) rhoα_2 alone (closed boxes, solid line) and rhoα_2 co-injected with wild-type GABA$_A$ β_1 (open triangles, dashed line). See table 1 for specific fitted parameters.

librium or changes in efficacy of the transduction mechanism for channel gating cannot be determined from these data. Rhogly and rhoα_2 showed qualitatively similar responses to VAs despite different agonist sensitivities. Therefore, such differences in agonist sensitivity may not be important to the VA effects demonstrated here.

The failure to detect functional receptors in oocytes injected with $\alpha_2$rho may result from one or more possible occurrences, including disruption of assembly or posttranslational processing, assembly with failure to insert properly in the cell membrane, or assembly with cell surface expression but loss of function. The extracellular domains of ligand-gated ion channels are crucial for assembly of functional receptors.[11] The extracellular domains of chimera $\alpha_2$rho were contributed by a GABA$_A$ α subunit, which does not form functional homomeric receptors,[18,19] possibly accounting for the observed lack of expression. In contrast, the rhoα_2 chimera, which has an extracellular component from the "homomer-native" GABA ρ, did form functional homomeric receptors. To facilitate possible assembly of an $\alpha_2$rho–GABA$_A$ β heteromeric receptor, GABA$_A$ β subunit cRNAs were coinjected with $\alpha_2$rho. The fact that this heteromer strategy was also unsuccessful suggests that the chimera $\alpha_2$rho, even if translated, most likely had sufficient distortion of its tertiary structure as to also preclude effective interaction with the β subunit. In an attempt to distinguish between failed assembly and assembly–surface expression without function, limited studies using radioligand binding of [^{3}H]-muscimol (a GABA agonist) were performed. Binding studies failed to detect any specific binding of either homomer or heteromer combination (data not shown). However, this result cannot distinguish between lack of surface expression and altered affinity for the radioligand. These results are consistent with previously published data showing inability to achieve expression of chimeric GABA–glycine receptors with large 5' domains, which include M1 contributed by GABA$_A$ receptors.[25]

In oocytes expressing chimeric receptors constructed for this study, two patterns of VA modulation were observed. The pattern of chloroform or halothane modulation for all expressed chimeras resembled that observed in the whole native receptor, which had contributed the portion of the receptor 5' to the splice point. Thus, when GABA ρ contributed the 5' portion (as in chimera rhogly or rhoα_2), the resultant chimera showed negative modulation by halothane or chloroform, like wild-type GABA ρ. When a glycine receptor subunit donated the 5' portion (as in chimera glyrho), chloroform or halothane positively modulated agonist gating, as they would in the wild-type glycine receptor. In contrast, isoflurane or enflurane influences on chimeras uniformly resembled those observed in the portion of the receptor 3' to the splice. If GABA ρ contributed the 3' portion (chimera glyrho), the resultant chimera showed negative modulation by enflurane or isoflurane, like the wild-type GABA ρ. However, if a glycine or GABA$_A$ receptor contributed the 3' portion (chimera rhogly or rhoα_2), the resultant chimera showed positive modulation by enflurane or isoflurane, like the wild-type "parents."

Our results suggest that the VAs studied here fall into two groups that appear to have different molecular interactions with this family of receptors. To the best of our knowledge, these are the first data demonstrating functional divergence of VA action on a single protein target.

The magnitude of negative modulation of these chimeras by VAs was consistent with that reported for GABA ρ

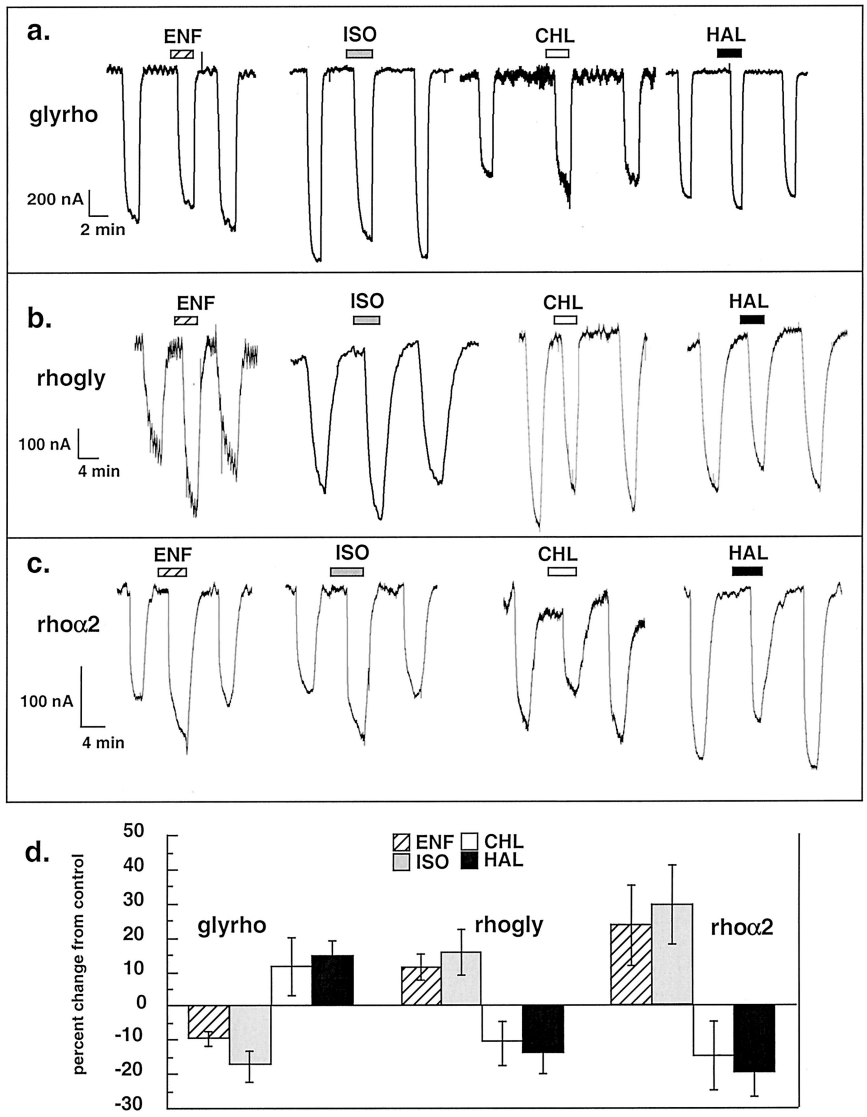

Fig. 5. Effects of volatile anesthetics (VAs) on agonist-evoked Cl^- currents in *Xenopus* oocytes expressing chimeric cRNAs. Representative traces from single oocytes expressing chimera (*A*) glyrho, (*B*) rhogly, and (*C*) rhoα_2. Bars indicate duration of coapplication of anesthetics with EC_{10} for appropriate agonist. ENF = enflurane; ISO = isoflurane; CHL = chloroform; HAL = halothane. (*D*) Summary data (mean ± SD) for effects of VAs on chimeras.

wild-type, since previous studies of VA effects on GABA ρ were performed at approximately twice the concentrations of VA that were used here.[6] The magnitude of positive modulation by VAs was less pronounced with our chimeras than the twofold to threefold potentiation of agonist responses previously reported in wild-type recombinant $GABA_A$[1] or glycine[13] receptors. Because the direction of modulation (*i.e.*, whether positive or negative) for individual VAs was consistent for each chimera in numerous oocytes tested, our results are important in the pursuit of the mechanism of VA actions in native receptors. Chimeras with components from "parent" receptors whose pharmacology is as different as those used here can be expected to show complex responses. Indeed, our chimeric receptors do not reproduce perfectly the pharmacology of their native components.

In experiments on chimera glyrho where VA concentration was varied from one to three times MAC, the magnitude of VA effects appeared to be greater at the lowest range of concentrations, although one cannot determine from these data a precise VA dose–response pattern. However, the pattern of modulation was generally consistent with that observed at 1.5 MAC (*i.e.*, isoflurane inhibited this receptor, whereas halothane enhanced), suggesting that the 1.5-MAC data for this chimera can be viewed as representative. Variance of

Table 2. Effect of VA Concentration on GABA-gated Currents in *Xenopus* Oocytes Expressing Chimera Glyrho

	% Change from Control	
MAC Multiple	Halothane (n)	Isoflurane (n)
1	NS (4)	−20 ± 8.6 (5)
1.5	15 ± 4.2 (5)	−17 ± 4.4 (6)
2	9 ± 4.7 (4)	−13 ± 5.1 (5)
3	NS (4)	NS (5)

Mean ± SD.

VA = volatile anesthetic; GABA = γ-aminobutyric acid; MAC = minimum alveolar concentration; NS = no significant effect of anesthetic coapplication; n = sample size (number of oocytes assayed).

these responses from those observed with wild-type receptors is, as previously discussed, to be expected.

Our results are in agreement with reports that have identified two residues in M2 and M3, respectively (both found in the 3' domains of the chimeras used here), which are critical for the action of enflurane[26] or isoflurane[27] in $GABA_A$ and glycine receptors. These results are in apparent conflict with a study[28] using a nicotinic acetylcholine–serotonin receptor, in which nicotinic acetylcholine subunit α_7 contributed the extracellular N-terminal, and serotonin-3_A the transmembrane and C-terminal domains. Halothane or isoflurane inhibited responses in that chimera, leading the investigators to conclude that the extracellular N-terminal domain was critical for action of these VAs. This discrepancy may be attributable, in part, to their use of isoflurane and halothane concentrations (> 10 MAC) far in excess of the clinically relevant concentrations used here. It is well known that high VA concentrations can inhibit agonist-evoked currents in GABA or glycine receptors.

The VAs studied here can also be cosegregated into the same grouping based on chemical properties. Halothane and chloroform are halogen-substituted alkanes, whereas isoflurane and enflurane are halogenated methy-ethyl ether derivatives. It has long been a matter of controversy how the mechanism of general anesthesia could involve chemically distinct anesthetic compounds that apparently exert similar end effects on a putative molecular target. To explain the discrepancy between these results and the observation that all of these VAs can positively modulate these receptors, we suggest the following hypothesis. Rather than hosting a unitary molecular site for interaction with all VAs, a single protein may show multiple target domains, each of which possesses chemical attributes favoring interaction with a particular subgroup of anesthetics. It is beyond the scope of this study to determine the specific chemical properties that underlie the divergent effects observed here. Additional evidence supports the concept of segregation of chemically related groups of VAs to divergent central nervous system target sites. Specific binding sites on nicotinic acetylcholine receptors can discriminate between halothane and isoflurane in competitive assays using photoaffinity labels.[29] The existence of mutant strains of the nematode *Caenorhabditis elegans*, which are divergent for isoflurane *versus* halothane stereoselectivity, may also be interpreted as evidence that these VAs may each have a different site of action.[30]

In summary, our results show, for the first time, a divergence in VA interactions with chimeric GABA– glycine receptors. The halogenated alkane VAs studied here (chloroform or halothane) had a consistent pattern of modulation that appeared to depend on the identity of the native receptor donating the portion of the chimera that included the N-terminal extracellular domain and M1. Conversely, the substituted ether VAs (enflurane or isoflurane) showed a pattern of modulation that was dependent on the identity of the native receptor contributing the portion that included M2–M4 and the C-terminus. If these divergent effects can be extrapolated to the native receptor proteins, it suggests that different groups of VAs may interact with disparate functional domains within a single protein. Although there can be no identification of the actual molecular site of VA action in any relevant protein target until detailed ultrastructural information becomes available, our results support the view that highly specific interactions between VAs and proteins underlie the anesthetic effect.

This work is dedicated to the memory of the late Dolan B. Pritchett, Ph.D. (Department of Pharmacology and Pediatrics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania), who provided invaluable inspiration and guidance.

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