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The effects of a point mutation of the β_2 subunit of $GABA_A$ receptor on direct and modulatory actions of general anesthetics

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Abstract

The γ-aminobutyric acid type A receptor ($GABA_A$ receptor) sites involved in the direct and modulatory actions of general anesthetics remain to be elucidated. The mutation of tyrosine at position 157 in the β_2 $GABA_A$ receptor subunit was reported to reduce sensitivity to activation by GABA, but not pentobarbital. We examined whether this mutation of the β_2 subunit ($Tyr^{157} \rightarrow Phe$) affects the direct and modulatory actions of other general anesthetics such as propofol and etomidate. Using the two-electrode voltage clamp method, we recorded Cl^- current in *Xenopus* oocytes expressing $\alpha_1\beta_2\gamma_{2s}$ and α_1-mutated $\beta_2\gamma_{2s}$ subunits. The mutation of the β_2 subunit reduced the apparent affinity for propofol. However, the mutation had no effect on both the direct actions of pentobarbital and etomidate or on the modulatory actions of pentobarbital, propofol and etomidate. These results suggest that unique loci may exist for the direct action of propofol and that the GABA binding site may not mediate the modulatory actions of general anesthetics at $GABA_A$ receptors.

Keywords: GABA (γ-aminobutyric acid); $GABA_A$ receptor; General anesthetic; Clone; Oocyte; Electrophysiology

1. Introduction

The γ-aminobutyric acid type A receptor ($GABA_A$ receptor) is a major inhibitory receptor in the central nervous system and has attracted lots of interest as an important site of action of sedatives and anesthetics (Keane and Biziere, 1987; Sieghart, 1995; Whiting et al., 1995). GABA, the inhibitory neurotransmitter, induces an inward Cl^- current which results in membrane hyperpolarization and reduced neuronal excitability on binding to $GABA_A$ receptors. The major sedatives, such as benzodiazepines and general anesthetics, enhance the action of GABA at $GABA_A$ receptors (Franks and Lieb, 1993; Tanelian et al., 1994; Sieghart, 1995). The intravenous anesthetics, such as barbiturates, etomidate, propofol and alphaxalone, and volatile anesthetics, like isoflurane and enflurane, directly activate $GABA_A$ receptors at high concentrations and at lower concentrations, these drugs positively modulate the action of GABA as seen with benzodiazepines (Orser et al., 1994; Sieghart, 1995). Accumulated evidence suggests that the sites for the modulatory actions of these anesthetics may be distinct from the GABA binding sites and that these anesthetics exert their actions by binding to allosteric sites at $GABA_A$ receptors (DeLorey and Oslen, 1992; Tanelian et al., 1994; Sieghart, 1995; Lavoie and Twyman, 1996; Davies et al., 1997a).

The $GABA_A$ receptor is presumably a hetero-pentameric receptor and 19 genes of the subunits have been identified (Barnard et al., 1987; Wang et al., 1994; Sieghart, 1995; Whiting et al., 1995; Chang et al., 1996; Davies et al., 1997b). Although molecular biology studies have revealed the heterogeneity of $GABA_A$ receptors, the sites of action of GABA and anesthetic drugs remain to be elucidated. By using recombinant techniques and molecular engineering to change the primary structure of the cloned $GABA_A$ receptor subunit, it is possible to deduce the subunit and even the specific sites of subunits involved in the action of general anesthetics.

It has been reported that point mutations in the amino-terminal region of the β_2 subunit result in impaired activation of $GABA_A$ receptors by GABA but not by pentobarbital, suggesting that GABA and pentobarbital occupy distinct sites to cause the direct activation of $GABA_A$

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receptors (Amin and Weiss, 1993). In order to clarify whether the site involved in the action of GABA is related to the site at which anesthetics other than pentobarbital exert a direct action and also to the site of modulatory action of anesthetics, we electrophysiologically examined the effects of a point mutation of the β_2 subunit (tyrosine to phenylalanine at position 157 of the amino acid sequence) on the agonistic as well as modulatory actions of anesthetic drugs. The $\alpha_1\beta_2\gamma_{2s}$ or α_1-mutated $\beta_2(m\beta_2)\gamma_{2s}$ subunit of the $GABA_A$ receptor was expressed in *Xenopus* oocytes. First, the effects of the mutation on the agonistic action of GABA, as well as on the direct action of anesthetics, such as pentobarbital, propofol and etomidate, were studied. Secondly, the effects of the mutation on the modulatory action of the anesthetics on GABA-induced Cl^- currents were analyzed.

2. Materials and methods

2.1. Expression vectors and in vitro transcription

Mouse cDNAs encoding for α_1, β_2, and γ_{2s} $GABA_A$ receptor subunits were kindly provided by Dr. J. Yang (University of Rochester, Rochester, USA). All subunits were subcloned into transcription vector, pBluescriptMXT, in which multiple cloning sites were flanked by β-globins of *Xenopus laevis* for better expression in *Xenopus* oocytes. The cloned DNAs were confirmed as coding for each subunit by identification of the size of the fragments digested by restriction enzymes. The pBluescriptMXT containing α_1, β_2 and γ_{2s} $GABA_A$ receptor subunits were linearized by restriction enzymes (PVU II for α_1 and γ_{2s}, *Bgl*I β_2) to create the template cDNAs. Capped mRNAs were synthesized by the T3 RNA polymerase in vitro transcription kit (mMessage mMachine™, Ambion, TX). Each mRNA stock in RNAse-free water was stored at −80°C until use.

2.2. Site-directed mutagenesis of β_2 subunit

Sequential polymerase chain reaction (PCR) steps were used for the site-directed mutagenesis of the β_2 subunit (tyrosine to phenylalanine at position 157 of the amino acid sequence). For the first PCR step, two primer pairs of (A) 5′-AAAGCTTTGGCTACACAACTGA-3′ and 5′-AGCTCTCTGGCTAACTAGAG-3′ and (B) 5′-GTAGCCAAAGCTTTCAAT-3′ and 5′-TGCAGCAGAGGCATCATAGT-3′, were designed to incorporate the base sequence for phenylalanine instead of tyrosine (the mismatched base pairs are underlined) at one end of amplified DNA sequence. The resultant DNA fragments of approximately 300 and 700 bases were then used as templates for the second PCR step to synthesize DNA of about 1 kbase containing the desired mutation. The mutated DNA fragment was subcloned to the original β_2 subunit with the restriction enzymes, *Pfl*mI and *Pst*I. The PCR amplification was limited to 12 thermal cycles of 95°C, 30 s; 58°C, 30 s; and 72°C, 30 s. The DNA sequence created by PCR was verified by an automatic sequencer (Applied Biosystems).

2.3. Oocyte expression

Xenopus laevis were anesthetized with ice and 0.5% tricaine (3-aminobenzoic acid ethyl ester) and oocytes were surgically removed and manually defolliculated with forceps. Oocytes were treated with 1.5 mg/ml collagenase (type 1A from Sigma, Tokyo, Japan) in Ca^{2+}-free ND96 (88 mM NaCl, 2 mM KCl, 5 mM HEPES, 1 mM $MgCl_2$, pH 7.5.) for 1 h at room temperature. Stage IV–V oocytes were isolated and thoroughly rinsed with ND96. The mRNAs for each subunit were diluted to the concentration of 1 mg/ml with RNAase-free water. The desired combination of subunit mRNA was mixed in equal ratios and 50 to 100 nl was injected into an oocyte with a glass capillary, using Nanoject injector (Drummond, PA). Oocytes were incubated at 20°C in ND96 containing 1.8 mM Ca^{2+}, 2.5 mM sodium pyruvate, 50 u/ml penicillin and 50 mg/ml streptomycin until the electrophysiological experiment.

2.4. Electrophysiology

Forty-eight to 72 h after mRNA injection, oocytes were placed in a 0.5-ml chamber and continuously perfused with frog's Ringer solution (115.5 mM NaCl, 2.0 mM KCl, 1.3 mM Na_2HPO_4, 0.7 mM NaH_2PO_4 1.8 mM $CaCl_2$, pH 7.4) at 7–10 ml/min. The electrophysiological recordings were made by using the two electrode-voltage clamp technique. Oocytes were impaled with 1 to 5 MΩ electrodes filled with 3 M KCl and voltage-clamped at −80 mV. Drugs dissolved in frog's Ringer solution were applied in the perfusate. Each application of the agents was separated by intervals of a few minutes and by longer intervals after high concentrations to eliminate the desensitization of the receptor. To test cumulative desensitization, the same low concentration of GABA was applied after every application. All experiments were done at room temperature (25°C). Data were monitored with a digital oscilloscope and digitally recorded with Pclamp6 (Axon Instruments, CA) running on IBM computer.

2.5. Data analysis

Peak amplitudes of the current elicited by drugs were measured directly from the digital recordings stored in Pclamp6. For dose–response curves, observed peak amplitudes were normalized and plotted, then best-fitted with the following equation using Sigmaplot software (Jandel Scientific, CA):

$$I = I_{max} / \left\{1 + \left(ED_{50}/[A]\right)^{n_H}\right\},$$

where I is the peak current at a given concentration of agonist A (as percentage of the maximum peak current observed), I_{max} is the maximum current, ED_{50} is the concentration of agonist eliciting a half-maximum response and n_H is the Hill coefficient. All data are expressed as means ± S.E. Statistical significance was determined when $P < 0.05$ using two-sided Student's t-test.

2.6. Chemicals

GABA, pentobarbital, diazepam, bicuculline methodide and picrotoxin were purchased from Wako (Tokyo, Japan). *R*-(+)-etomidate was kindly provided by Abbott Labs. (IL, USA). 2,6-Disopropylphenol (Propofol) was purchased from Sigma–Aldrich Japan (Tokyo, Japan). Stock solutions of etomidate, diazepam, propofol and picrotoxin were made up in dimethylsulfoxamide (DMSO). The final concentration of DMSO did not exceed 0.5% in the experiment. All agents were prepared on the day of experiment.

3. Results

Tyrosine at position 157 in the β_2 subunit was mutated to phenylalanine. Either $\alpha_1\beta_2\gamma_{2s}$ (wild-type) or $\alpha_1 m\beta_2\gamma_{2s}$ $GABA_A$ receptor subunit combinations were expressed in oocytes. The expression of functional recombinant receptors was confirmed by the presence of GABA-induced Cl^- currents which were blocked by bicuculline and picrotoxin (Sieghart, 1995; Whiting et al., 1995). The expression of

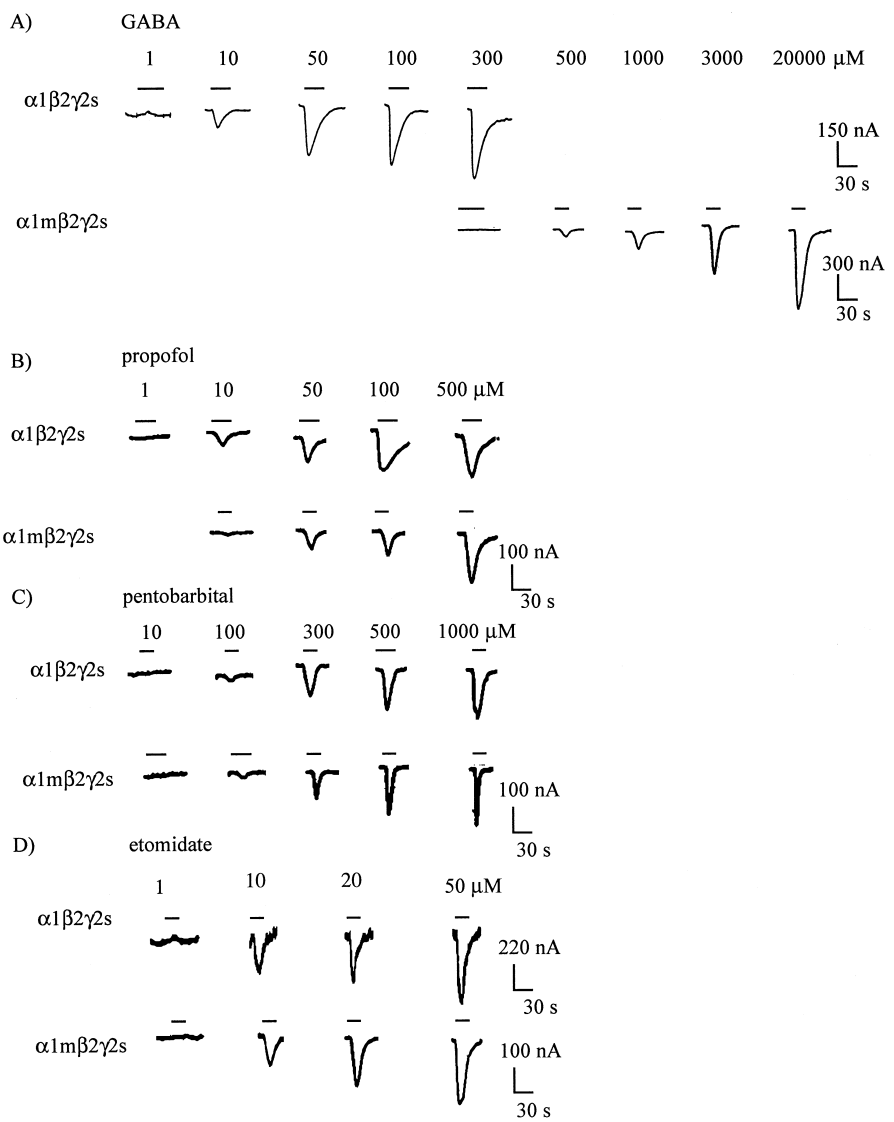

Fig. 1. Both GABA and anesthetics induce currents in a dose-dependent manner in oocytes expressing $\alpha_1\beta_2\gamma_{2s}$ and $\alpha_1 m\beta_2\gamma_{2s}$ $GABA_A$ receptors. The oocytes were voltage-clamped at −80 mV. Increasing concentrations of GABA (A), propofol (B), pentobarbital (C) and etomidate (D) were applied in the perfusate. The duration of the drug application is denoted by a bar over the trace.

the γ_{2s} subunit in the recombinant receptors was confirmed by the positive modulation of the GABA-activated Cl^- current by 1 μM diazepam (Verdoorn et al., 1990; Mihic et al., 1994; Lüddens et al., 1995; McKernan et al., 1995).

3.1. The direct activation by GABA and general anesthetics

The Cl^- currents induced by GABA and the general anesthetics, pentobarbital, propofol and etomidate, in oocytes expressing $\alpha_1\beta_2\gamma_{2s}$ or $\alpha_1 m\beta_2\gamma_{2s}$ subunits are shown in Fig. 1. The dose–response curves for GABA, pentobarbital, propofol and etomidate were obtained for both subunit combinations (Fig. 2). GABA, as well as the general anesthetics, increased the current in a dose-dependent manner. However, much higher concentrations of GABA were needed to induce the same response when the receptor contained the mutated β_2 subunit. As Amin and Weiss (1993) reported, this mutation of the β_2 subunit did not affect the sensitivity to pentobarbital. Interestingly, the action of propofol was also affected by the β_2 subunit mutation but to a lesser extent than the action of GABA. In contrast, this mutation did not affect the response induced by etomidate, as was also observed with pentobarbital. Fig. 3 shows that the dose–response curve for GABA was shifted to the right in oocytes expressing the mutated receptor. The mutation of tyrosine to phenylalanine produced a comparable decrease in sensitivity to GABA, increasing the half-maximal effective concentration (ED_{50}) from 20.3 ± 2.1 to 1845.0 ± 62.6 μM, which is about 90-fold (Fig. 2). Similarly, the mutation produced a decrease in sensitivity to propofol, shifting the dose–response curve to the right and increasing the ED_{50} from 24.0 ± 4.7 to 80.1 ± 7.9 μM, but the decrease was smaller than that for GABA. The dose–response curves for pentobarbital and etomidate showed that the mutation in the β_2 subunit had no effect on the direct action of these agents.

3.2. The effect of β_2 mutation on modulatory action of the anesthetics

Based on the dose–response curves for GABA in oocytes expressing the $\alpha_1\beta_2\gamma_{2s}$ and $\alpha_1 m\beta_2\gamma_{2s}$ subunit

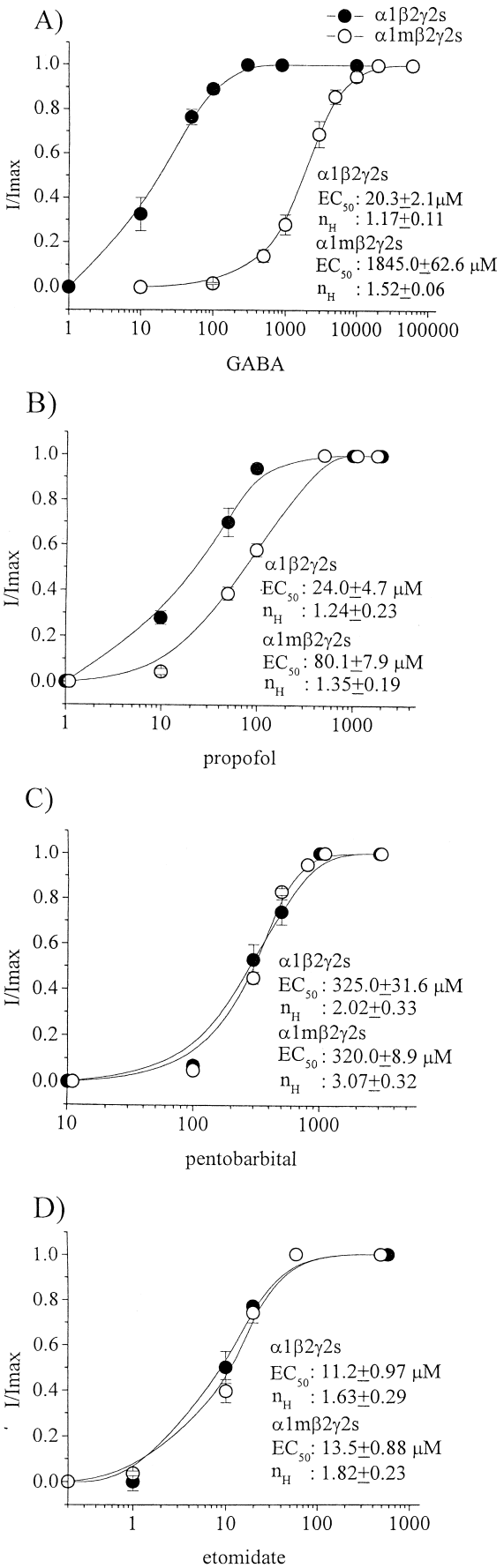

Fig. 2. The dose–response curves of GABA (A), propofol (B), pentobarbital (C) and etomidate (D) for the direct activation of $\alpha_1\beta_2\gamma_{2s}$ and $\alpha_1 m\beta_2\gamma_{2s}$ $GABA_A$ receptors. The Cl^- currents are expressed as ratios to the maximum currents induced by GABA and anesthetics. Each point represents the mean ± S.E. determined from four to eight oocytes. (A) The dose–response curve of GABA was shifted to the right as a result of the β_2 mutation. (B) The dose–response curve of propofol was shifted to the right as a result of the β_2 mutation, but the effect of the mutation was not as large as that observed with GABA. (C, D) The β_2 mutation had no effect on the dose–response curves of pentobarbital and etomidate. The data for ED_{50} and Hill coefficient (n_H) were determined by fitting the Hill equation as described Section 2.

combinations, GABA was used to induce a Cl^- current at concentrations approximately equal to the EC_{20} (5 μM for $\alpha_1\beta_2\gamma_{2s}$ and 500 μM for $\alpha_1 m\beta_2\gamma_{2s}$). This was considered the control response. Low concentrations of anesthetics (pentobarbital 5 μM, etomidate 5 μM and propofol 1 μM) were applied with GABA (Franks and Lieb, 1993; Orser et al., 1994; Zimmerman et al., 1994; Davies et al., 1997a). At these concentrations, the anesthetics induced only a slight Cl^- current by themselves. Co-application of GABA and the low doses of the anesthetics yielded a greater current than that obtained by addition of the currents induced by GABA and the anesthetics (Fig. 3). This

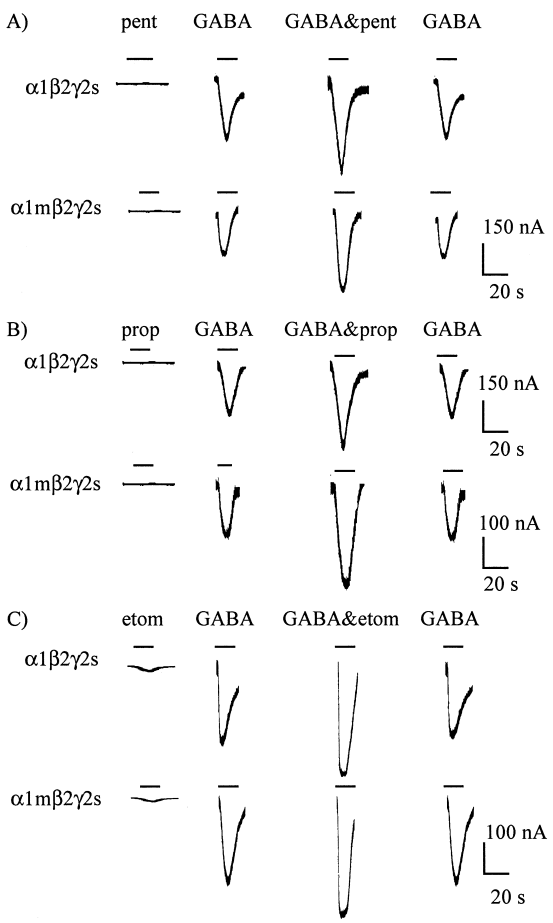

Fig. 3. The modulatory effects by pentobarbital (A), propofol (B) and etomidate (C) on GABA-induced currents elicited by activation of $\alpha_1\beta_2\gamma_{2s}$ and $\alpha_1 m\beta_2\gamma_{2s}$ $GABA_A$ receptors. Control currents were induced by GABA at EC_{20} (5 μM for $\alpha_1\beta_2\gamma_{2s}$ and 500 μM for $\alpha_1 m\beta_2\gamma_{2s}$) obtained from the dose–response curves. The concentrations of pentobarbital, propofol and etomidate were 5 μM, 1 μM and 5 μM, respectively. Application of the low concentrations of these anesthetics alone induced no or only a trace Cl^- current. Co-application of the anesthetics and GABA yielded a positive similar modulation of currents in cells expressing $\alpha_1\beta_2\gamma_{2s}$ or $\alpha_1 m\beta_2\gamma_2$ subunits. The mutation of the β_2 subunit did not affect the modulation by pentobarbital, propofol and etomidate of GABA-induced currents. The duration of drug application is denoted by a bar over the trace. pent = pentobarbital, prop = propofol, etom = etomidate.

Table 1
Modulation by the anesthetics of $\alpha_1\beta_2\gamma_{2s}$ and $\alpha_1 m\beta_2\gamma_2$ $GABA_A$ receptor activation

Anesthetics	$GABA_A$ receptor	Potentiation (percentage of control)	Number of oocytes tested
Pentobarbital	$\alpha_1\beta_2\gamma_{2s}$	140 ± 11.1	5
	$\alpha_1 m\beta_2\gamma_{2s}$	147 ± 12.5	4
Propofol	$\alpha_1\beta_2\gamma_{2s}$	149 ± 12.9	5
	$\alpha_1 m\beta_2\gamma_{2s}$	153 ± 11.7	4
Etomidate	$\alpha_1\beta_2\gamma_{2s}$	131 ± 3.2	5
	$\alpha_1 m\beta_2\gamma_{2s}$	141 ± 4.5	5

The effect of the mutation of the β_2 subunit on the modulatory actions on $GABA_A$ receptor activation by pentobarbital, etomidate and propofol is shown. The percentages of potentiations were compared to the control current induced by EC_{20} of GABA. No significant differences between $\alpha_1\beta_2\gamma_{2s}$ and $\alpha_1 m\beta_2\gamma_2$ $GABA_A$ receptors were observed among the three anesthetics.
Values are expressed as means ± S.E.

synergistic effect was seen both in cells expressing $\alpha_1\beta_2\gamma_{2s}$ and $\alpha_1 m\beta_2\gamma_{2s}$ receptors and the extent of the modulation was also similar. In cells expressing $\alpha_1\beta_2\gamma_{2s}$ receptors, pentobarbital, etomidate and propofol positively modulated the GABA-induced current to 140 ± 11.1%, 131 ± 3.2% and 149 ± 12.9% of the controls, respectively. In cells expressing $\alpha_1 m\beta_2\gamma_{2s}$ receptors, pentobarbital, etomidate and propofol also positively modulated the GABA-induced current to 145 ± 31.7%, 141 ± 4.5% and 136 ± 29.9% of the controls, respectively. The mutation of the β_2 subunit did not significantly influence the extent of the potentiation elicited by pentobarbital, etomidate and propofol (Table 1).

4. Discussion

General anesthetics are known to directly activate and modulate $GABA_A$ receptors at different concentration ranges; however, the exact sites of these dual actions are still not known (MacDonald, 1994; Zimmerman et al., 1994). Amin and Weiss (1993) showed that the mutation of the tyrosine (Tyr) residue to phenylalanine (Phe) in the β_2 subunit at position 157 of the amino-terminal region dramatically reduced GABA sensitivity, but had no effect on pentobarbital sensitivity. The results suggest that there are distinct sites for GABA and pentobarbital in the $GABA_A$ receptor. Other investigators also provided evidence for the presence of barbiturate sites distinct from GABA-binding sites (Thompson et al., 1996). Although the GABA site is distinct from the site at which pentobarbital exerts its direct action, it may be involved in the modulatory action of pentobarbital and/or the dual actions of other intravenous anesthetic agents. The Tyr residue at 157 of the β_2 subunit is close to the cysteine–cysteine loop at the N-terminus which has attracted considerable

attention in relation to channel function (Amin et al., 1994). The amino acid sequence in the vicinity of the cysteine–cysteine loop is conserved in all α, β and γ subunit isoforms. The point mutation of the corresponding amino acid, Tyr at 157 of the β_2 subunit, in the α subunit has recently been reported to impair diazepine sensitivity (Amin et al., 1997). The point mutation of the γ subunit in the vicinity of the cysteine–cysteine loop is also reported to alter benzodiazepine binding site specificity (Buhr and Sigel, 1997). Because this area seems to possess several functions, the point mutation of Tyr at 157 of the β_2 subunit may influence the modulatory action of pentobarbital and the dual actions of intravenous anesthetic agents other than pentobarbital. To clarify such possibilities, we examined the influence of the mutation on both the direct and modulatory actions of pentobarbital, etomidate and propofol.

4.1. The effect of the β_2 mutation on the direct action of general anesthetics

When the dose–response curves for GABA and the general anesthetics (pentobarbital, etomidate and propofol) were compared, the point mutation of the β_2 subunit largely impaired the ability of GABA to activate the receptor, as reported previously (Amin and Weiss, 1993). The mutation also significantly reduced the apparent affinity of propofol, but not as much as that of GABA. Unlike propofol, pentobarbital and etomidate did not show a change in sensitivity in cells expressing the mutation. The Tyr at 157 of β_2 subunit is apparently related with direct activation by propofol, and the mode of action of propofol is different from that of GABA and other two anesthetics. It was recently proposed that the β_3 subunit possesses sites for both pentobarbital and propofol that are distinct from the GABA binding site because rat β_3 homomers are activated by pentobarbital and propofol but are insensitive to GABA (Davies et al., 1997a,b). Our results for the mutation of the β_2 subunit, together with the above reports, suggest that there are at least two loci for direct activation by propofol.

We previously reported that GABA and the intravenous general anesthetics, such as pentobarbital and etomidate, act at near by but non-identical sites on the $GABA_A$ receptor of rat hippocampal neurons and that these sites could be distinguished by two different $GABA_A$ receptor antagonists, bicuculline and SR95531 (Uchida et al., 1996). Recent data suggest that etomidate binds to sites on the β_2 and β_3 subunits, and most probably involves the second transmembrane domain of the subunits for its direct actions, but conflicting evidence exists regarding the involvement of these sites in its modulatory action (Belelli et al., 1997; Hill-Venning et al., 1997; Moody et al., 1997). These previous reports and our results suggest that different anesthetics cause receptor activation by different mechanisms.

4.2. The effects of β_2 mutation on modulatory action of general anesthetics

It is generally accepted that a common feature of general anesthetics is their positive modulation of $GABA_A$ receptors, which contributes to the clinical anesthetic state (Franks and Lieb, 1993; Sieghart, 1995). At lower concentrations, the anesthetics, pentobarbital, propofol and etomidate, potentiated GABA-induced currents, but by themselves, did not induce or induced only a Cl^- current. In our experiment, the oocytes expressing the mutated receptors showed positive modulation by the three anesthetics at their lower concentrations to the same extent as the oocytes expressing wild-type receptors. The observation, that the point mutation of the β_2 subunit involved in the GABA binding site had no effect on the modulatory actions of pentobarbital, etomidate and propofol, confirms the recent general consensus that GABA binding and modulatory sites are at distinct loci in the $GABA_A$ receptor complex (Sieghart, 1995).

The question arises whether the direct and modulatory actions of anesthetics are mediated via different sites or the same site. Our results suggest that the modulatory and direct actions of propofol appear to involve different sites on the $GABA_A$ receptor. Other investigators also suggest the distinct loci for direct and modulatory actions for another anesthetic, etomidate (Moody et al., 1997). Recent studies suggest the involvement of the α subunit in the modulatory action of propofol. Our report revealed that the extracellular amino-terminal domain of the α_1 subunit was sufficient to support the propofol-induced potentiation of the Cl^- current (Uchida et al., 1997). Krasowski et al. (1997) reported that α subunit isoforms may be important in the allosteric modulation by propofol, observing a greater modulatory effect in α_1 subunit-containing receptors and a greater direct activation effect in α_6 subunit-containing receptors, but suggested that the direct binding of propofol was to the β subunit of the $GABA_A$ receptor.

The presence of the β subunit is said to be necessary to reveal the positive modulatory actions of intravenous anesthetics (Verdoorn et al., 1990; Harris et al., 1995). Both pentobarbital and alphaxalone showed positive modulatory effects in cells expressing $GABA_A$ receptors with the β_2 or β_3 subunits, but not in cells expressing $GABA_A$ receptors without any β subunit (Harris et al., 1995). Recently, Mihic et al. (1997) reported that two specific amino acid residues in the second and third transmembrane domains of the α_1 and β_1 subunits are critical for the allosteric modulation of $GABA_A$ receptors by alcohol and volatile anesthetics. The modulatory action of lorecrezole was also reported to be highly dependent on the second and third transmembrane domain of β_2 or β_3 subunits and the point mutation of β_1 in that domain abolished the potentiation by pentobarbital (Wingrove et al., 1994; Birnir et al., 1997). Accumulating evidence suggests that α and β subunits, especially the transmembrane area, are important

in the modulation of receptor function by anesthetics. Our result, that the Tyr^{157} of the β_2 subunit, which is not in the transmembrane area, was not associated with modulation of receptor activation by pentobarbital, propofol and etomidate, confirms this suggestion.

Our results indicate that tyrosine at position 157 of the β_2 subunit is involved with direct activation of $GABA_A$ receptors by propofol but its mode of action appears to be different from that of GABA. This site for propofol is not related to the site(s) involved in the direct activation of $GABA_A$ receptor by pentobarbital and etomidate. This mutation had no effect on the modulation by pentobarbital, propofol and etomidate of GABA-induced currents. Thus, the direct activation produced by pentobarbital, propofol and etomidate is exerted by mechanisms different from those of GABA and these mechanisms also differ among the anesthetics. Modulation by anesthetics is not associated with the locus crucial to the GABA binding site.

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