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ACCELERATED COMMUNICATION

Influence of Recombinant γ-Aminobutyric Acid-$_A$ Receptor Subunit Composition on the Action of Allosteric Modulators of γ-Aminobutyric Acid-Gated Cl^- Currents

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SUMMARY

γ-Aminobutyric acid (GABA)-activated Cl^- currents in neonatal rat cortical neurons and in cultured cells engineered for the expression of specific molecular forms of the $GABA_A$ receptor α, β, and γ subunits, were recorded with the patch-clamp technique in the whole-cell configuration. The effects of various allosteric modulators of $GABA_A$ receptors were determined. Diazepam and clonazepam showed greater efficacy as positive modulators of GABA-elicited currents in $\alpha2\beta1\gamma2$ or $\alpha3\beta1\gamma2$ receptors than in $\alpha1\beta1\gamma2$ or $\alpha5\beta1\gamma2$ receptors or in cortical neurons. Alpidem was more efficacious at $\alpha1\beta1\gamma2$ or $\alpha2\beta1\gamma2$ receptors than at $\alpha1\beta1\gamma2$ or $\alpha5\beta1\gamma2$ receptors or in cortical neurons. Conversely, zolpidem was equally efficacious for all these receptors except for $\alpha5\beta1\gamma2$. Both imidazopyridines (alpidem and zolpidem) were virtually ineffective at modulating the GABA response of $\alpha5\beta1\gamma2$ receptors and in almost all the receptors assembled from α1, α2, α3, or α5 subunits together with β1 and γ1 subunits. The β-carboline derivatives methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) and methyl-β-carboline-3-carboxylate (β-CCM) elicited a positive allosteric modulation of $\alpha1\beta1\gamma1$ or $\alpha2\beta1\gamma1$ receptors, whereas they acted as negative allosteric modulators at nearly all other receptors tested, as they do in cortical neurons. Although the positive allosteric modulation by β-carbolines never exceeded a doubling of the GABA response, DMCM was more efficacious at $\alpha1\beta1\gamma1$ receptors and β-CCM was more efficacious at $\alpha2\beta1\gamma1$ receptors. DMCM was inactive at $\alpha3\beta1\gamma1$ receptors, whereas β-CCM was virtually inactive at $\alpha5\beta1\gamma1$ receptors. The benzodiazepine 4′-chlorodiazepam, which is a negative modulator resistent to flumazenil inhibition, acted at all the various $GABA_A$ receptors that contained a γ subunit.

The $GABA_A$ receptor is the most abundant inhibitory receptor in mammalian brain; this receptor has a heteropolymeric structure that forms a Cl^- channel (1). To date, 12 distinct receptor subunits have been identified (six α, three β, two γ, and one δ) (2–10). The biophysical properties of receptors assembled from a number of subunit combinations have been defined (11).

GABA binding to its specific recognition site on the $GABA_A$ receptor opens the ion channel and allows an inward flow of Cl^- into the cell. This Cl^- current can be regulated both by drugs and by endogenous ligands that act as positive or negative allosteric modulators. GABA action can be modified by isosteric receptor antagonists, which are thought to bind to the extracellular domain of the receptor. Allosteric modulation is elicited by two classes of compounds, those that act on the extracellular domain and those that act on the channel domain of the receptor (1), with both classes including positive and negative allosteric modulators. The positive modulators acting on the extracellular domain include BZs, IZs, and triazolopyridazines, whereas the negative modulators mainly comprise BC derivatives. These positive and negative allosteric modulators act on the extracellular domain of the $GABA_A$ receptor and are inhibited by flumazenil (1). The only BZ known to act as negative modulator is 4′-chlorodiazepam, but its action is flumazenil resistant (12). The presence or absence of the γ2 subunit in the $GABA_A$ receptor structure can influence the action of positive allosteric modulators (4). In contrast, the effects of drugs that bind within the channel domain and act as positive (barbiturates and steroid hormone derivatives) or negative (pregnenolone sulfate and picrotoxin) allosteric modulators do not appear to depend on the structural configuration of the receptor (3, 4, 13, 14). These compounds also act on homopolymeric receptors

ABBREVIATIONS: $GABA_A$ receptor, type A γ-aminobutyric acid receptor; DMCM, methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate; GABA, γ-aminobutyric acid; β-CCM, methyl-β-carboline-3-carboxylate; zolpidem, *N,N*-6-trimethyl-2-(4-methylphenyl)imidazo[1,2-a]-pyridine-3-acetamide hemitartrate; alpidem, *N,N*-dipropyl-6-chloro-2-(4-chlorophenyl)imidazo[1,2-a]-pyridine-3-acetamidehemitartrate; BZ, benzodiazepine; BC, β-carboline; IZ, imidazopyridine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

(3, 13, 14). The activities of BCs and BZs, including 4′-chlorodiazepam, require a heteropolymeric structure containing α, β, and γ subunits (4, 12).

Recently, the distinction between the type I and type II classes of BZs based on binding studies (15) was shown to be related to different molecular forms of the α subunit (16). However, *in situ* hybridization studies (5, 17–19) of different brain areas and binding studies with various $GABA_A$ receptor subunit assemblies (4, 7, 13) provided evidence that the natural heterogeneity of $GABA_A$ receptor subunits is more complex, and the assumption that there are only two natural types of BZ receptor appears to be reductionistic.

Our present studies address the pharmacological profiles of three BZs (diazepam, clonazepam, and 4′-chlorodiazepam), two BCs (DMCM and β-CCM), and two IZs (alpidem and zolpidem). We describe the effects of subunit compositions of heteropolymeric recombinant $GABA_A$ receptors on the efficacy and potency of these allosteric modulators and on the direction of the allosteric modulation they elicit.

Materials and 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 $0.8–1 \times 10^6$ on 35-mm Nunc dishes that were 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), 100 μg/ml gentamicin (GIBCO), for 1 to 3 weeks. After 24 hr, the incubation medium was replaced and 1 μM cytosine arabinofuranoside was added to inhibit replication of nonneuronal cells.

Culture of kidney embryonic cell line and cDNA transfection. Transformed human embryonic kidney 293 cells (American Type Culture Collection no. CRL 1573) were grown in minimal essential medium (GIBCO), supplemented with 10% fetal bovine serum, 100 units/ml penicillin (GIBCO), and 100 units/ml streptomycin (GIBCO), in a 6% CO_2 incubator. Exponentially growing cells were dispersed with trypsin and seeded at 2×10^5 cells/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, and γ2) and rat (α5) $GABA_A$ receptor subunit cDNAs singly inserted into the eukaryotic expression vector pCIS2 (22) were used to perform the transfections. Cells were incubated in the presence (3 μg/35-mm dish) of one or more supercoiled plasmids for 12–16 hr at 37°, under 3% CO_2. The medium was removed, and the cells were rinsed twice with culture medium and then incubated in the same medium for 24 hr at 37°, under 6% CO_2, before electrophysiological studies. Cells that were analyzed expressed the following subunit combinations: $\alpha1\beta1\gamma2$, $\alpha2\beta1\gamma2$, $\alpha3\beta1\gamma2$, $\alpha5\beta1\gamma2$, $\alpha1\beta1\gamma1$, $\alpha2\beta1\gamma1$, $\alpha3\beta1\gamma1$, and $\alpha5\beta1\gamma1$.

Electrophysiology. Primary cultures of cortical neurons or cultures of transfected cells were studied with the single-electrode voltage-clamp technique in the whole-cell configuration (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 nM 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 mOs M with sucrose. GABA (0.5 M in H_2O, adjusted to pH 4 with HCl) was applied by iontophoresis with 30-msec pulses of positive current. With GABA iontophoretic currents in the 25–50 nA range, outward currents were generated in neurons or transfected cells in such a way as to obtain a peak amplitude of 150–200 pA. BZs were a gift from Hoffman La Roche, DMCM and β-CCM were from Ferrosan, and the IZs were from Syntelabo. All drugs were dissolved in bath solution containing dimethylsulfoxide at a maximal final concentration of 0.01%. Drugs were applied by pressure (2 to 4 psi), in the proximity of the cell body, with micropipettes of 5–10 μm diameter. The application of dimethylsulfoxide (0.01% in bath medium) failed to modify GABA responses. To avoid uncontrolled drug leakage, we kept the pipette outside the bath before the pressure injection and brought it into proximity with the recorded cell just before drug application. Drugs were applied for 5 sec between two GABA pulses delivered every 10 sec. The maximal Cl^- current measured from each cell was larger (>1 nA) than the test response of 150–200 pA we used, indicating that the percentages of potentiation we observed were far below the maximal efficacy of the system. Current traces were recorded by a patch-clamp amplifier (EPC-7; List Electronics), filtered at 1500 Hz (eight-pole low-pass Bessel; Frequency Devices), and recorded on a chart recorder (Gould 2600S) for off-line analysis.

Often the GABA response was greater in cells adjacent to those from which currents were being recorded. This observation might relate to electrical coupling between 293 cells (13). This phenomenon made it easier to find cells expressing the $GABA_A$ receptor subunits. Recordings were performed within 3 days of transfection.

Results

Action of diazepam and alpidem on $\alpha x\beta1\gamma2$ receptors. Examples of the positive modulatory effects of diazepam and alpidem (both at 10 μM) on native $GABA_A$ receptors of cortical neurons and on various reconstituted $GABA_A$ receptors are shown in Fig. 1. As evident from these records, alpidem and diazepam showed very little activity toward $\alpha5\beta1\gamma2$ receptors, and the extent of facilitation by diazepam was much greater for $\alpha3\beta1\gamma2$ receptors than for other receptors. Alpidem appeared to be nearly as efficacious at $\alpha1\beta1\gamma2$ as at native receptors and lower at $\alpha3\beta1\gamma2$ receptors.

Differences in the efficacy and potency of diazepam at $\alpha x\beta1\gamma2$ receptors. We measured the allosteric modulation of the GABA response mediated by different concentrations (10^{-9} to 10^{-5} M) of diazepam (Fig. 2) in cortical and in $\alpha x\beta1\gamma2$ receptors. Diazepam had similarly low efficacy at $\alpha1\beta1\gamma2$, $\alpha5\beta1\gamma2$, and native $GABA_A$ receptors, and its lowest potency was at $\alpha5\beta1\gamma2$ receptors. Diazepam efficacy was nearly 3 times greater for the $\alpha3\beta1\gamma2$ assemblage than for $\alpha1\beta1\gamma2$, $\alpha5\beta1\gamma2$, and native receptors, and at $\alpha2\beta1\gamma2$ receptors diazepam showed an intermediate efficacy. The EC_{50} of diazepam was approximately the same for the native receptors and for the $\alpha1\beta1\gamma2$ and $\alpha2\beta1\gamma2$ receptors (~50 nM) but was much greater for the $\alpha5\beta1\gamma2$ receptor (~4000 nM).

Differences in IZ and BZ efficacy at various reconstituted $GABA_A$ receptors. Fig. 3A shows the effects elicited by two BZs and two IZs, all at a concentration of 10 μM, in cortical cells and in cells expressing $\alpha1\beta1\gamma2$, $\alpha2\beta1\gamma2$, $\alpha3\beta1\gamma2$, or $\alpha5\beta1\gamma2$ receptors. We chose the $\alpha1\beta1\gamma2$ combination as a reference point because such a receptor seems to be frequently expressed in brain (18). Diazepam and clonazepam were equally efficacious in cortical and in $\alpha1\beta1\gamma2$ receptors, but their efficacy increased considerably when the $\alpha2$ subunit was present and was even greater in $\alpha3\beta1\gamma2$ receptors. However, a significantly greater potentiation at the $\alpha3\beta1\gamma2$ receptors, compared with $\alpha1\beta1\gamma2$ receptors (Duncan test), could be observed.

Zolpidem and alpidem did not show significant differences in efficacy between $\alpha1\beta1\gamma2$, $\alpha2\beta1\gamma2$, $\alpha3\beta1\gamma2$, and cortical receptors (Fig. 3A) but failed to potentiate GABA responses at $\alpha5\beta1\gamma2$ receptors.

Decreased sensitivity to BZs and IZs at receptors containing the $\gamma1$ subunit. We further investigated the modulation of the GABA response by the two BZs and the two IZs in cells expressing the $\gamma1$ subunit together with $\alpha1\beta1$, $\alpha2\beta1$,

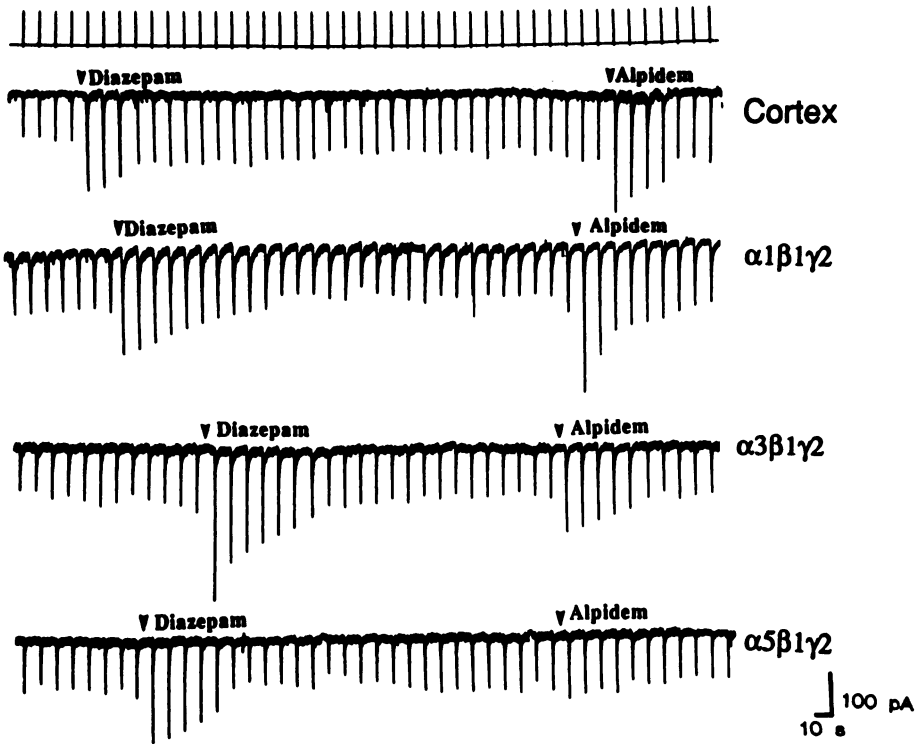

Fig. 1. Differential sensitivity to diazepam and alpidem of neurons expressing native GABA$_A$ receptors (cortex) and of cells engineered to express $\alpha1\beta1\gamma2$, $\alpha3\beta1\gamma2$, or $\alpha5\beta1\gamma2$ receptors. The *upper trace* marks the repetitive iontophoretic application of GABA (50 nA for 30 msec). GABA elicited an inward Cl^- current. Diazepam and alpidem, applied by pressure at a concentration of 10 μM for 2 sec (*arrowheads*), potentiated the inward current.

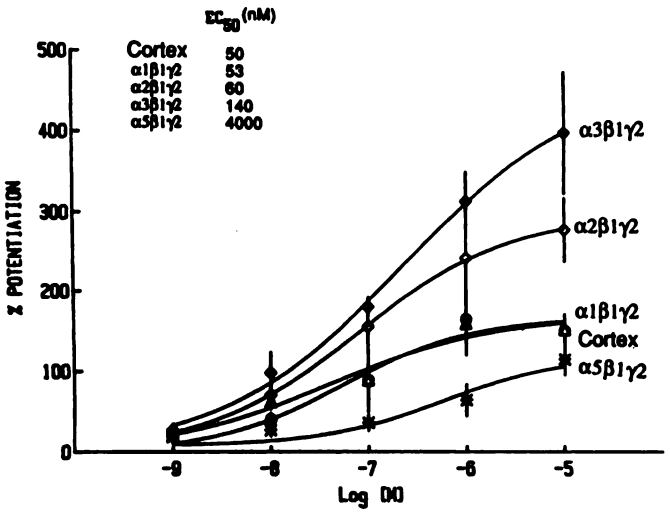

Fig. 2. Dose-dependent potentiation by diazepam of GABA-evoked Cl^- current in cortical cells and cells expressing $\alpha1\beta1\gamma2$, $\alpha2\beta1\gamma2$, $\alpha3\beta1\gamma2$, and $\alpha5\beta1\gamma2$ transfected receptors. Each concentration point is mean ± standard error of six different cells. The EC_{50} of each curve is shown in the *inset*. The maximal potentiation values for the different receptors are as follows: $\alpha5\beta1\gamma2$, 110%; $\alpha1\beta1\gamma2$ and cortex, 120%; $\alpha2\beta1\gamma2$, 270%; and $\alpha3\beta1\gamma2$, 400%.

$\alpha3\beta1$, or $\alpha5\beta1$ subunit combinations (Fig. 3B). Each drug was much more efficacious at receptors containing the $\gamma2$ rather than the $\gamma1$ subunit. The efficacies of diazepam and clonazepam at GABA$_A$ receptors containing the $\gamma1$ subunit were reduced by about 50%, compared with $\alpha1\beta1\gamma2$ receptors. Alpidem and zolpidem efficacies were markedly decreased (approximately 85% for alpidem and 75% for zolpidem) at $\alpha1\beta1\gamma1$ receptors, compared with $\alpha1\beta1\gamma2$ receptors, and there was virtually no potentiation of the GABA response in cells expressing $\alpha1\beta1\gamma1$, $\alpha2\beta1\gamma1$, or $\alpha3\beta1\gamma1$ receptors.

Negative modulatory activity at recombinant GABA$_A$ receptors. The BCs DMCM and β-CCM, as well as 4′-chlorodiazepam, were tested on receptors containing the $\gamma1$ or $\gamma2$ subunit together with $\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$, or $\alpha5\beta1$ subunit combinations (Fig. 4). 4′-Chlorodiazepam decreased GABA-activated Cl^- currents in cortical neurons and in all the recombinant receptors by 30–70%. In contrast, DMCM and β-CCM behaved as negative modulators in cortical neurons and at receptors containing the $\gamma2$ subunit but acted as positive modulators at $\alpha1\beta1\gamma1$ and $\alpha2\beta1\gamma1$ receptors. DMCM was more efficacious at $\alpha1\beta1\gamma1$ than at $\alpha2\beta1\gamma1$ receptors; conversely, β-CCM showed a higher efficacy at $\alpha2\beta1\gamma1$ than at $\alpha1\beta1\gamma1$ receptors. At $\alpha3\beta1\gamma1$ receptors, β-CCM was a negative modulator, whereas DMCM was almost inactive. DMCM elicited a weak positive modulation at $\alpha5\beta1\gamma1$ receptors, whereas β-CCM was inactive. Thus, BC derivatives might behave as positive or negative modulators according to the receptor structure. Fig. 5 shows the dose-dependent positive allosteric modulation by DMCM of GABA-activated Cl^- currents in $\alpha1\beta1\gamma1$ receptors and the dose-dependent negative allosteric modulation by the same drug of GABA-activated Cl^- currents in $\alpha1\beta1\gamma2$ receptors.

Discussion

Recent cloning of the cDNAs encoding for the mRNA for various subunits of the GABA$_A$ receptor (2–10) has prompted a search for the functional significance of this structural heterogeneity. Our experiments show that this heterogeneity causes a diversity in the pharmacological profiles of BZ, BC, and IZ acting as allosteric modulators of GABA-gated Cl^- currents. This diversity may arise from differences in the structure of the modulatory center where these drugs act. In spite of the fact that differences in the stochiometry or post-translational modifications of reconstituted receptors might underlie some of the diversity we observed, we believe that our

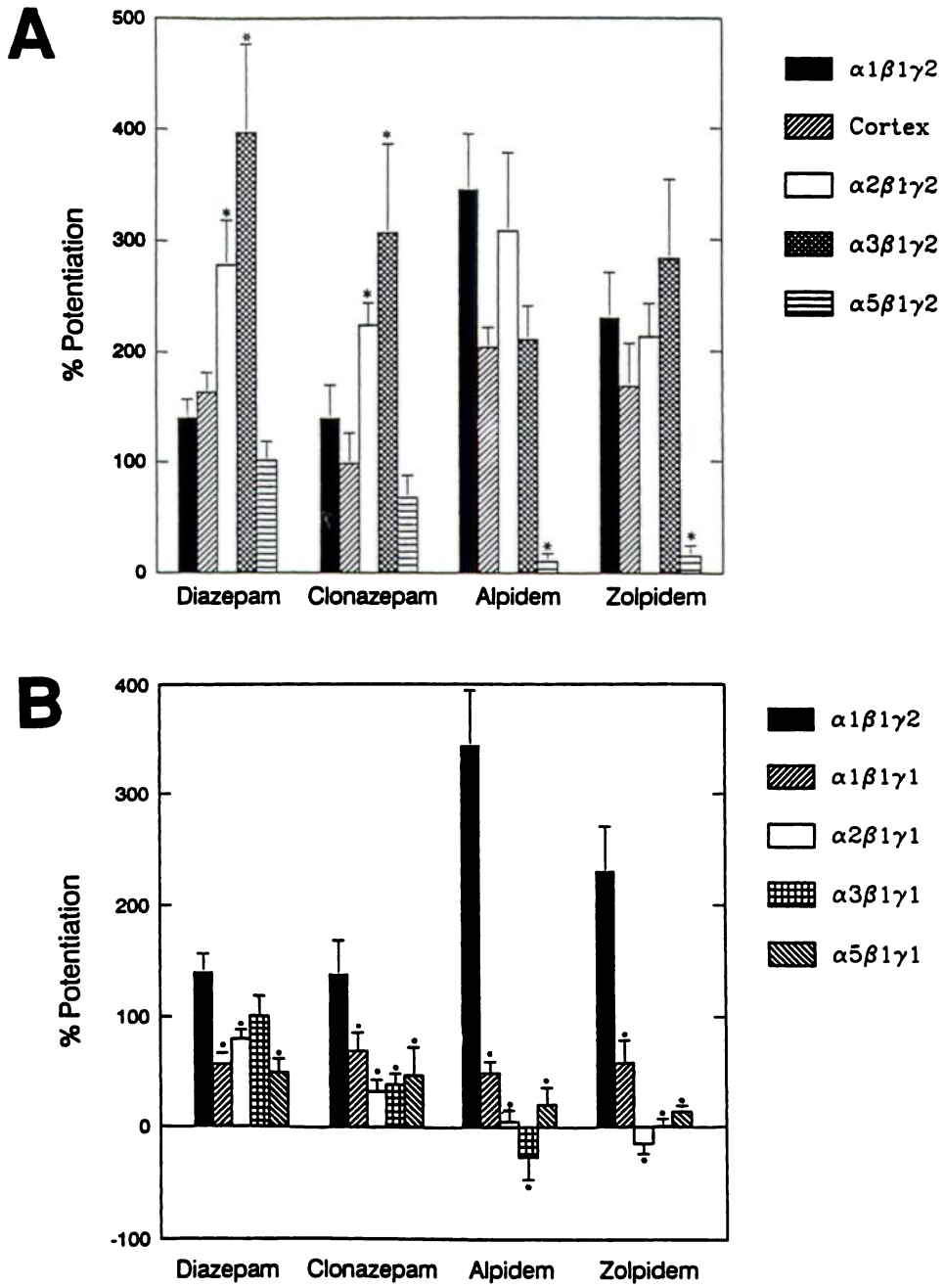

Fig. 3. A, BZ and IZ modulation of the GABA response of native receptors and of reconstituted receptors containing $\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$, and $\alpha5\beta1$ subunit combinations together with the $\gamma2$ subunit. B, BZ and IZ modulation of the GABA responses of $\alpha1\beta1\gamma1$, $\alpha2\beta1\gamma1$, $\alpha3\beta1\gamma1$, or $\alpha5\beta1\gamma1$ receptors. Each value is the mean ± standard error of 8–12 cells. *, Subunit combinations showing a significant ($p < 0.05$, Duncan test) difference in potentiation of the GABA response, compared with that measured for $\alpha1\beta1\gamma2$ receptors. Drug concentrations in all experiments were 10^{-5} M.

results are a good first approximation of structural differences in pharmacological profiles of $GABA_A$ receptors. The drug efficacy not only changes in relation to the molecular form of the α subunit but also depends on the molecular structure of the γ subunit. Indeed, the direction of the modulatory effect of BC derivatives seems to be markedly dependent on the type of γ subunit.

Drug potency was studied only for diazepam and appeared to be related to changes in the molecular form of the α subunit, in contrast to the efficacy of 4′-chlorodiazepam, a selective ligand for the peripheral BZ receptor that also decreases GABA-gated Cl^- currents in a manner that is insensitive to flumazenil (12). Moreover, the direction of its modulatory action appeared to be independent of the molecular form of α and γ subunits. This difference suggests that the molecular mechanisms that are operative in the two types of allosteric modulation may not be similar; 4′-chlorodiazepam modulation may act via the transmembrane domain of the receptor, which is known to be insensitive to flumazenil inhibiton.

Ligand binding experiments have indicated that at least two distinct classes of BZ receptors, termed type I and type II (15), exist in the brain. These binding differences have been reproduced with receptors containing $\beta1$, $\gamma2$, and either $\alpha1$ (type I) or $\alpha2$ or $\alpha3$ (type II) $GABA_A$ receptor subunits (16). Furthermore, in those receptors, diazepam binding was facilitated by the presence of GABA to a greater extent if the $\alpha3$ subunit, rather than $\alpha1$ or $\alpha2$ subunits, was present. This not only was the first indication that distinct receptor subtypes have different BZ sensitivities but also suggested that BZ type II receptors are not uniform. Our results verify the existence of such functional differences and expand on the relation between struc-

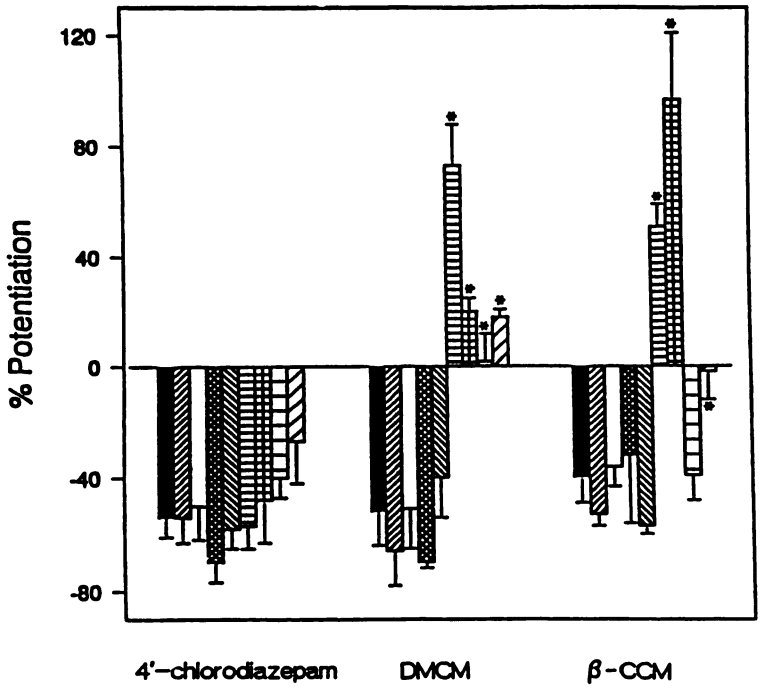

Fig. 4. DMCM and β-CCM modulation of GABA responses in cells expressing different subunit assemblies. Drugs were applied at a concentration of 10^{-6} M. *, Significant ($p < 0.05$, Duncan test) difference relative to the effect on α1β1γ2 receptors. Each value is an average of six cells.

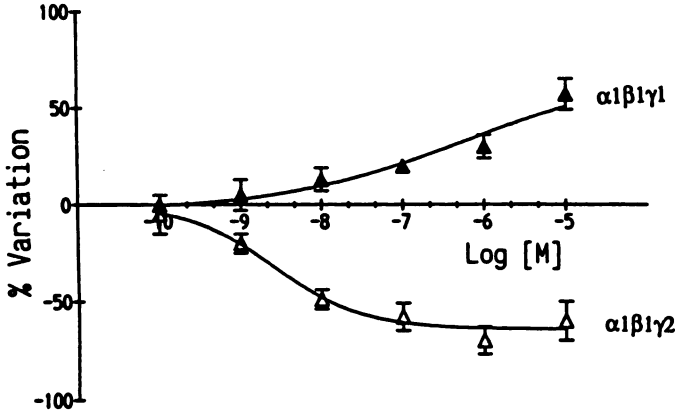

Fig. 5. Dose-response to DMCM of GABA-evoked Cl^- current in cells expressing α1β1γ2 and α1β1γ1 transfected receptors. Each concentration point is mean ± standard error of four different cells.

tural heterogeneity and functional diversity in the allosteric modulation of the GABA$_A$ receptor by BZs, BCs, and IZs.

The extent of GABA activity potentiation by maximally efficacious doses of diazepam and clonazepam (10 μM) was similar in rat cortical neurons expressing native receptors and in cells engineered for the expression of α1β1γ2 and α5β1γ2 receptors. However, both drugs were more efficacious when α2 substituted for α1, and even more so when α3 substituted for α1. In fact, the diazepam dose-response curves for potentiation of GABA activity in cortical neurons and in cells expressing α1β1γ2 or α5β1γ2 receptors demonstrate similar low efficacies of the drug, whereas diazepam efficacy was much greater (4-fold potentiation versus 1.5-fold) in α3β1γ2 receptors. One might surmise that this difference could be due to a greater number of cellularly expressed α3β1γ2 receptors than α1β1γ2 or α5β1γ2 receptors. However, this possibility can be excluded because, if this were the case, all the drugs tested should have produced a maximal potentiation of GABA activity in cells expressing α3β1γ2 receptors. Alpidem did not show maximal potentiation with α3β1γ2 receptors; the efficacy of this IZ was greatest for α1β1γ2 and α2β1γ2 receptors.

Very likely, GABA$_A$ receptors expressed in cortical neurons consist of a heterogeneous receptor population with regard to subunit compositions, and the responses measured at these native receptors probably reflect an average of the drug interactions with diverse GABA$_A$ receptor subtypes.

Our data suggest that certain subunit assemblages are more susceptible to modulation by IZs than by BZs and support the results of previous binding studies (7) showing that the affinity of zolpidem for receptors containing α5 is lower than for α2 or α3 subunit-containing receptors and that zolpidem shows the highest affinity for receptors containing the α1 subunit.

The modulation of Cl^- currents by both BZs and IZs was markedly reduced in GABA$_A$ receptors that carried the γ1 subunit (10) instead of γ2 (Fig. 3B). Furthermore, there was a tendency for zolpidem and alpidem to elicit negative modulation when applied to cells expressing α2β1γ1 or α3β1γ1 receptors. Such a subunit combination might be expressed in astroglial cells, but a complete study of IZ and BZ modulation of GABA action on these cells is lacking. However, a positive modulation of the GABA response by DMCM was described in primary cultures of astrocytes (24), which suggests that the γ1 subunit may be present in the GABA$_A$ receptors of glial cells.

Because certain GABA$_A$ receptor subtypes seem to be located preferentially in certain brain structures (17–19), the relation we have demonstrated between receptor structural heterogeneity and the efficacy and potency of various anxiolytic and anxiogenic drugs may have important implications for the therapeutic targeting of these drugs to specific GABA$_A$ receptor subtypes, perhaps with a preference for certain brain structures. Recently, the diversity of responses to BZs related to the molecular structure was investigated in GABA$_A$ receptors expressed in *Xenopus* oocytes (25). Differences in molecular structure, conferring sensitivity to BZs, between our reconstitution

model and the *Xenopus* oocytes might be related to the intrinsic difference between the two expression systems and/or to species differences in recombinant subunit structures (rat versus human).

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