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Different Subunit Requirements for Volatile and Nonvolatile Anesthetics at γ-Aminobutyric Acid Type A Receptors

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SUMMARY

The ability of volatile (halothane and isoflurane) and nonvolatile (alphaxalone and pentobarbital) general anesthetics to modulate radioligand binding to γ-aminobutyric acid $(GABA)_A$ receptors was examined in an immortalized cell line (WSS-1) expressing rat α1 and γ2 subunits. Volatile anesthetics enhance [^{3}H]flunitrazepam binding to WSS-1 cells in a concentration-dependent manner, with potencies and efficacies comparable to those found with native $GABA_A$ receptors. Transfection of these cells with cDNAs encoding rat β2 or β3 subunits had a significant influence on anesthetic efficacy but not potency in this assay. Thus, transfection with the β2 subunit reduced the efficacy of both isoflurane and halothane, whereas transfection with the β3 subunit increased the efficacy of isoflurane but not halothane, compared with values obtained in WSS-1 cells. In contrast, alphaxalone (an anesthetic steroid) had no effect, whereas at high concentrations pentobarbital (an anesthetic barbiturate) produced a modest inhibition of [^{3}H]flunitrazepam binding to $GABA_A$ receptors in WSS-1 cells. Transfection of WSS-1 cells with cDNAs encoding either β2 or β3 subunits resulted in a concentration-dependent enhancement of [^{3}H]flunitrazepam binding by these nonvolatile anesthetics. Moreover, pentobarbital was significantly more potent in enhancing [^{3}H]flunitrazepam binding to WSS-1 cells transfected with the β2 subunit, compared with the β3 subunit. The difference in subunit requirements between volatile and nonvolatile anesthetics for enhancement of [^{3}H]-flunitrazepam binding indicates that these classes of agents affect $GABA_A$ receptor function at distinct loci. These studies also provide evidence that the β subunit is required for these nonvolatile anesthetics to positively modulate $GABA_A$ receptors.

Converging lines of evidence indicate that proteins, and in particular ion channels, are the primary targets of general anesthetics (reviewed in Refs. 1 and 2). Among these structurally diverse proteins, the $GABA_A$ receptor family has long been considered a potential locus of anesthetic action. Thus, during the past 15 years, both direct and correlative evidence indicates that the ability of both nonvolatile [e.g., barbiturates (3–6) and steroids (7–10)] and volatile (e.g., halothane and isoflurane) (2, 11, 12) agents to potentiate transmission at $GABA_A$ receptors results in general anesthesia.

Significant differences among brain regions have been reported for both the potencies and efficacies of general anesthetics, including barbiturates (3, 13), steroids (8), and inhalation agents (14), to modulate $GABA_A$ receptor function. Although neither the composition nor the stoichiometry of native $GABA_A$ receptors has been determined, the distribution of mRNAs encoding $GABA_A$ receptor subunits throughout the central nervous system (15) indicates that receptor heterogeneity could contribute to these regional differences described for both volatile and nonvolatile anesthetics. If such regional differences are attributable to $GABA_A$ receptor heterogeneity, then the effects of general anesthetics on $GABA_A$ receptors may involve actions at specific protein sequences, rather than perturbation of lipid bilayers (1, 2). Studies with various combinations of αγ and αβγ subunits expressed in mammalian cell lines (10, 16, 17) and *Xenopus* oocytes (18, 19) are generally consistent with the hypothesis that subunit composition can affect the extent to which general anesthetics, including inhalation agents (19, 20), modulate $GABA_A$ receptors.

The objective of the present study was to compare the potencies and efficacies of prototypic agents from three major classes of anesthetic agents (barbiturates, steroids, and inhalation agents) to modulate [^{3}H]flunitrazepam binding to $GABA_A$ receptors in a cell line (WSS-1) stably expressing α1γ2 subunits. These anesthetics produce a robust and reliable enhancement of ^{3}H-labeled benzodiazepine binding to native $GABA_A$ receptors (4, 10, 13, 14, 21). When expressed in mammalian cell lines, α1γ2 subunits produce benzodiazepine receptor-modulated, GABA-gated, chloride currents (22–24) with pharmacology that closely resembles that of native "type I" $GABA_A$ receptors (25). Transfection of WSS-1 cells with cDNAs encoding β2 and β3 subunits also permitted an examination of the relative contribution of these β subunits to the effects of general anesthetics at $GABA_A$ receptors.

ABBREVIATION: GABA, γ-aminobutyric acid.

Materials and Methods

Cell culture. WSS-1 cells (CRL 2029; American Type Culture Collection, Rockville, MD) are derived from human embryonic kidney 293 cells that have been co-transfected with and stably express rat cDNAs encoding α1 and γ2 $GABA_A$ subunits (23). The presence of the corresponding cDNAs and mRNAs was confirmed by Southern and Northern blotting, respectively (23). WSS-1 cells were maintained at 37° in 5% CO_2 in Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin. Cells were transfected with rat β2 or β3 subunits by calcium phosphate precipitation, as described previously (26). Rat β2 subcloning was performed as described previously (23). The β3 insert was produced by *Sfu*I digestion of pCDβ3 (provided by L. Mahan, National Institute of Neurological Disorders and Stroke), followed by formation of blunt ends with Klenow and subsequent digestion with *Xba*I. The insert was then subcloned into the *Eco*RV and *Xba*I sites of pcDNAI (Invitrogen). Rat β2 and β3 subunits were expressed in WSS-1 cells by transfection of RC/CMV (β2) (23) or pcDNAI (β3) (Invitrogen) containing the cloned cDNAs [3–5 μg of DNA/10-cm^2 dish (~4 × 10^6 cells)] (23). pRSVZ (plasmid 37495; American Type Culture Collection) encoding β-galactosidase was co-transfected to monitor transfection efficiency, which was typically ~20%. [^{3}H]Flunitrazepam binding to WSS-1 cell membranes obtained from mock-transfected cells did not differ from that to membranes from nontransfected cells. The β2 and β3 cDNA clones were kindly supplied by Dr. L. Mahan (National Institute of Neurological Disorders and Stroke).

Radioligand binding. Cells (typically five 10-cm^2 dishes) were removed with 15 ml of 50 mM Tris-citrate buffer and disrupted with a Polytron homogenizer (setting 6, for 3 sec). The homogenates were centrifuged at 20,000 × *g* (4°). This "washing" procedure (disruption followed by centrifugation) was performed a total of five times. Assays (total volume, 500 μl) consisted of 300 μl of tissue suspension (~0.2–0.4 mg of protein/assay), 50 μl of [^{3}H]flunitrazepam (final concentration, ~1 nM for studies with alphaxalone or ~2 nM for studies with volatile anesthetics and pentobarbital), 50 μl of drug solution, 50 μl of 2 M NaCl, and 50 μl of buffer. In assays examining the effects of isoflurane and halothane on [^{3}H]flunitrazepam binding, the assay volumes were increased to 750 μl to accommodate higher concentrations of anesthetic. In these assays, tissue was added in 100-μl aliquots (0.2–0.4 mg of protein/assay) and 50 μl of 3 M NaCl were added. Anesthetic concentrations in these assays were determined by gas chromatography, as described below. Nonspecific binding was defined with diazepam (10 μM) and, under these assay conditions, represented ~20% of total binding in the absence of general anesthetics. Assays were performed in Beckman 96-well polypropylene plates and were terminated after 1 hr (4°) by rapid filtration over GF/B filters, using a Brandel M-48R cell harvester (Brandel Instruments, Gaithersberg, MD), followed by washing with two 5-ml aliquots of ice-cold Tris-citrate buffer. The radioactivity retained on the filters was measured with a Beckman LS5801 liquid scintillation counter. Assays were performed in duplicate or triplicate, and all experiments were repeated three to nine times. Protein concentrations were determined using the bicinchoninic acid assay (Pierce, Rockford, IL).

Determination of anesthetic concentrations. Volatile anesthetic (halothane and isoflurane) concentrations were measured by methods similar to those described previously (11). Stock solutions of isoflurane (20.2 ± 0.8 mM) and halothane (26.1 ± 1.5 mM) were prepared in 50 mM Tris-citrate buffer at 4° and added as aliquots to the assay. Under these assay conditions, anesthetic concentrations were determined by direct sampling. Using a gas-tight Hamilton syringe, direct sample measurement was performed by sampling the assay buffer directly through a nonpermeable membrane and injecting the sample into the gas chromatograph (HP 5880-A) with an HP-1 column. Anesthetic concentrations were calculated by linear regression of the peak areas obtained from known standard concentrations with the same injection volume (2 μl) and comparison with values obtained from each sample. Anesthetic concentrations were measured under assay conditions at least three or four times, with similar results.

Materials. [^{3}H]Flunitrazepam (specific activity, 83.4 Ci/mmol) was purchased from DuPont-NEN (Boston, MA). Diazepam was donated by Hoffmann-LaRoche (Nutley, NJ). Other compounds and reagents used were obtained from standard commercial suppliers.

Curve fitting and statistics. Nonlinear curve fitting (Inplot4; Intuitive Software, La Jolla, CA) was used to determine parameter values. Values were estimated by using nonlinear iterative curve fitting to a sigmoid function, $Y = [A + (B - A)]/[1 + [(10^X)^D/(10^C)^D]]$, where A is the bottom plateau, B is the top plateau, C is the log IC_{50} or EC_{50}, and D is the slope. Data were fitted by constraining A to basal (i.e., in the absence of anesthetics) [^{3}H]flunitrazepam binding (in femtomoles/assay) and were subsequently expressed as percentage of control (basal) binding.

In studies comparing the effects of β subunits on volatile agents, data were analyzed by analysis of variance and Newman-Keuls *post hoc* test. Student's t test was used to compare the effect of transfection with β2 and β3 subunits on nonvolatile agents.

Results

The inhalational agents isoflurane and halothane enhanced [^{3}H]flunitrazepam binding to WSS-1 cells in a concentration-dependent manner (Figs. 1 and 2). Transfection of WSS-1 cells with either the β2 or β3 subunit did not significantly affect the potency of isoflurane to enhance [^{3}H]flunitrazepam binding (Table 1). However, the maximal enhancement (E_{max}) in [^{3}H]-flunitrazepam binding produced by isoflurane was modestly but statistically significantly altered by the presence of either the β2 subunit or the β3 subunit. The presence of the β3 subunit resulted in a significant increase in the maximal binding, relative to the β2 subunit ($p < 0.05$, Newman-Keuls test). Com-

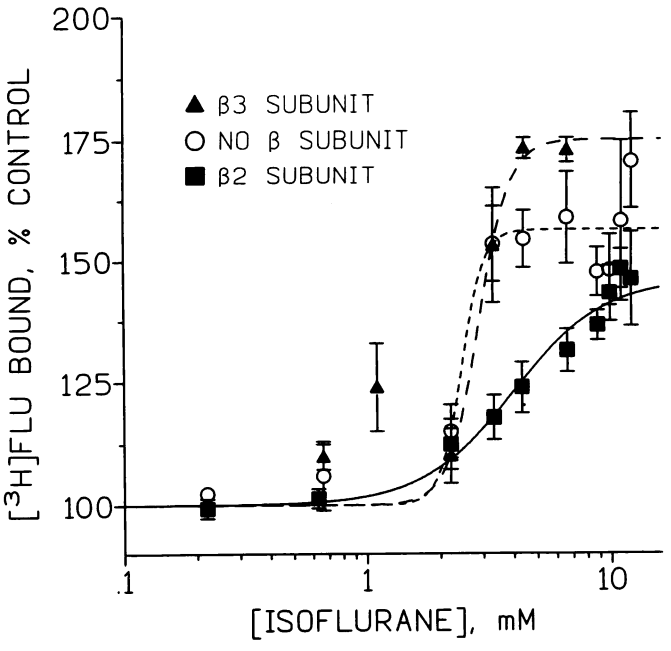

Fig. 1. Enhancement by isoflurane of [^{3}H]flunitrazepam (*[^{3}H]FLU*) binding to WSS-1 cells and effects of β subunits. *Symbols*, mean ± standard error of at least three independent experiments. No statistically significant differences in anesthetic potency were observed in cells expressing either $\alpha1\gamma2$, $\alpha1\beta2\gamma2$, or $\alpha1\beta3\gamma2$ subunits, whereas significant differences in efficacy were manifested. See Table 1 for statistical analyses. Basal binding in these series of experiments was 94 ± 13, 69 ± 5, and 76 ± 4 fmol/mg of protein in cells expressing $\alpha1\gamma2$, $\alpha1\beta2\gamma2$, and $\alpha1\beta3\gamma2$ subunits, respectively. The EC_{50} and E_{max} values in this figure (calculated using the mean ± standard error values obtained at each anesthetic concentration) are 2.4, 4.1, and 2.8 mM and 56, 47, and 73% for cells expressing the $\alpha1\gamma2$, $\alpha1\beta2\gamma2$, and $\alpha1\beta3\gamma2$ subunits, respectively.

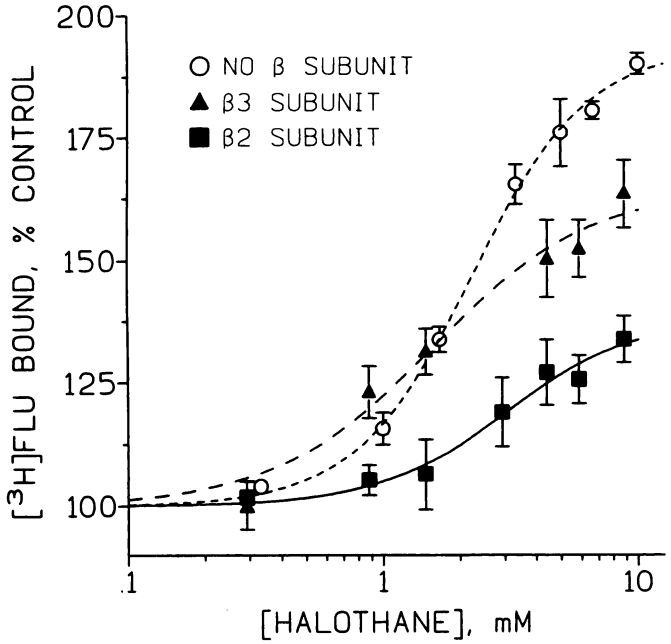

Fig. 2. Enhancement by halothane of [^{3}H]flunitrazepam (*[^{3}H]FLU*) binding to WSS-1 cells and effects of β subunits. *Symbols*, mean ± standard error of at least three independent experiments. No statistically significant differences in anesthetic potency were observed in cells expressing either $\alpha1\gamma2$, $\alpha1\beta2\gamma2$, or $\alpha1\beta3\gamma2$ subunits, whereas significant differences in efficacy were manifested. See Table 1 for statistical analyses. Basal binding in these series of experiments was 50 ± 4, 65 ± 3, and 74 ± 3 fmol/mg of protein in cells expressing $\alpha1\gamma2$, $\alpha1\beta2\gamma2$, and $\alpha1\beta3\gamma2$ subunits, respectively. The EC_{50} and E_{max} values in this figure (calculated using the mean ± standard error values obtained at each anesthetic concentration) are 1.8, 3.0, and 1.6 mM and 95, 38, and 65% for cells expressing the $\alpha1\gamma2$, $\alpha1\beta2\gamma2$, and $\alpha1\beta3\gamma2$ subunits, respectively.

TABLE 1

Effects of volatile and nonvolatile anesthetics on [^{3}H]flunitrazepam binding to WSS-1 cells and influence of β subunits

EC_{50} and E_{max} values for isoflurane and halothane were compared using analysis of variance followed by Newman-Keuls test. EC_{50} and E_{max} values for nonvolatile anesthetics were compared using Student's *t* test.

Anesthetic	EC_{50}	E_{max}	No. of experiments
		%	
Volatile	*mM*		
Isoflurane			
WSS-1	2.3 ± 0.3	61 ± 5[a]	4
WSS-1 ± β2	3.3 ± 0.4	43 ± 3[a]	5
WSS-1 + β3	2.0 ± 0.6	75 ± 4[a]	3
Halothane			
WSS-1	1.5 ± 0.1	80 ± 8	4
WSS-1 + β2	3.0 ± 0.8	30 ± 6[b]	4
WSS-1 + β3	2.1 ± 0.5	68 ± 9	4
Nonvolatile	*μM*		
Alphaxalone			
WSS-1	NC[c]	NC	9
WSS-1 + β2	0.49 ± 0.07[d]	38 ± 9	3
WSS-1 + β3	2.32 ± 0.12	58 ± 6	3
Pentobarbital			
WSS-1	640 ± 140	−37 ± 2	3
WSS-1 + β2	150 ± 30[e]	30 ± 2	3
WSS-1 + β3	410 ± 70	35 ± 4	4

[a] $p < 0.05$, WWS-1 plus β2 versus WSS-1 plus β3.
[b] $p < 0.01$, WWS-1 plus β2 versus WSS-1 plus β3.
[c] NC, no consistent effect on [^{3}H]flunitrazepam binding was seen (see Fig. 3).
[d] $p < 0.001$, WSS-1 plus β2 versus WSS-1 plus β3.
[e] $p < 0.05$, WSS-1 plus β2 versus WSS-1 plus β3.

pared with WSS-1 cells, E_{max} values were significantly lower ($p < 0.05$, Newman-Keuls test) in WSS-1 cells transfected with β2 subunits and significantly higher in cells transfected with β3 subunits ($p < 0.05$, Newman-Keuls test) (Table 1). Although transfection of WSS-1 cells with β subunits had no statistically significant effect on the potency of halothane to enhance [^{3}H]-flunitrazepam binding, the E_{max} was significantly diminished by the presence of the β2 subunit ($p < 0.01$, Newman-Keuls test) and modestly but not significantly reduced in the presence of the β3 subunit, compared with WSS-1 cells (Fig. 2; Table 1).

Alphaxalone (100 nM to 20 μM) had no consistent effect on [^{3}H]flunitrazepam binding to WSS-1 cells but enhanced radioligand binding in a concentration-dependent manner in the presence of either the β2 or β3 subunit (Fig. 3; Table 1). Alphaxalone was 4–5 times more potent in cells transfected with cDNA encoding the β2 subunit, compared with the β3 subunit ($p < 0.001$, *t* test).

Pentobarbital (10–2000 μM) produced a concentration-dependent but partial inhibition of [^{3}H]flunitrazepam binding to WSS-1 cells, with an IC_{50} of 645 ± 140 μM. In contrast, pentobarbital enhanced [^{3}H]flunitrazepam binding in cells transfected with the β2 construct, with an EC_{50} of 150 ± 30 μM (Fig. 4; Table 1). Pentobarbital enhanced [^{3}H]flunitrazepam binding in WSS-1 cells transfected with the β3 construct with similar efficacy but significantly lower potency (410 ± 70 μM, $p < 0.05$, *t* test), compared with cells transfected with the β2 construct (Fig. 4; Table 1).

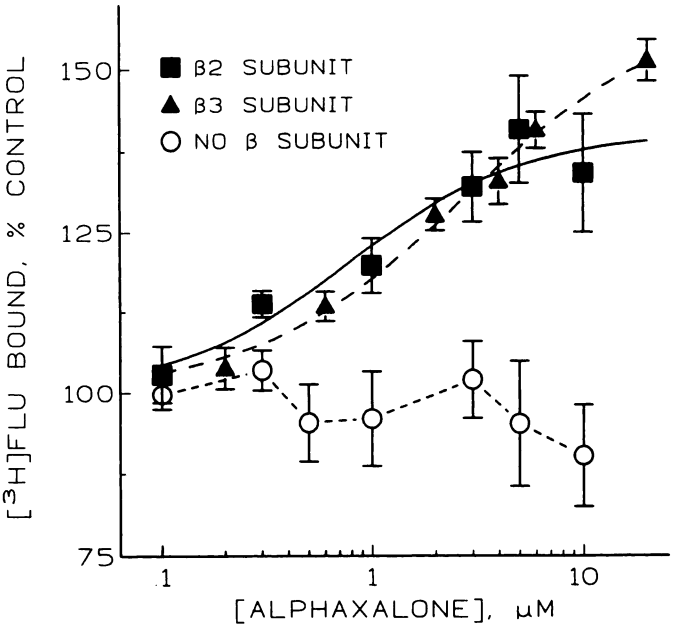

Fig. 3. Enhancement by alphaxalone of [^{3}H]flunitrazepam (*[^{3}H]FLU*) binding to WSS-1 cells and effects of β subunits. *Symbols*, mean ± standard error of at least three independent experiments. No significant concentration-dependent effect of alphaxalone on [^{3}H]flunitrazepam binding was observed in WSS-1 cells. Transfection with β2 or β3 subunits resulted in concentration-dependent enhancement of [^{3}H]flunitrazepam binding. The potency of alphaxalone was significantly greater in cells expressing β2 subunits, compared with β3 subunits. See Table 1 for statistical analyses. Total binding in these experiments was 44 ± 3, 39 ± 1, and 60 ± 3 fmol/mg of protein in $\alpha1\gamma2$-, $\alpha1\beta2\gamma2$-, and $\alpha1\beta3\gamma2$-expressing cells, respectively. The EC_{50} and E_{max} values in this figure (calculated using the mean ± standard error values obtained at each anesthetic concentration) are 0.77 and 2.65 μM and 40 and 60% increases for cells expressing the $\alpha1\beta2\gamma2$ and $\alpha1\beta3\gamma2$ subunits, respectively.

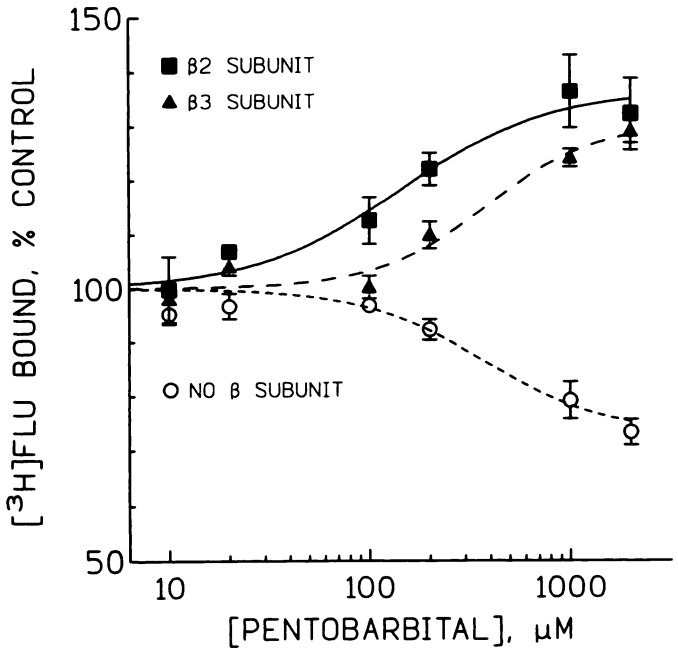

Fig. 4. Enhancement by pentobarbital of [^{3}H]flunitrazepam (*[^{3}H]FLU*) binding to WSS-1 cells and effects of β subunits. *Symbols*, mean ± standard error of at least three independent experiments. Pentobarbital inhibited [^{3}H]flunitrazepam binding in WSS-1 cells. Transfection with β2 or β3 subunits resulted in a concentration-dependent enhancement of [^{3}H]flunitrazepam binding. The potency of pentobarbital was significantly greater in cells expressing β2 subunits, compared with β3 subunits. See Table 1 for statistical analyses. Total binding in these experiments was 81 ± 8, 79 ± 2, and 101 ± 2 fmol/mg of protein in $\alpha1\gamma2$-, $\alpha1\beta2\gamma2$-, and $\alpha1\beta3\gamma2$-expressing cells, respectively. The EC_{50} and E_{max} values in this figure (calculated using the mean ± standard error values obtained at each anesthetic concentration) are 371, 142, and 390 μM and 27, 36, and 31% increases for cells expressing the $\alpha1\gamma2$, $\alpha1\beta2\gamma2$, and $\alpha1\beta3\gamma2$ subunits, respectively.

Discussion

The demonstration that general anesthetics have different subunit requirements to enhance [^{3}H]flunitrazepam binding was unexpected, in view of the many common neurochemical and electrophysiological actions shared by inhalation agents, steroids, and barbiturates at native $GABA_A$ receptors (3, 7, 27–29). Thus, consonant with data obtained using native $GABA_A$ receptors (11, 14, 21), inhalation agents (halothane and isoflurane) enhance [^{3}H]flunitrazepam binding to cells expressing $\alpha1\gamma2$ subunits (Figs. 1 and 2; Table 1) by decreasing the apparent K_d of this radioligand (data not shown). This observation is also consistent with the recent report of Harrison *et al.* (20), who concluded that a β subunit was not required for isoflurane to augment GABA responses in human embryonic kidney 293 cells expressing $\alpha2\gamma2$ subunits. Nonetheless, introduction of the β2 subunit significantly reduced the efficacy of both halothane and isoflurane to enhance [^{3}H]flunitrazepam binding, compared with either WSS-1 cells or WSS-1 cells transfected with β3 cDNA. These findings, together with the demonstration that isoflurane augments GABA responses in *Xenopus* oocytes expressing $\alpha1\beta1$, $\alpha1\beta1\gamma2L$, or $\alpha1\beta1\gamma2S$ subunits (19), indicate that the subunit requirements for modulation of $GABA_A$ receptors by inhalation agents are less stringent than for other anesthetics, including benzodiazepines (30), barbiturates, and steroids (Figs. 3 and 4). Although these data could be interpreted as a "nonspecific" perturbation (e.g., by disruption of either membrane lipids bordering these proteins or common hydrophobic sequences on the proteins) of $GABA_A$ receptors by volatile anesthetics, the stereoselective effects of isoflurane at $GABA_A$ receptors (11, 12, 31) suggest that this is not the case. Instead, the ability of inhalation anesthetics to activate $GABA_A$ receptors containing $\alpha\beta$, $\alpha\gamma$, and $\alpha\beta\gamma$ subunits may be attributable to an effect at a common sequence (or sequences) in the highly conserved (and relatively hydrophobic) putative membrane-spanning domains of these subunits (32). This explanation is also consistent with both the ability of β subunits to modulate anesthetic efficacy in WSS-1 cells and the regional differences in the potency and efficacy of inhalation agents to act as positive modulators of $GABA_A$ receptors (14). Moreover, because WSS-1 cells transfected with β subunits express $GABA_A$ receptors composed of both $\alpha\gamma$ and $\alpha\beta\gamma$ subunits, the apparent contribution of β2 and β3 subunits to the anesthetic potency and efficacy in this measure may be underestimated.

The ability of both neurosteroids and barbiturates to act as positive modulators at $GABA_A$ receptors constituted by various $\alpha\beta$ or $\alpha\beta\gamma$ subunit combinations has been documented (10, 17, 18, 33–35). Despite the presence of GABA-gated, benzodiazepine receptor-modulated, chloride currents in these and other cells expressing $\alpha1\gamma2$ subunits (22–24, 36), the present findings demonstrate an apparent requirement for the β subunit to reveal the positive modulatory actions of two prototypic nonvolatile anesthetics. Although both drug and transmitter responses in recombinant $GABA_A$ receptors are strongly dependent upon both the expression system (e.g., *Xenopus* oocytes versus mammalian cell lines) (16–18, 35–37) and the source of DNA (e.g., human versus rodent) (33) used, these findings indicate that, when receptors are expressed in a mammalian cell line, the subunit requirements for activation of $GABA_A$ receptors by nonvolatile anesthetics are more stringent than those for volatile agents; this may be compared with the requirement for a γ subunit to manifest an effect of benzodiazepine receptor ligands (30). In this regard, the ability of pentobarbital to inhibit [^{3}H]flunitrazepam binding to WSS-1 cells also merits comment. Although this effect of pentobarbital was concentration dependent, the potency (IC_{50} of ~645 μM) was well above pharmacologically active concentrations and the inhibition was incomplete. Nonetheless, based on the these findings, it would be predicted (38) that pentobarbital would reduce GABA-mediated chloride currents in WSS-1 cells (i.e., it would act as a negative modulator). Although concentrations of pentobarbital sufficient to produce negative modulation of GABA receptors were not used, pentobarbital was ineffective in either potentiating GABA-induced chloride currents or eliciting a direct hyperpolarizing response at concentrations of up to 100 μM in WSS-1 cells.[1] Although it is not known whether this latter effect of pentobarbital results from an action at a specific amino acid sequence or sequences on the $\alpha1$ or $\gamma2$ subunits, the ability of subunit composition to produce qualitative differences in ligand efficacy at $GABA_A$ receptors has previously been documented (39, 40).

Both pentobarbital and alphaxalone were significantly more potent in enhancing [^{3}H]flunitrazepam binding to WSS-1 cells transfected with β2 subunits, compared with β3 subunits (Figs. 3 and 4; Table 1). These findings indicate that the β subunit can affect anesthetic potency at $GABA_A$ receptors, providing a potential basis for the potency differences among brain regions

[1] A. Valeyev, personal communication.

described for barbiturates and steroids (3, 8, 9, 13). Although these potency differences are modest, the qualitative difference in response to nonvolatile anesthetics, relative to cells that do not contain a β subunit, is remarkable.

In view of the high degree of sequence conservation between the β2 and β3 subunits (particularly in the hydrophobic, membrane-spanning domains) (32), these data indicate that alphaxalone and pentobarbital act at specific protein (presumably extracellular) sequences on the β subunit, rather than through a nonspecific perturbation of hydrophobic domains. These data also suggest that these drugs act on the extracellular aspect of the receptor. Although these data do not differentiate between identical sites and discrete sites on the β subunit for these drugs, studies with native GABA$_A$ receptors are not consistent with a common locus of action for barbiturates and steroids (41). Site-directed mutagenesis studies may provide better definition of the sequences on β subunits critical to the action of these nonvolatile agents.

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