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Molecular mechanisms of the partial allosteric modulatory effects of bretazenil at γ-aminobutyric acid type A receptor

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ABSTRACT **In central nervous system γ-aminobutyric acid (GABA) inhibits neuronal activity by acting on GABA type A ($GABA_A$) receptors. These heterooligomeric integral membrane proteins include a GABA-gated Cl^- channel and various allosteric modulatory sites where endogenous modulators and anxiolytic drugs act to regulate GABA action. *In vivo*, various anxiolytic drugs exhibit a wide range of variability in their modulatory efficacy and potency of GABA action. For instance, bretazenil modulatory efficacy is much lower than that of diazepam. Such low efficacy could be due either to a preferential modulation of specific $GABA_A$ receptor subtypes or to a low modulatory efficacy at every $GABA_A$ receptor subtype. To address these questions we studied drug-induced modifications of GABA-activated Cl^- currents in native $GABA_A$ receptors of cortical neurons in primary cultures and in recombinant $GABA_A$ receptors transiently expressed in transformed human embryonic kidney cells (293) after transfection with cDNAs encoding different molecular forms of α, β, and γ subunits of $GABA_A$ receptors. In cortical neurons the efficacy of bretazenil was lower than that of diazepam, whereas the potency of the two drugs was similar. In cells transfected with γ2 subunits and various molecular forms of α and β subunits bretazenil efficacy was always lower than that of diazepam. However, in cells transfected with γ1 or γ3 subunits and various forms of α and β subunits the efficacy of both diazepam and bretazenil was lower and always of similar magnitude. When bretazenil and diazepam were applied together to $GABA_A$ receptors including a γ2 subunit, the action of diazepam was curtailed in a manner related to the dose of bretazenil.**

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system. This inhibition is mediated by the ability of GABA to gate Cl^- channels included in GABA type A ($GABA_A$) receptors (1). The binding of two molecules of the transmitter to each $GABA_A$ receptor triggers an inward flux of Cl^- that hyperpolarizes the neuron, preventing activation by incoming depolarizing stimuli. The $GABA_A$ receptor is an heterooligomeric integral membrane protein (2). Two subunits of the $GABA_A$ receptor (α and β) were cloned in 1987 (3); since then, several additional subunits (γ, δ, and ρ) and subunit isoforms have been cloned, so that the total number of identified subunits now stands at 15 (4–13). The transfection of eukaryotic cell lines with cDNAs encoding various $GABA_A$ receptor subunits allows the biophysical (14), physiological (15), and pharmacological (16) characterization of every possible subtype of $GABA_A$ receptor.

$GABA_A$ receptors include a site that mediates flumazenil-sensitive allosteric modulation of GABA action by benzodiazepines (BZs) and β-carbolines (17). Such modulation of GABA efficacy at native $GABA_A$ receptors can be either facilitatory (BZs), resulting in an increase in GABA-activated Cl^- current, or inhibitory (β-carbolines), leading to a decrease in Cl^- current (18). Studies with reconstituted $GABA_A$ receptors have shown that the potency and efficacy of BZs and β-carbolines depend on the structural configuration of the receptor, and that the type of BC modulation (positive or negative) can be determined by the molecular nature of the γ subunit included in the receptor structure (16). Moreover, within each class of modulatory compounds the chemical configuration of a drug plays a role in determining its intrinsic efficacy (16). The intrinsic efficacy of these modulators changes within the limits imposed by the maximal efficacy of GABA for a given $GABA_A$ receptor configuration. With this provision the efficacy of a drug can be defined as the ratio between the number of receptors modulated by the drug and the number of receptors occupied by the drug. Because for the same degree of receptor occupancy, low-efficacy modulators (partial agonists) induce a smaller response in their target cells than do full-efficacy modulators (full agonists), one wonders about the molecular nature of their low efficacy. Bretazenil (Ro 16-6028) is the prototypical low-efficacy allosteric modulator of the $GABA_A$ receptor; *in vivo* studies with this compound have shown that its intrinsic activity is much lower than that of several BZs and imidazopyridines (19). The partial efficacy of bretazenil could result from a prefential activation of a selected population of receptor subtypes or from a reduced activity at every $GABA_A$ receptor subtype. Here we have addressed this question by comparing the activities of diazepam and bretazenil at a number of $GABA_A$ receptors with different structural configurations.

METHODS

Primary Culture of Cortical Neurons. Neonatal rat cortical neurons were prepared as described (20). Briefly, cells were dispersed with trypsin (0.25 mg/ml; Sigma) and plated at a density of 1×10^6 cells per plate on 35-mm Nunc dishes coated with poly(L-lysine) (10 μg/ml; Sigma). The cultures were maintained in basal Eagle's medium/10% fetal bovine serum (GIBCO)/25 mM KCl/2 mM glutamine (Sigma)/gentamicin (100 μg/ml; GIBCO) for 1–3 weeks. Twenty-four hours after plating, the incubation medium was replaced, and 1 μM cytosine arabinofuranoside was added to inhibit replication of nonneuronal cells.

Culture of Embryonic Kidney Cell Line 293 and cDNA Transfection. Transformed human embryonic kidney 293 cells (American Type Culture Collection CRL 1573) were grown in minimal essential medium (GIBCO)/10% fetal bovine serum/penicillin at 100 units/ml (GIBCO)/streptomycin at 100 units/ml (GIBCO) in a 6% CO_2 incubator. Exponentially growing cells were dispersed with trypsin and seeded at 2×10^5 cells per 35-mm dish in 2 ml of culture medium. Transfection was performed with the calcium phosphate precipitation technique (21). Human (α1, α2, α3, β1, γ1, γ2

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Abbreviations: GABA, γ-aminobutyric acid; $GABA_A$, GABA type A; BZ, benzodiazepine.

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short-form) and rat ($\alpha 5$, $\beta 2$, $\beta 3$, $\gamma 3$) $GABA_A$ receptor subunit cDNAs singly inserted into the eukaryotic expression vector pCIS2 (22) were used to transfect the 293 cells. Cells were incubated in the presence of the supercoiled plasmids (3 μg of each plasmid per 35-mm dish) for 12–16 hr at 37°C under 3% CO_2. The medium was then removed, and the cells were rinsed twice with culture medium and incubated in fresh medium for 24 hr at 37°C under 6% CO_2 before electrophysiological studies.

Electrophysiology. Primary cultures of cortical neurons or cultures of transfected cells were studied with the whole-cell configuration of the patch-clamp technique (23) on the stage of an inverted microscope (Zeiss IM-35) at room temperature. The recording pipette contained 145 mM CsCl, 1 mM $MgCl_2$, 11 mM EGTA, and 10 mM Hepes·CsOH (pH 7.2). Cells were bathed in 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 5 mM Hepes·NaOH (pH 7.4); osmolarity was adjusted to 325 mosM with sucrose. GABA (0.5 M in H_2O adjusted to pH 4 with HCl) was applied by iontophoresis with 30-ms pulses of positive current. With GABA iontophoretic currents in the 25–50 nA range, outward currents were generated in neurons or transfected cells so as to obtain a peak amplitude of 150–200 pA. The Cl^- current measured in response to a maximally effective concentration of GABA was larger (>1 nA) than the test response of 150–200 pA, indicating that the maximal percentage potentiation we observed was far below the maximal efficacy of the system.

BZs were from Hoffman–La Roche. Drugs were dissolved in dimethyl sulfoxide and diluted in bath solution so that the maximal final concentration of dimethyl sulfoxide was 0.01%. Drugs were applied by pressure (2–4 psi; 1 psi = 6.9 kPa) from micropipettes of 5- to 10-μm diameter positioned in the proximity of the cell body. The application of dimethyl sulfoxide (0.01% in bath medium) failed to modify GABA responses. To avoid uncontrolled drug leakage, we kept the drug pipette outside the bath before the pressure injection and brought it in proximity of the recorded cell just before drug application. Drugs were applied for 5 s between two GABA pulses, which were delivered every 10 s.

Current traces were recorded with a patch-clamp amplifier (EPC-7; List Electronics, Darmstadt, F.R.G.), filtered at 1500 Hz (8-pole low-pass Bessel; Frequency Devices, Haverhill, MA) and recorded on a chart recorder (Gould 2600S) for off-line analysis. Recordings were performed within 3 days of transfection.

RESULTS

Dose–Response Curves of Bretazenil and Diazepam in Primary Cultures of Cortical Neurons. Under the ionic conditions of our experiments (symmetrical Cl^- concentrations), iontophoretic application of GABA to cortical neurons elicited an outward flux of Cl^-, which was measured as an inward current (Fig. 1). Pressure application of bretazenil (10^{-5} M) produced a positive allosteric modulation of the GABA-activated Cl^- current that was inhibited by flumazenil. When, on the same neuron, we first applied bretazenil (10^{-5} M) and then, after the GABA response has returned to the basal condition, diazepam (10^{-5} M), the modulatory efficacy of diazepam was always greater than that of bretazenil (Fig. 1). The bretazenil-positive modulation was always smaller than that of diazepam independently of the drug application order. The dose–response relations for the allosteric modulation of GABA-evoked Cl^- currents by the two drugs revealed bretazenil to be much less efficacious than diazepam at every dose tested (Fig. 2). In contrast, the bretazenil potency (EC_{50} = 60 nM) was only slightly lower than that of diazepam (EC_{50} = 30 nM).

Bretazenil Modulation of Structurally Diverse $GABA_A$ Receptor Subtypes. Because on native $GABA_A$ receptors the

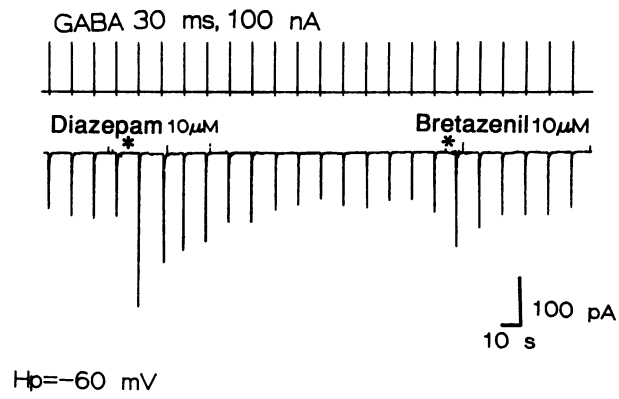

FIG. 1. Effect of bretazenil (10^{-5} M) and diazepam (10^{-5} M) on GABA-activated Cl^- currents in cortical neurons. The holding potential (Hp) was −60 mV. Asterisks indicate the start of drug application (30 ms).

modulatory efficacy of bretazenil was lower than that of diazepam, in the next series of experiments we studied the modulation of GABA action by maximally (10^{-5} M) and intermediate (10^{-7} M) effective doses of the two BZs in transfected $GABA_A$ receptors. Cultures of the human kidney cell line 293 were transfected with plasmids containing cDNAs encoding one molecular form of α subunit ($\alpha 1$, $\alpha 2$, $\alpha 3$, or $\alpha 5$) and the $\beta 1$ and $\gamma 2$ subunits. Thus the positive modulation of GABA-activated Cl^- currents by the same concentrations (10^{-5} or 10^{-7} M) of diazepam and bretazenil were compared at $\alpha 1\beta 1\gamma 2$, $\alpha 2\beta 1\gamma 2$, $\alpha 3\beta 1\gamma 2$, and $\alpha 5\beta 1\gamma 2$ receptors (Fig. 3). At a concentration of 10^{-5} M, diazepam was much more efficacious than bretazenil at every $GABA_A$ receptor subtype tested (Fig. 3*A*); furthermore, unlike diazepam, bretazenil did not show preferential facilitation at the $\alpha 2$ and $\alpha 3$ $GABA_A$ receptor variants.

The dose–response curve of diazepam and bretazenil in recombinant $\alpha 1\beta 1\gamma 2$ receptors led to the calculation of an EC_{50} of 50 nM for diazepam and of 10 nM for bretazenil.

On comparison of recombinant $GABA_A$ receptors that included $\alpha 1$ and $\beta 1$ together with different molecular forms of the γ subunit ($\gamma 1$, $\gamma 2$, or $\gamma 3$), differences between the modulatory efficacies of the two drugs were detectable only at $\alpha 1\beta 1\gamma 2$ receptors at both 10^{-5} M (Fig. 4*A*) and 10^{-7} M (Fig. 4*B*).

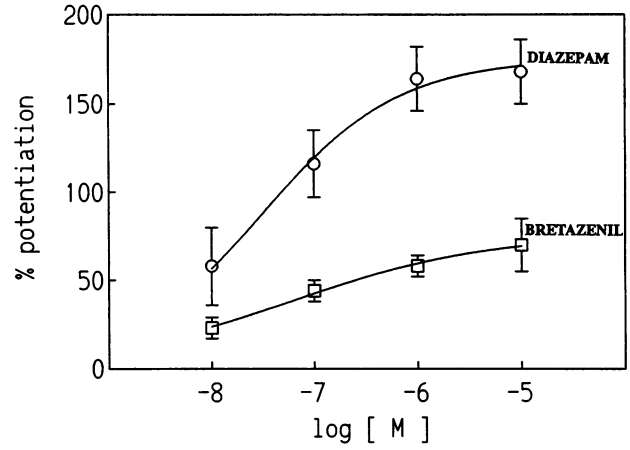

FIG. 2. Dose–response curves for the positive modulation of GABA-gated Cl^- current by diazepam (○) and bretazenil (□) in cortical neurons. Each value represents the mean ± SEM for six cells. The EC_{50} of diazepam (EC_{50diaz}) was 30 nM and that of bretazenil (EC_{50bret}) was 60 nM. Maximal potentiation of the GABA response was 170% for diazepam and 70% for bretazenil.

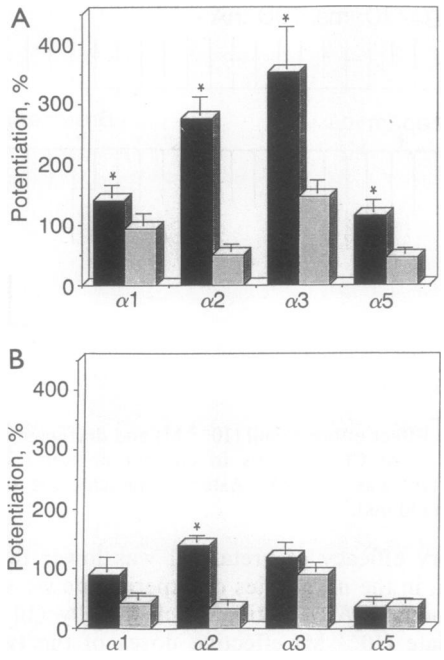

FIG. 3. Effect of diazepam (black bars) and bretazenil (gray bars) on GABA-gated Cl^- currents at $GABA_A$ receptors reconstituted in 293 cells by cotransfection with cDNAs encoding for $\beta 1$ and $\gamma 2$ subunits and various molecular forms of the α subunit. Each bar represents the mean ± SEM for six to eight cells. (*A*) Asterisks indicate that application of maximally efficacious doses (10^{-5} M) of diazepam and bretazenil yielded significantly different activities (Student's *t* test, $P < 0.05$) at all the receptor configurations. (*B*) The differences between the activities of diazepam and bretazenil at 10^{-7} M were significant only for $\alpha 2\beta 1\gamma 2$ receptor (Student's *t* test, $P < 0.05$).

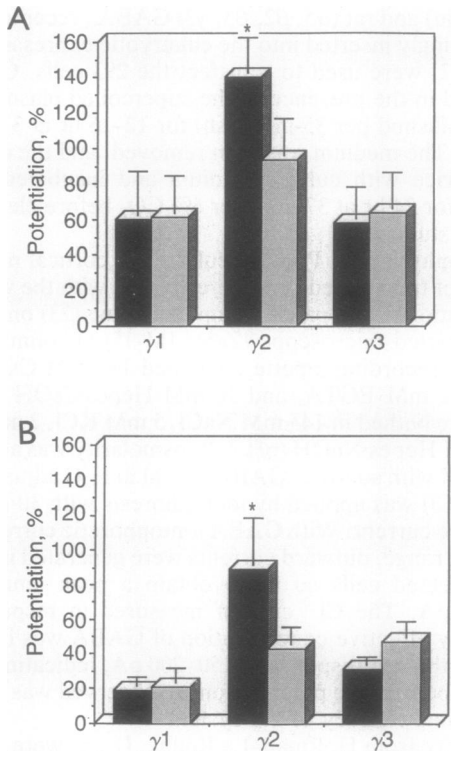

FIG. 4. Effect of diazepam (black bars) and bretazenil (gray bars) on GABA-activated Cl^- currents at $GABA_A$ receptors in 293 cells transfected with cDNAs encoding various molecular forms of the γ and the $\alpha 1$ and $\beta 1$ subunits. Each bar represents the mean ± SEM for six to eight cells. At a concentration of 10^{-5} M (*A*) and 10^{-7} M (*B*), there was a significant difference between the activities of the two drugs only at $\alpha 1\beta 1\gamma 2$ receptors (Student's *t* test, $P < 0.05$).

It should be noted that diazepam is clearly less efficacious in $GABA_A$ receptor configurations that include $\gamma 1$ and $\gamma 3$ receptor subunits than in those containing $\gamma 2$. In 293 cells transfected with cDNAs encoding one of three distinct forms of β subunit ($\beta 1$, $\beta 2$, $\beta 3$) and $\alpha 1$ and $\gamma 2$ subunits, diazepam was always more efficacious than bretazenil (Fig. 5).

Antagonism of Diazepam Action by Bretazenil in $\alpha 1\beta 1\gamma 2$. To determine whether the differences in the efficacies of bretazenil and diazepam were due to different occupancy of the receptors, diazepam (10^{-5} M) was applied from pipettes that also contained various concentrations of bretazenil. The diazepam effect was reduced in a dose-dependent manner by bretazenil (Fig. 6). At equal concentrations of the two drugs (10^{-5} M), diazepam modulation never exceeded the basal modulatory action of bretazenil (compare data of Fig. 6 with those of Fig. 5, configuration $\beta 1$).

DISCUSSION

With the use of the Vogel test to evaluate the anticonflict and anti-proconflict potencies of positive modulators of $GABA_A$ receptors, Giusti *et al.* (24) showed that bretazenil was as potent as diazepam at reducing conflict responses caused by punishment. However, the bretazenil potency was 10 times that of diazepam in reducing the proconflict action of pentamethylentetrazol (24). Moreover, when diazepam and bretazenil were compared with regard to their potencies in inhibiting pentamethylentetrazol-induced convulsions, the bretazenil potency was significantly greater than that of diazepam. However, the efficacy of diazepam was three times that of bretazenil. The lower efficacy of bretazenil could not be attributed to a preferential modulatory action of the drug at $GABA_A$ receptor subtypes different from those modulated by diazepam because maximally efficacious doses of bretazenil reduced the efficacy of diazepam to inhibit pentamethylentetrazol-induced convulsions (24). Thus, it could be concluded that bretazenil acts as a partial allosteric modulator of the same $GABA_A$ receptors that are used by diazepam to inhibit convulsions.

There are, however, differences between the pharmacological profiles of diazepam and bretazenil that could be interpreted as due to a lack of bretazenil action at a $GABA_A$ receptor subunit that is modulated by diazepam. For instance, diazepam causes ataxia, bretazenil does not (25); diazepam potentiates ethanol sedation, bretazenil does not (19); and three daily doses of diazepam repeated for several

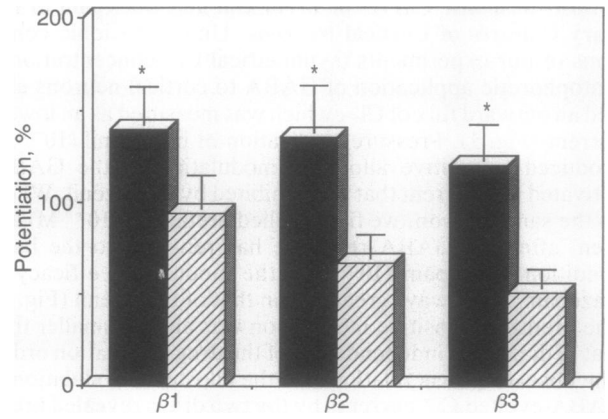

FIG. 5. Effect of bretazenil (10^{-5} M; hatched bars) and diazepam (10^{-5} M; black bars) on GABA-activated Cl^- currents at reconstituted $GABA_A$ receptors containing various molecular forms of β subunit together with the $\alpha 1$ and $\gamma 2$ subunits. Each value represents the mean ± SEM for *n* cells. Asterisks indicate significant differences (Student's *t* test, $P < 0.05$) between the effects of the two drugs.

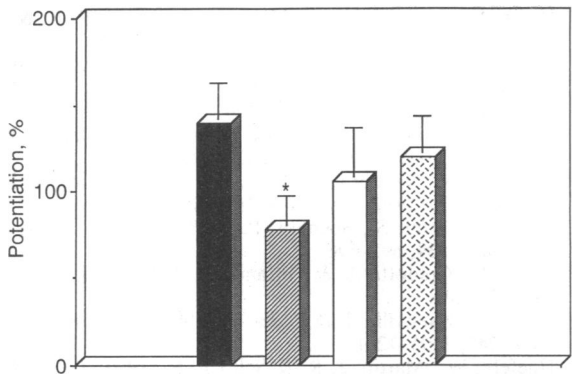

FIG. 6. Antagonism of bretazenil on the positive modulatory effect of diazepam on GABA-gated Cl^- currents in $\alpha1\beta1\gamma2$ receptors. Coapplication of bretazenil (10^{-5} M) with diazepam (10^{-5} M) (hatched bar) reduced the diazepam effect (black bar) by almost 50%. The antagonistic action of bretazenil was dose-dependent (white bar = diazepam plus 1 μn bretazenil; speckled bar = diazepam plus 0.1 μn bretazenil) but was significantly different from the modulation of diazepam alone only at 10^{-5} M (Student's t test, $P < 0.05$). Each bar represents the mean ± SEM for five cells.

days can induce tolerance to the behavioral and anticonvulsant actions of the drug, whereas an identical treatment schedule with bretazenil fails to induce tolerance (26). Moreover, the activity of bretazenil persists in animals that are tolerant to diazepam (A. Guidotti, personal communication). Collectively, these data raise the question of whether bretazenil behaves like a low-efficacy allosteric modulator because it has no intrinsic activity on a subset of $GABA_A$ receptors or because it has low efficacy on all $GABA_A$ receptor subtypes.

Although mRNA expression studies have shown that at least 15 genes encoding for $GABA_A$ receptor subunits are expressed *in vivo*, the subunit composition and stoichiometry of native $GABA_A$ receptors are not known. Although various assemblies of $GABA_A$ receptor subunits can be expressed in eukaryotic cells after transfection with cDNAs encoding for selected $GABA_A$ receptor subunits, the exact stoichiometry of the transfected $GABA_A$ receptors is unknown. We have used the transient expression of various cDNAs encoding for different molecular forms of α, β, and γ subunits to address the following questions: (*i*) Does diazepam elicit the maximal modulatory effect at all $GABA_A$ receptor subtypes? (*ii*) Does bretazenil act like a partial agonist because it is devoid of modulatory activity at specific $GABA_A$ receptor subtypes? (*iii*) Does bretazenil possess a limited modulatory efficacy at all $GABA_A$ receptor subtypes?

We addressed, in part, the first question in previous experiments (16) by showing that the diazepam modulatory efficacy, like that of other $GABA_A$ receptor modulators, depends on the subunit composition of the $GABA_A$ receptor. For instance, diazepam showed greater efficacy at $\alpha3\beta1\gamma2$ and $\alpha2\beta1\gamma2$ receptors than at $\alpha1\beta1\gamma1$ receptors (16). In the latter receptor assembly zolpidem and alpidem were more efficacious than diazepam (16). Thus, even diazepam, which was considered the prototypical high-efficacy allosteric modulator at all $GABA_A$ receptors, did not behave in the predicted manner on every $GABA_A$ receptor subtype.

Although several investigators have studied extensively the pharmacological profile of bretazenil *in vivo*, its binding characteristics and some electrophysiological properties (27) *in vitro*, the modulatory action of this drug at various $GABA_A$ receptor subtypes had not been investigated. We first studied the effect of bretazenil on native $GABA_A$ receptors in primary cultures of cortical neurons and showed that the bretazenil efficacy was significantly lower than that of diazepam, whereas the potencies of the two drugs were similar (Fig. 2). This is in agreement with a binding affinity of bretazenil greater than that of diazepam (24). Because cortical neurons (28) express a heterogeneous population of $GABA_A$ receptors, we addressed our third question by examining the diazepam and bretazenil modulatory efficacy and potency on recombinant $GABA_A$ receptors obtained by transfecting the 293 cell line with α, β, and γ subunits and alternatively inserting several molecular variants of these subunits. We found that, whereas diazepam showed different efficacies at structurally different $GABA_A$ receptors (16), bretazenil consistently expressed an almost uniform low modulatory efficacy at all the different $GABA_A$ receptors tested (Fig. 3).

The presence of a γ subunit in the $GABA_A$ receptor is an essential requirement for the functional expression of the allosteric modulatory site at which BZs act. However, only at receptors including the $\gamma2$ subunit did diazepam and bretazenil show different potencies and efficacies. In $GABA_A$ receptors, including the $\gamma1$ or the $\gamma3$ subunit, both drugs revealed low absolute modulatory efficacy (Fig. 4); moreover in these receptors the properties of the two drugs were virtually identical. Thus, it can be surmised that the different efficacies of diazepam and bretazenil in eliciting anticonvulsant action, ataxia, ethanol-potentiating effects, impairment of motor activity, asthenia, and tolerance do not result from drug actions at receptors including $\gamma1$ or $\gamma3$ subunits. The differences between the pharmacological profile of bretazenil and diazepam may not be due to reduced occupancy by bretazenil of the $GABA_A$ receptor modulatory sites responding to diazepam because the coapplication of diazepam and bretazenil (Fig. 6) showed a clear competition for the same site within the $GABA_A$ receptor ($\alpha1\beta1\gamma2$) tested.

The low-efficacy modulators of $GABA_A$ receptors are important because their pharmacological profiles indicate that they may have clinical relevance. They fail to induce tolerance (26), they do not depress motor activity (24), they do not potentiate the effects of ethanol (19), and they do not appear to elicit physical dependence liability. It is also believed that modulators with low intrinsic activity, such as bretazenil, may possess therapeutical efficacy for panic disorders and phobias, again with minimal tolerance and dependence liabilities. Moreover, because high-efficacy allosteric modulators tend to raise any response of $GABA_A$ receptor to physiologically released GABA to the maximal level, one can suggest that the high-efficacy modulators obliterate physiologically graded responses, thereby leveling GABA responses to their plateau level. Because high-efficacy modulators cause tolerance, dependance, and other unwanted side effects, one can conclude that the obliteration of physiologically graded response might be considered the source of many problems encountered in therapy.

It was reported that blockade of GABA uptake, which also abolishes physiologically graded responses of $GABA_A$ receptors, induces psychotic behavior (29). Probably also high-efficacy BZs with low degree of preference for selective $GABA_A$ receptor subtypes, such as triazolam, should be avoided because of a possible psychotogenic liability. In contrast, low-efficacy allosteric modulators, probably because they fail to obliterate physiologically graded responses of $GABA_A$ receptors, appear to have a basic pharmacological profile of potential therapeutic interest. In fact, in animal experiments pharmacologically active doses of low-efficacy modulators appear to be devoid of or to provide only limited sedative action, ataxia, and potentiation of alcohol sedation.

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