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Positive Modulation of Human γ-Aminobutyric Acid Type A and Glycine Receptors by the Inhalation Anesthetic Isoflurane

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SUMMARY

The interactions of the inhalation anesthetic agent isoflurane with ligand-gated chloride channels were studied using transient expression of recombinant human receptors in a mammalian cell line. Isoflurane enhanced γ-aminobutyric acid (GABA)-activated chloride currents in cells that expressed heteromeric $GABA_A$ receptors consisting of combinations of α1 or α2, β1, and γ2 subunits and in cells that expressed receptors consisting of combinations of only α and β subunits. Receptors consisting of α2 and γ2 subunits were poorly expressed but were sensitive to isoflurane. Receptors consisting of β1 and γ2 subunits were not expressed. Isoflurane also enhanced glycine-activated chloride currents through homomeric α glycine receptors but did not enhance GABA currents in cells expressing homomeric ρ1 receptors. These results show that not all ligand-gated chloride channel receptors are sensitive to isoflurane and, therefore, that the anesthetic interacts with specific structural determinants of these ion channel proteins.

It is now accepted that many general anesthetic agents, such as barbiturates (1), propofol (2), and the anesthetic steroids (3), enhance inhibitory synaptic transmission in the brain via allosteric modulation of $GABA_A$ receptors. Ligand-gated chloride channels form one branch of the ligand-gated ion channel superfamily. The recent cloning and expression of many subunits of the $GABA_A$ receptor family (reviewed in Ref. 4) has significantly advanced the understanding of mechanisms of receptor modulation by these agents (5–10). Recently, the modulation of neuronal $GABA_A$ receptors by volatile anesthetics has also been described (11–13). We have used transient expression of specific receptor subunits in a non-neuronal cell line, the HEK 293 cell line (7), to study the modulation of human ligand-gated chloride channels by the inhalation anesthetic isoflurane. We demonstrate here that positive modulation of ligand-gated chloride channel function by isoflurane can be observed in αβ, αγ, or αβγ heteromeric human $GABA_A$ receptors and in homomeric α2 glycine receptors but not in homomeric receptors composed of the structurally related ρ1 subunit.

Materials and Methods

HEK 293 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in culture. The following human $GABA_A$ receptor subunit cDNAs were used for transient expression studies: α1, α2, β1, and γ2 (8, 14). Also used were the human glycine receptor α2 subunit cDNA (15) and the human ρ1 cDNA (16). The α1 (or α2) and β1 $GABA_A$ receptor subunit cDNAs were often included together in one plasmid; the other cDNAs were included in separate plasmids. All plasmids (CIS2 or PRK7) contained one copy of the cytomegalovirus promoter, controlling the transcription of the receptor subunit cDNAs, together with an SV40 polyadenylation sequence. Transfection was performed using a modification of the calcium phosphate precipitation technique (7). Electrophysiological recordings were made using the whole-cell patch-clamp technique (17). Recordings were made from single isolated HEK 293 cells where possible; in the case of the human $GABA_A$ receptor α2γ2 subunit combination, currents were recorded from clusters of cells, because single-cell currents were barely detectable. Patch pipettes contained (in mM) 145 *N*-methyl-D-glucamine hydrochloride, 5 K_2ATP, 2 $MgCl_2$, 5 HEPES/KOH, pH 7.2, 0.1 $CaCl_2$, and 1.1 EGTA. Pipette-to-bath resistance was 4–5 MΩ. The extracellular medium contained (in mM) 145 NaCl, 3 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 6 D-glucose, and 10 HEPES/NaOH, pH 7.4. Under these conditions of almost symmetrical chloride, the chloride equilibrium potential was close to 0 mV; cells were normally held at −60 mV in voltage clamp. GABA or glycine was applied to the cell under study by brief pressure application (10–100 msec, <6 kPa) (12) from blunt-tipped micropipettes containing 100 μM GABA or glycine, eliciting transient inward currents. Current responses were low-pass filtered at 2 kHz and digitized (TL-1–125 interface; Axon Instruments, Foster City, CA) for off-line analysis using AXOBASIC (Axon Instruments). Peak amplitudes of the GABA responses were measured, and the data are presented as percentages of the control GABA current response

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ABBREVIATIONS: GABA, γ-aminobutyric acid; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

and, where possible, as mean ± standard error. Note that, because it was not technically possible to reproduce the concentrations of isoflurane in successive experiments, the data for isoflurane are given as a range. Bicuculline, strychnine, zinc, and isoflurane were dissolved in the extracellular medium and applied by bath perfusion. Isoflurane was measured directly using gas chromatography (12). Isoflurane was obtained from Anaquest (Madison, WI). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Results

Co-transfection of HEK 293 cells with plasmid DNA encoding human $\alpha1\beta1$ and $\gamma2$ subunits resulted in the expression of functional $GABA_A$ receptors, as described previously (8). GABA-activated chloride currents in 293 cells co-transfected with plasmids containing $\alpha1\beta1$ or $\alpha2\beta1$ and another plasmid containing the $\gamma2$ subunit cDNA were blocked by the prototypical $GABA_A$ receptor antagonist bicuculline (Fig. 1a) but were insensitive to blockade by 5 μM Zn^{2+} (Fig. 1b), indicating successful incorporation of the $\gamma2$ subunit (18). In four experiments with 5 μM Zn^{2+}, $\alpha1\beta1\gamma2$ receptor responses were inhibited by 10 ± 7% (not significant); in five experiments, $\alpha2\beta1\gamma2$ receptor responses were inhibited by 1 ± 2% of control (not significant). $\alpha x\beta1\gamma2$ receptor currents were strongly inhibited by 20 μM bicuculline; in four experiments, $\alpha1\beta1\gamma2$ receptor responses were reduced by 84 ± 8% and, in four experiments, $\alpha2\beta1\gamma2$ receptor responses were reduced by 80 ± 12%. $\alpha x\beta1\gamma2$ receptor currents were strongly potentiated by isoflurane (Fig. 1c); in four experiments with isoflurane concentrations between 0.70 and 1.38 mM, $\alpha1\beta1\gamma2$ receptor responses were increased by 40–414% and, in four experiments with isoflurane concentrations between 0.20 and 2.22 mM, $\alpha2\beta1\gamma2$ receptor responses were increased by 47–128%.

Transient expression of $\alpha1\beta1$ or $\alpha2\beta1$ subunit combinations in the absence of $\gamma2$ subunits resulted in the expression of $GABA_A$ receptors that were strongly inhibited by both 20 μM bicuculline (Fig. 2a) and 5 μM Zn^{2+} (Fig. 2b). With 20 μM bicuculline, $\alpha1\beta1$ receptor responses were reduced by 87 ± 8%, relative to control (four experiments); $\alpha2\beta1$ receptor responses were reduced by 92 ± 5% (four experiments). With 5 μM zinc, $\alpha1\beta1$ receptor responses were inhibited by 89 ± 5% (four experiments); $\alpha2\beta1$ receptor responses were inhibited by 94 ± 6% (four experiments). GABA responses in cells expressing $\alpha1\beta1$ or $\alpha2\beta1$ receptor subunit combinations were also potentiated by isoflurane (Fig. 2c). In four experiments with isoflurane concentrations between 1.16 and 2.30 mM, $\alpha1\beta1$ receptor responses were increased by 69–104%; in four experiments with isoflurane concentrations between 1.73 and 2.30 mM, $\alpha2\beta1$ receptor responses were increased by 29–266%.

Transient expression of $\alpha2\gamma2$ subunit combinations resulted in the expression of $GABA_A$ receptors. The currents were of very small amplitude (<100 pA) and were blocked by 20 μM bicuculline but were insensitive to 5 μM Zn^{2+}. GABA responses in cells expressing $\alpha2\gamma2$ receptor subunit combinations were also potentiated by isoflurane (0.89–0.95 mM) in three experiments, by 33–142% (data not shown). GABA responses were not detected in cells transfected with $\beta1$ and $\gamma2$ subunit cDNAs. Expression of $\rho1$ subunits produced receptors that were sensitive to GABA and elicited Cl^- currents that were not blocked by bicuculline (Fig. 3a); $\rho1$ responses were reduced by 0 ± 1% with 20 μM bicuculline (four experiments). Currents through $\rho1$ channels were insensitive to isoflurane. In nine experiments with isoflurane concentrations between 0.61 and 2.26 mM, $\rho1$ responses were not significantly altered by the anesthetic (e.g.,

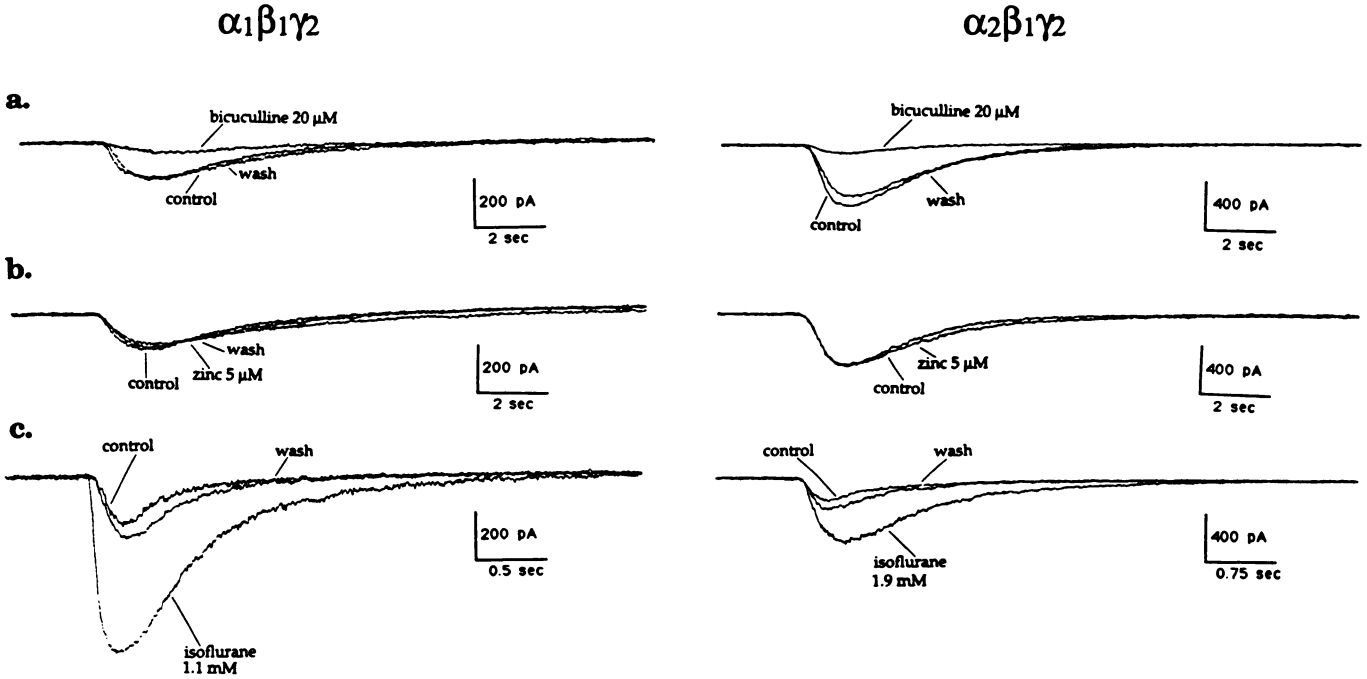

Fig. 1. Isoflurane enhances bicuculline-sensitive, Zn^{2+}-insensitive, GABA-induced membrane currents in HEK 293 cells expressing $\alpha1\beta1\gamma2$ and $\alpha2\beta1\gamma2$ receptors. a, GABA-activated chloride currents in 293 cells expressing $\alpha1\beta1\gamma2$ or $\alpha2\beta1\gamma2$ receptor subunit combinations are blocked by 20 μM bicuculline. Note that here and in Figs. 2 and 3 the cells were held at −60 mV in voltage clamp and GABA was applied by pressure from a micropipette containing 100 μM GABA. b, GABA responses in cells expressing $\alpha1\beta1\gamma2$ or $\alpha2\beta1\gamma2$ receptor subunit combinations are insensitive to 5 μM Zn^{2+}. c, GABA responses in cells expressing $\alpha1\beta1\gamma2$ or $\alpha2\beta1\gamma2$ receptor subunit combinations are potentiated by isoflurane, to 281% and 176% of control, respectively, in these examples.

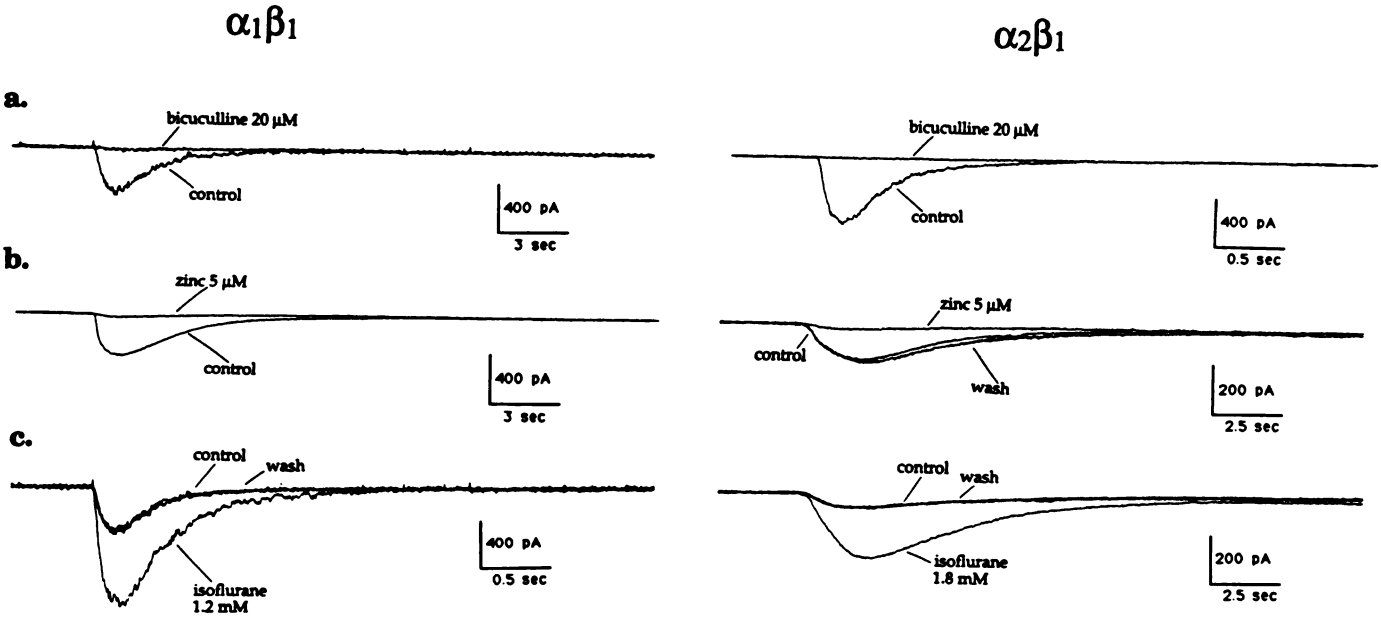

Fig. 2. Isoflurane enhances Zn^{2+}-sensitive GABA-induced membrane currents in HEK 293 cells expressing $\alpha1\beta1$ and $\alpha2\beta1$ receptors. a, GABA-activated chloride conductance responses in 293 cells expressing $\alpha1\beta1$ or $\alpha2\beta1$ receptor subunit combinations are blocked by 20 μM bicuculline. b, GABA responses in cells expressing $\alpha1\beta1$ or $\alpha2\beta1$ receptor subunit combinations are also blocked by 5 μM Zn^{2+}. c, GABA responses in cells expressing $\alpha1\beta1$ or $\alpha2\beta1$ receptor subunit combinations are potentiated by isoflurane.

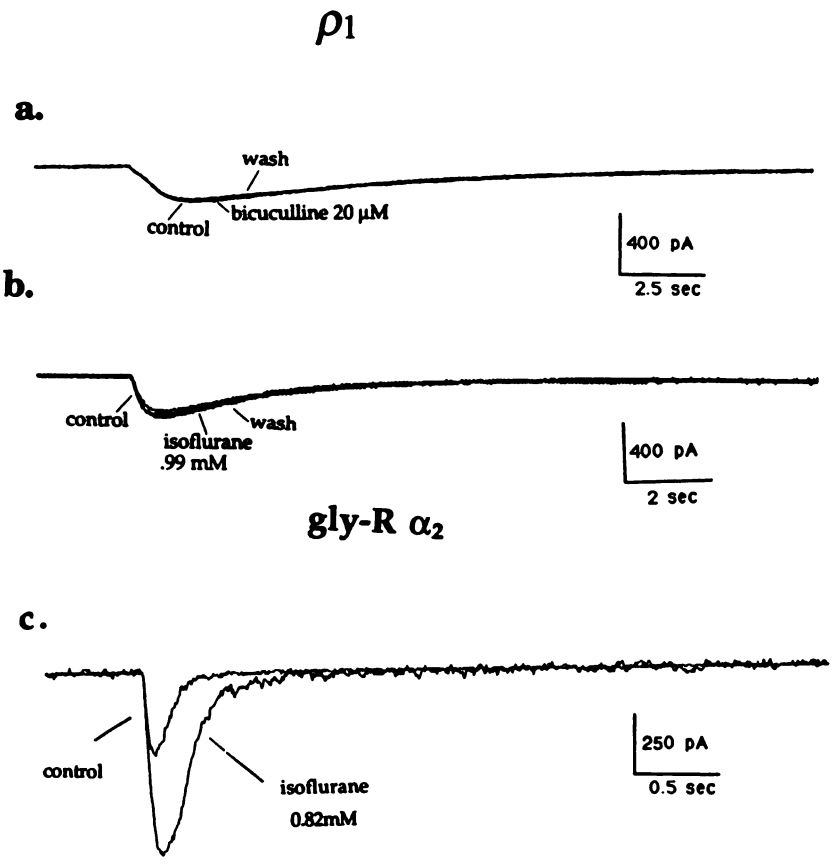

Fig. 3. Isoflurane modulates glycine receptors but not "$GABA_C$ receptors." a, GABA-activated chloride conductance responses in 293 cells expressing $\rho1$ subunits are insensitive to bicuculline. b, GABA responses in cells expressing the $\rho1$ subunit are not potentiated by isoflurane. c, Glycine responses in cells expressing $\alpha2$ subunits are potentiated by isoflurane.

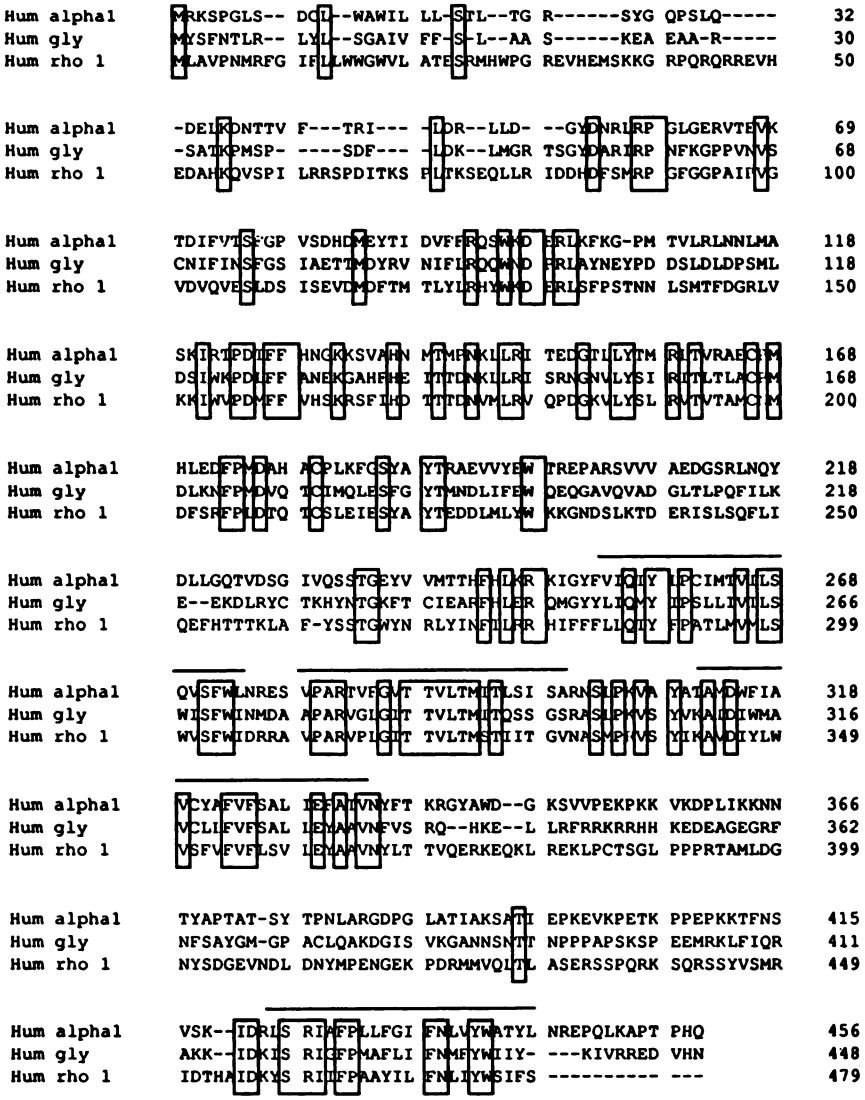

Fig. 4. Sequence comparison between the human α1 GABA$_A$ receptor subunit, α2 glycine receptor subunit, and ρ1 subunit. The amino acid sequences of the three human chloride channel subunits are aligned here to highlight the homology among these subunits within the four transmembrane domains (*overlined*). Note the substantial heterogeneity among the subunits in the amino-terminal extracellular domain and in the long cytoplasmic loop between the third and fourth transmembrane domains. Enclosed areas indicate regions of homology among all three subunits.

see Fig. 3b). Transient expression of glycine receptor α2 subunits produced glycine-activated Cl^- currents that were completely blocked by low concentrations of strychnine (<1 μM). In six experiments with isoflurane concentrations between 0.54 and 1.15 mM, glycine responses were increased by 45–208% (Fig. 3c).

Discussion

There has been considerable debate regarding the question of whether anesthetics achieve their effects via interactions with lipid or protein components of neuronal membranes (19, 20). The data presented here show clearly that volatile anesthetics are capable of modulating human recombinant GABA$_A$ and glycine receptors expressed in a non-neuronal mammalian cell line. The ρ1 subunit forms a GABA-gated chloride channel that is insensitive to isoflurane, indicating that channel structure is important in the action of the anesthetic. The transfection procedure isolates the receptor from other neuronal elements that may be altered by volatile anesthetics. These observations therefore provide further evidence in favor of sites on ligand-gated channel proteins as targets for general anesthetics. Enhancement of GABA-activated currents was observed at isoflurane concentrations in the clinically useful range between 0.5 and 1.0 mM, equivalent to 1–2 times the minimum alveolar concentration (21), as described previously for the native GABA$_A$ receptors of hippocampal neurons (12). Modulation by higher anesthetic concentrations has also been reported for native GABA$_A$ receptors of dorsal root ganglion neurons (11) and in *Xenopus* oocytes injected with whole-brain mRNA (13).

Sensitivity to Zn^{2+} has been previously shown to be dependent on the presence or absence of a γ subunit in recombinant rat GABA$_A$ receptors (18); the data presented here suggest that human GABA$_A$ receptors carry a similar Zn^{2+} binding site. We have used sensitivity to Zn^{2+} primarily as an assay to establish the efficiency of our co-transfection of plasmids containing $\alpha\beta$ and γ2 cDNAs. In every set of experiments, successful co-transfection was achieved, as evidenced by the ineffectiveness of 5 μM Zn^{2+} in blocking GABA currents. Our data show clearly that isoflurane potentiates GABA$_A$ receptor function independently of the presence of the γ subunit. This is similar to results described previously for the barbiturate and steroidal (22) modulators of the GABA$_A$ receptor and clearly distinct from the modulation by benzodiazepines (8).

Our results with transfection of the α2γ2 and β1γ2 subunit

combinations are similar to those of Verdoorn *et al.* (23). Angelotti *et al.* (24) could not detect expression of $\alpha\gamma$ or $\beta\gamma$ receptors in L929 cells, but our measurements of $\alpha2\gamma2$ responses were made on clusters, indicating a very low density of functional receptors in single cells. The sensitivity of $\alpha2\gamma2$ receptors to isoflurane suggests that the β subunit is not required for modulation. This in turn implies either that the binding site for isoflurane modulation resides on the α subunit or that isoflurane may bind to any of the $GABA_A$ receptor subunits in a degenerate manner.

Both the human glycine receptor $\alpha2$ subunit and the GABA-gated chloride channel formed by the $\rho1$ subunit were expressed in HEK 293 cells. The strychnine-sensitive glycine-activated currents were positively modulated by isoflurane. This is an interesting observation because, in contrast to the myriad of substances that modulate the $GABA_A$ receptor, the glycine receptor has appeared pharmacologically less complex. Glycine receptors are, for example, insensitive to both benzodiazepines and barbiturates. The GABA-gated currents recorded in cells transfected with the $\rho1$ subunit cDNA could be large (up to 6 nA) in amplitude. These currents were often larger than the currents recorded from cells expressing heteromeric $GABA_A$ receptor channels. The $\rho1$ currents are completely insensitive to bicuculline and thus have been termed "$GABA_C$ receptors," rather than $GABA_A$ receptor subunits (25). $\rho1$ currents were insensitive to isoflurane concentrations up to 2.26 mM; $\rho1$ currents are also insensitive to modulation by both benzodiazepines and barbiturates (25). This is an important piece of information, because it suggests that not all GABA-gated chloride channels are sensitive to anesthetic modulation. Thus, there must be structural features of the $GABA_A$ receptor and glycine receptor channel proteins that permit isoflurane modulation and are not shared by $\rho1$ homomeric channels. $\rho1$ has 64% sequence homology within the transmembrane domains, but only ~25% sequence homology overall, with representatives of the subunit families of the $GABA_A$ and glycine receptors (Fig. 4). Future efforts will be directed toward identification of the components of these ligand-gated chloride channels that are responsible for modulation by isoflurane.

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