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ACCELERATED COMMUNICATION

Novel Properties of Homomeric β1 γ-Aminobutyric Acid Type A Receptors: Actions of the Anesthetics Propofol and Pentobarbital

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SUMMARY

In this study we determined the influence of γ-aminobutyric acid $(GABA)_A$ receptor subunit composition on the direct effects of the general anesthetics propofol, pentobarbital, and alphaxalone, using recombinant receptors expressed in *Xenopus* oocytes. cDNAs coding for human β1, α1β1, or α1β1γ2S $GABA_A$ receptor subunits were injected into *Xenopus* oocytes, and responses induced by either GABA or anesthetics were measured by two-electrode voltage-clamp recording. Expression of homomeric β1 receptors resulted in the formation of a Cl^- channel that was sensitive to picrotoxin and strychnine and could be activated, albeit with relatively low potency, by GABA. However, GABA-induced currents of homomeric β1 receptors were completely insensitive to the $GABA_A$ receptor antagonist bicuculline. Homomeric β1 receptors showed marked direct activation by propofol or pentobarbital, but not by alphaxalone. In contrast, these three anesthetics induced much weaker direct activation of Cl^- currents in oocytes expressing α1β1 or α1β1γ2S receptors. These data indicate that the β1 subunit of the $GABA_A$ receptor forms a functional Cl^- channel that contains sites for the direct activating effects of GABA, propofol, and pentobarbital and this GABA site is not blocked by bicuculline.

Although anesthesia is a complex phenomenon, a common feature of general anesthetic agents is positive modulation of the inhibitory function of the neurotransmitter GABA through $GABA_A$ receptors (1, 2). Both electrophysiological and neurochemical studies indicate that volatile anesthetics such as halothane, isoflurane, and enflurane (3–5) and injectable anesthetics such as pentobarbital (6), anesthetic steroids (7, 8), and propofol (9–12) enhance the function of $GABA_A$ receptors, suggesting that this neurochemical event may be crucial for the production of their pharmacological effects. In addition, general anesthetics, such as propofol and pentobarbital, can directly activate the Cl^- channel coupled to $GABA_A$ receptors, in the absence of GABA (11, 12). The molecular mechanism of these actions is not understood, and the question of whether there exists a specific site for these agents on the $GABA_A$ receptor remains to be determined (2).

The $GABA_A$ receptor is a multimeric complex that is formed by different glycoprotein subunits (α, β, γ, and δ) and contains an integral Cl^- channel (13). Expression studies revealed that the actions of several classes of drugs, such as benzodiazepine agonists and inverse agonists, on $GABA_A$ receptors depend upon the subunit composition of the receptors (14, 15). During the past few years, our laboratory has investigated the importance of the receptor subunit structure in the modulatory actions of general anesthetics on $GABA_A$ receptors (16–18). This work indicates that potentiation of GABA action by anesthetics occurs with a variety of receptor subunits, and it does not support the existence of subunit specificity for the actions of general anesthetics. In contrast to potentiation of GABA responses, we now report that the direct activation of $GABA_A$ receptors by two anesthetics, propofol and pentobarbital, requires a β subunit. This is, to our knowledge, the first report showing that an action of these anesthetics may be mediated by a specific subunit of the $GABA_A$ receptor.

Experimental Procedures

Materials. Adult *Xenopus laevis* female frogs were purchased from Xenopus I (Ann Arbor, MI). Plasmid purification kits were obtained

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ABBREVIATIONS: GABA, γ-aminobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

from Qiagen (Chatsworth, CA). Propofol (2,6-diisopropylphenol) was purchased from Aldrich Chemical Co. (Milwaukee, WI) and then purified by distillation. GABA and bicuculline methiodide were obtained from Research Biochemicals (Natick, MA). All other reagents were of analytical grade and were obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Propofol was purified by distillation, divided into aliquots, and stored at −20° under argon. Propofol dilutions were prepared daily using dimethylsulfoxide (final concentration in buffer, 0.01–0.1%). Determination of propofol concentrations in the recording chamber was carried out as described by Lin *et al.* (16).

Preparation of cDNAs. cDNAs coding for the human $\alpha 1$, $\beta 1$, and $\gamma 2S$ $GABA_A$ receptor subunits were subcloned into the pCDM8 expression vector (Invitrogen, San Diego, CA) (19). cDNAs were purified using the Qiagen plasmid kit, resuspended in sterile water, divided into aliquots, and stored at −20° until use.

Isolation of *Xenopus* oocytes and microinjection of cDNAs. Stage V and VI oocytes were isolated and then exposed to collagenase (type IA; Sigma) as described elsewhere (20). Solutions containing $\beta 1$ cDNA or mixtures of $\alpha 1\beta 1$ or $\alpha 1\beta 1\gamma 2S$ cDNAs (1.5 ng/30 nl) were injected into the oocyte nuclei using a micropipette (10–15-μm diameter). Injected oocytes were incubated in modified Barth's solution (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, pH 7.5), supplemented with 2 mM sodium pyruvate, 10,000 units/liter penicillin, 10 units/liter streptomycin, 50 units/liter gentamicin, and 0.5 mM theophylline, at 16–19°.

Electrophysiological recording. Electrophysiological recording began approximately 24 hr after cDNA injection. Oocytes were placed in a rectangular recording chamber (100-μl volume) and continuously perfused with modified Barth's solution, at a flow rate of 2 ml/min, at room temperature (21). Oocytes were impaled with two microelectrodes (0.5–3 MΩ/3 M KCl) and voltage-clamped at −70 mV, using an Axoclamp 2A amplifier (Axon Instruments, Burlingame, CA). Drugs were perfused for 20 sec, and 5–10-min intervals were allowed between applications of GABA or anesthetics.

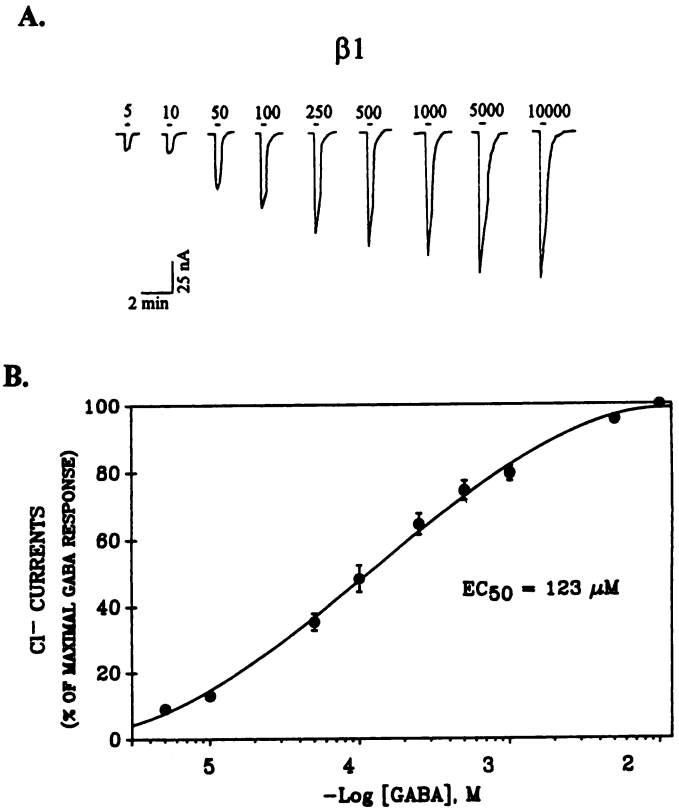

Fig. 1. GABA activation of Cl^- currents in oocytes expressing $\beta 1$ homomeric receptors. A, Tracings were obtained from a single oocyte and show Cl^- currents evoked by perfusion with GABA (concentrations are indicated in μM). *Horizontal bars*, drug applications. B, Values represent currents induced by GABA and are expressed as mean ± standard error of the percentage of the maximum current from four different oocytes.

Results

Activation of $\beta 1$ homomeric receptors by GABA. Microinjection into *Xenopus* oocytes of cDNA coding for the $\beta 1$ subunit of the human $GABA_A$ receptor resulted in the formation of a GABA-sensitive ion channel. Before experiments with anesthetics were carried out, it was necessary to characterize the properties of this homomeric receptor. As shown in Fig. 1A, perfusion with GABA (5–10,000 μM) produced inward currents whose amplitude was dependent upon the GABA concentration. The responses showed desensitization in the presence of GABA, a phenomenon that increased with increasing concentrations of GABA. The reversal potential of GABA-induced currents was found to be close to −20 mV, indicating that these currents were carried by Cl^-. In addition, uninjected or water-injected oocytes did not respond to any concentration of GABA (data not shown). The EC_{50} value for GABA-induced currents was 123 μM, and the Hill coefficient was 0.73 (Fig. 1B). The steady state current due to spontaneously opening channels was not subtracted from the values in Fig. 1B. We were unable to obtain GABA responses in oocytes injected with only $\alpha 1$, $\alpha 2$, $\alpha 5$, or $\gamma 2S$ subunit cDNAs.

Sensitivity of $\beta 1$ homomeric receptors to picrotoxin and strychnine but not to bicuculline. Sigel *et al.* (22) showed that $\beta 1$ homomeric receptors, although insensitive to GABA, could be affected by the $GABA_A$ receptor-coupled Cl^- channel blocker picrotoxin. In agreement with their data, we found that perfusion with picrotoxin (10 μM) induced outward Cl^- currents (Fig. 2). Similarly to picrotoxin, $\beta 1$ homomeric receptors could be affected also by the glycine receptor Cl^- channel blocker strychnine (100 μM), and responses produced by both drugs showed slow reversal during wash-out (Fig. 2). It is noteworthy that these effects were not detected in oocytes expressing $\alpha 1\beta 1$ or $\alpha 1\beta 1\gamma 2S$ receptors (data not shown).

To further characterize the pharmacological properties of $\beta 1$ homomeric receptors, the capability of the $GABA_A$ receptor antagonist bicuculline to inhibit GABA-induced Cl^- currents was studied and compared with its actions on $\alpha 1\beta 1$ or $\alpha 1\beta 1\gamma 2S$ receptors. As illustrated in Fig. 3, GABA-induced responses measured in oocytes expressing $\beta 1$ homomeric receptors were completely insensitive to blockade by 10 μM (or 100 μM) (data not shown) bicuculline. However, in a few cells bicuculline elicited small inward Cl^- currents (membrane potential, −70 mV) in the absence of GABA. As expected, this antagonist almost completely inhibited GABA responses with $\alpha 1\beta 1$ or $\alpha 1\beta 1\gamma 2S$ receptors.

Homomeric $\beta 1$ receptors and general anesthetics. The ability of anesthetics to directly activate the Cl^- channel coupled to the $GABA_A$ receptor was tested in oocytes expressing $\beta 1$ homomeric receptors as well as those expressing $\alpha 1\beta 1$ or $\alpha 1\beta 1\gamma 2S$ receptors. Cl^- currents induced by anesthetics were compared with those induced by 5 μM GABA. As shown in Fig. 4C, pentobarbital (50 μM), propofol (25 μM), or alphaxalone (10 μM) perfusion of oocytes expressing $\alpha 1\beta 1\gamma 2S$ receptors produced Cl^- currents whose amplitude was 32 ± 4%, 11 ± 1%, and 42 ± 5% ($n = 5$) of the control GABA (5 μM) response,

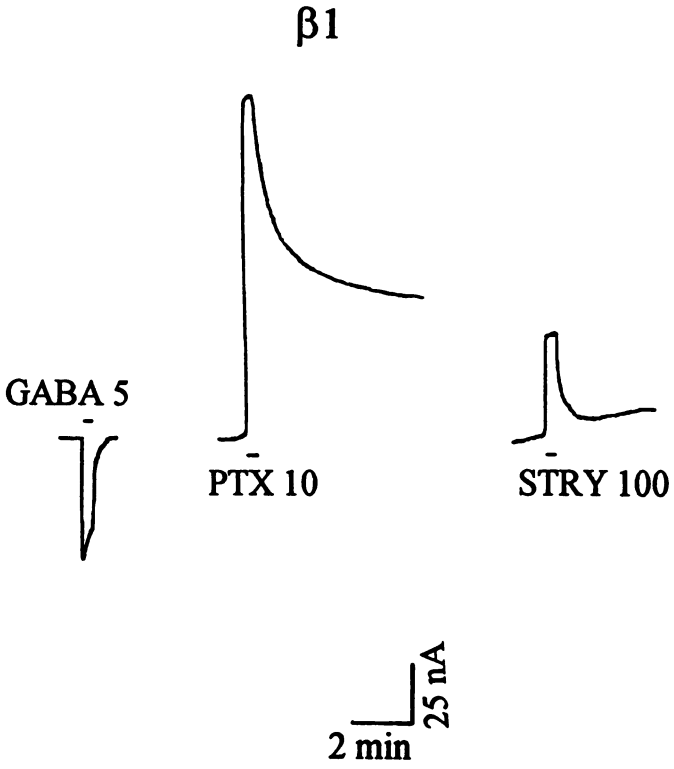

Fig. 2. Sensitivity of β1 homomeric receptors to picrotoxin and strychnine. Tracings were obtained from a single oocyte and show the effect of perfusion with 10 μM picrotoxin (*PTX*) or 100 μM strychnine (*STRY*) on β1 homomeric receptors. These are representative of similar results obtained in five to seven different cells.

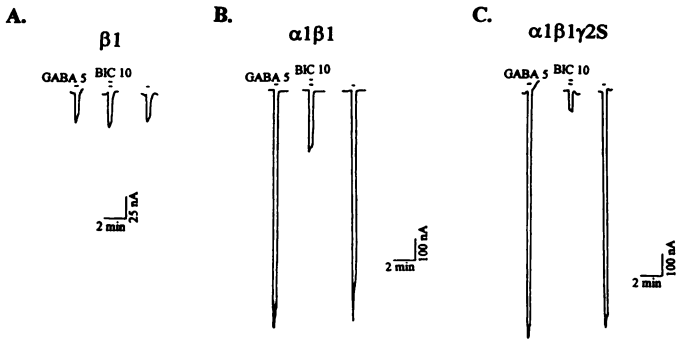

Fig. 3. Evidence that GABA-evoked Cl$^-$ currents in oocytes expressing β1 homomeric receptors are not blocked by bicuculline. Tracings represent the effects of 10 μM bicuculline (*BIC*) on Cl$^-$ currents evoked by 5 μM GABA, measured in oocytes expressing β1 homomeric (A), $\alpha1\beta1$ (B), or $\alpha1\beta1\gamma2S$ (C) receptors. These results are representative of similar results obtained in four to eight different cells.

respectively. Receptors containing only $\alpha1\beta1$ subunits were less sensitive to the direct effects of the anesthetics than were $\alpha1\beta1\gamma2S$ receptors (Fig. 4B). In contrast, β1 homomeric receptors showed a dramatic sensitivity to direct activation by propofol (744 ± 77% of the GABA response, n = 29) or pentobarbital (940 ± 112% of the 5 μM GABA response, n = 12) (Fig. 4A). Effects of both drugs were seen with concentrations as low as 1–5 μM. The effect appeared to plateau at 1–5 mM, but it was not possible to determine the true maximal effect because of solubility limitations. In contrast to the striking effects of pentobarbital and propofol, the response induced by alphaxalone was quite weak, amounting to only 12 ± 2% (n = 10) of the control GABA response. In most oocytes, the response to

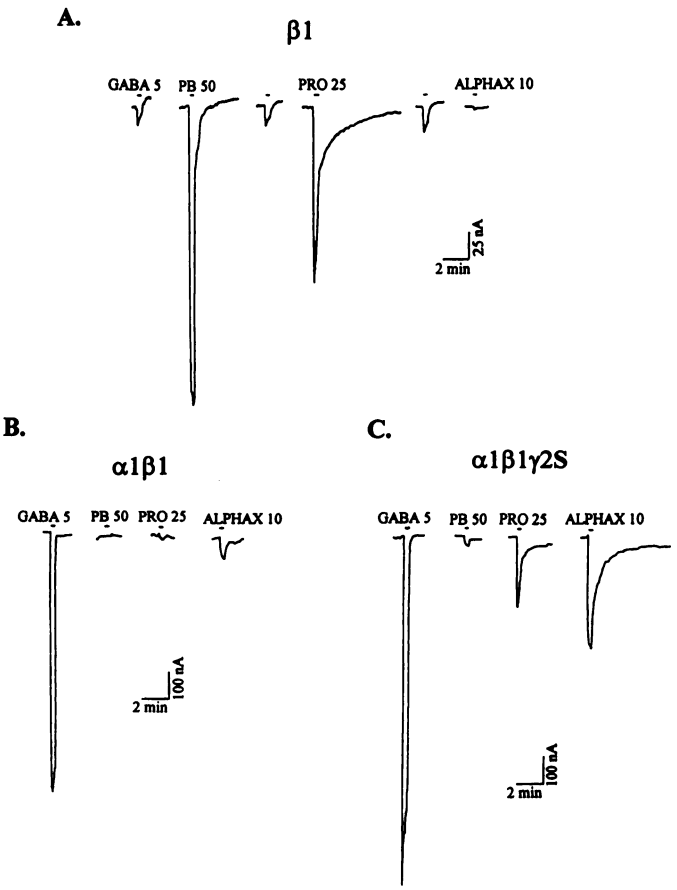

Fig. 4. Ability of general anesthetics to directly activate Cl$^-$ currents in oocytes expressing different GABA$_A$ receptor constructs. Tracings represent the Cl$^-$ currents evoked by pentobarbital (*PB*) (50 μM), propofol (*PRO*) (25 μM), or alphaxalone (*ALPHAX*) (10 μM), compared with those induced by GABA (5 μM), in oocytes expressing β1 homomeric (A), $\alpha1\beta1$ (B), or $\alpha1\beta1\gamma2S$ (C) receptors. These are representative of results obtained with 10–29 different oocytes.

alphaxalone was either undetectable or barely detectable (Fig. 4A). Activation of the receptor by propofol (25 μM) was not blocked by bicuculline (10 μM) (data not shown).

The direct effects of propofol and the potentiation of GABA responses were compared for four different subunit combinations (Fig. 5). The direct action of propofol was most pronounced for homomeric β1 receptors, completely absent for $\alpha1\gamma2S$ receptors, and small for $\alpha1\beta1$ and $\alpha1\beta1\gamma2S$ receptors. In contrast, potentiation of GABA responses by propofol was similar (e.g., 3–6-fold) for all four receptor constructs (Fig. 5).

Because β1 homomeric receptors were sensitive to the direct actions of propofol and pentobarbital but not to alphaxalone, it was important to determine whether alphaxalone could potentiate the Cl$^-$ currents induced by GABA, i.e., whether alphaxalone had any effect on homomeric β1 receptors. As shown in Fig. 6, alphaxalone potentiated the action of GABA, although it did not produce any direct action. The effects of these concentrations of pentobarbital and propofol were somewhat smaller in the presence of GABA than in its absence. However, lower concentrations were able to potentiate the effects of GABA on homomeric β1 receptors; for example, 5 μM propofol produced only a small direct effect but potentiated the action of GABA by 5.8-fold (482 ± 72%, n = 13) (Fig. 5).

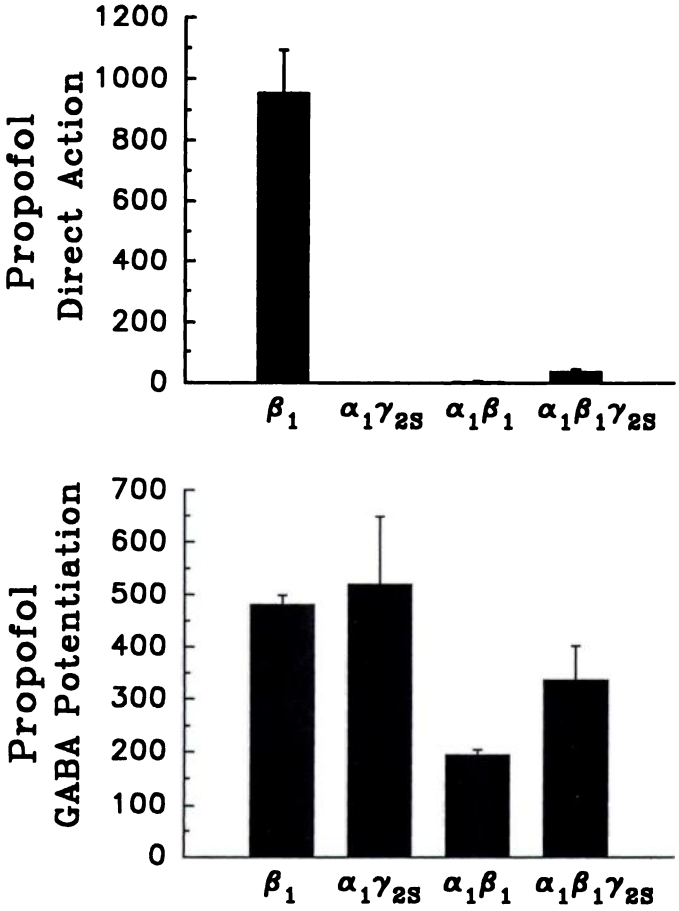

Fig. 5. Effects of propofol on $GABA_A$ receptor subunit combinations expressed in *Xenopus* oocytes. *Upper*, direct activation of chloride conductance by 25 μM propofol, expressed as a percentage of the current produced by 5 μM GABA. *Lower*, propofol (5 μM) potentiation of GABA (5 μM) action, expressed as percentage increase. Values are the mean ± standard error of seven to 14 different oocytes.

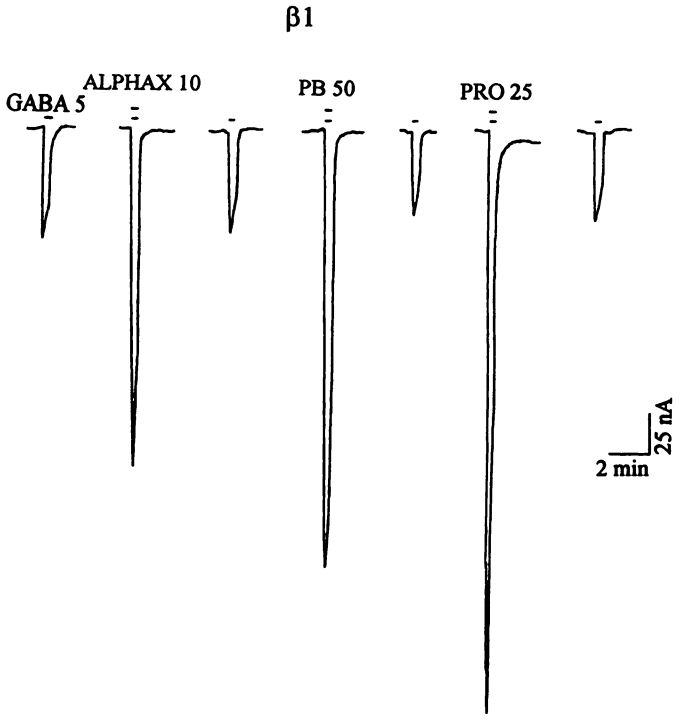

Fig. 6. Potentiation of GABA responses by anesthetics with β1 homomeric receptors. Tracings were obtained from a single cell and represent the effects of alphaxalone (*ALPHAX*) (10 μM), pentobarbital (*PB*) (50 μM), and propofol (*PRO*) (25 μM) on Cl^- currents induced by 5 μM GABA.

Discussion

Expression of functional monomeric β receptors was first shown in oocytes by Blair *et al.* (23), who demonstrated that GABA could activate Cl^- currents and that these were potentiated by pentobarbital and blocked by pertussis toxin. In contrast, Sigel *et al.* (22) showed that β1 monomeric receptors can form functional channels that are sensitive to pertussis toxin (outward currents) but cannot be activated by GABA. In view of our data and those of Blair *et al.* (23), it is surprising that Sigel *et al.* (22) were unable to detect any action of 0.1 mM GABA. Our work supports the idea of Sigel *et al.* (22) that these channels are spontaneously active and that picrotoxin produces an outward current because of inhibition of channel activity, and it extends these observations to include strychnine. The spontaneous opening of the channel may also contribute to the low potency of GABA in activating chloride currents, although it is also possible that homomeric β1 receptors display a low affinity for GABA. The inability of bicuculline to block the actions of GABA in our study was surprising, because bicuculline is a competitive antagonist of GABA actions and is assumed to act at the GABA binding site (15, 24). Our data support the idea (25) that the β subunit contains a GABA binding site, but they suggest that another subunit is required to provide a functional bicuculline site.

The finding that direct actions of propofol and pentobarbital can be observed with homomeric β1 receptors demonstrates that these anesthetics act directly on this subunit. The specificity of the interaction is confirmed by the finding that propofol directly activates α1β1γ2S, α1β1, and β1γ2S but not α1γ2S receptors. However, the direct actions of alphaxalone do not appear to be due to actions on the β1 subunit. This is consistent with reports that the site of action of alphaxalone is distinct from that of barbiturates (26), but it is in contrast to the study of Puia *et al.* (27), who found that 3α,21-dihydroxy-5α-pregnan-20-one and 3α-hydroxy-5α-pregnan-20-one directly activated homomeric β1 receptors expressed in human embryonic kidney 293 cells. The potentiation of GABA action produced by alphaxalone, pentobarbital, and propofol likely occurs at a different site than the direct actions. In support of this conclusion, alphaxalone was able to potentiate the GABA responses of homomeric receptors even though it produced no direct effect. In addition, heteromeric receptors show potentiation of GABA responses at concentrations of propofol or pentobarbital that produce little or no direct action. For example, propofol potentiates the action of GABA on α1γ2S receptors despite the lack of any direct action (and the lack of a β1 subunit).[1] Blair *et al.* (23) reported that pentobarbital (50 μM) potentiated the action of GABA on homomeric β1 receptors, but no mention was made of direct effects, and it is not clear whether pentobarbital was tested alone. If not, the direct effects may have been misinterpreted as potentiation. To our knowledge, there is only one other report of a drug binding site that is unique to a β subunit. Wafford *et al.* (28) found that the anticonvulsant loreclezole requires a β2 or β3 subunit to poten-

[1] E. Sanna, unpublished observations.

tiate GABA action. However, loreclezole does not act on receptors containing the β1 subunit, and its site of action must be distinct from that of pentobarbital or propofol.

It is interesting to note that the β subunits appear to be phylogenetically the oldest of the GABA$_A$ receptor subunits and may have existed for one billion years before the α subunits arose (29). Thus, homomeric β receptors may reflect a primitive subtype with sensitivity to GABA, but not to bicuculline. In view of the marked sensitivity of this receptor to pentobarbital, it is tempting to speculate that a similar molecule (e.g., a pyrimidine) was an early neurotransmitter and retention of this site leads to the sensitivity to modern anesthetics seen in this study.

Although there is presently no evidence for homomeric receptors in brain, it is worth considering the pharmacological relevance of the concentrations of anesthetics required to interact with sites on β1 subunits. The EC_{50} values of pentobarbital and propofol for general anesthesia in mammals are about 50 and 0.4 μM, respectively (2). Thus, homomeric β1 subunits are markedly affected by anesthetic concentrations of pentobarbital but not by propofol. These observations, combined with construction of subunit chimeras and site-directed mutagenesis, should help to define the molecular site of action of pentobarbital and propofol on the GABA$_A$ receptor.

Note Added in Proof:

We have also found oocytes that express homomeric β_3 receptors that are sensitive to pentobarbitol and picrotoxin but not GABA. The lack of GABA action may be due to a low level of receptor expression in these oocytes.

Acknowledgments

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