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Comparison of Interactions of [^{3}H]Muscimol, *t*-Butylbicyclophosphoro[^{35}S]thionate, and [^{3}H]Flunitrazepam with Cloned γ-Aminobutyric Acid$_A$ Receptors of the $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_2$ Subtypes

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SUMMARY

The $\alpha1\beta2$ and $\alpha1\beta2\gamma2$ subtypes, common subtypes of γ-aminobutyric acid (GABA)$_A$ receptors in the brain, are known to share many ligands, but only the latter interacts with benzodiazepines. In this study, we attempted to examine whether the presence of the $\gamma2$ subunit in the cloned receptors alters the binding properties of GABA and t-butylbicyclophosphorothionate (TBPS) (a highly sensitive probe for conformational changes in the chloride ionophore of GABA$_A$ receptors) and their interactions. Using a high-level expression system of SF-9 cells infected with baculovirus, we produced a group of cloned GABA$_A$ receptors with variations in the ratio (0 to 3) of the virion carrying the cDNA for the $\gamma2$ subunit to those carrying the cDNAs for the $\alpha1$ and $\beta2$ subunits. The number of benzodiazepine binding sites increased as the level of the $\gamma2$ virion was raised and reached that of GABA high affinity sites at a $\gamma2$ to $\alpha1\beta2$ ratio of 0.5 or more. It appears that the conversion of the $\alpha1\beta2$ to the $\alpha1\beta2\gamma2$ subtype is favorable and complete in the presence of a sufficient level of the $\gamma2$ subunit, assuming the number of the GABA sites to be equal to the total number of the cloned GABA$_A$ receptors. In all preparations, the dissociation constants for flunitrazepam, muscimol, and TBPS were fairly constant, and the maximal number of binding sites for TBPS appeared to be equal to that for muscimol, with no dependence on the $\gamma2$ virion levels. The effect of GABA on TBPS binding, however, were markedly altered by the $\gamma2$ subunit. With the $\alpha1\beta2$ subtype GABA at concentrations occupying its high affinity sites markedly stimulated but at higher concentrations (micromolar ranges) inhibited TBPS binding, whereas with the $\alpha1\beta2\gamma2$ subtype GABA inhibited TBPS binding without the early stimulatory phase. We also confirmed the selective interaction of Zn^{2+} (50 μM) with the $\alpha1\beta2$ subtype, as probed with TBPS binding, and observed a progressive disappearance of Zn^{2+} sensitivity as the $\gamma2$ virion level increased. These results show that addition of the $\gamma2$ subunit to the $\alpha1\beta2$ subtype does not disturb the primary binding sites for GABA and TBPS but markedly modifies GABA-induced conformational changes in the chloride channel, as detected with TBPS binding, and alters allosteric sites (i.e., creating benzodiazepine sites and obliterating Zn^{2+} sites), in agreement with earlier studies [*Nature (Lond.)* **338**:582–585 (1989); *Neuron* **5**:781–788 (1990)].

GABA$_A$ receptors are supermolecular receptor-chloride channel complexes of multiple subunits; in mammalian brains, several classes of the receptor subunits exist, with a family of isoforms for each class (1–3). Expression of various combinations of cloned cDNAs for GABA$_A$ receptor subunits produced functional homomeric and heteromeric Cl^- channel-receptor complexes in human kidney cells or *Xenopus laevis* oocytes (1–6). The benzodiazepine receptor is a well known allosteric site on GABA$_A$ receptors (7–9). Its formation requires the $\gamma2$ subunit in addition to the α and β subunits (6, 10, 11); the $\alpha1\beta2$ and the $\alpha1\beta2\gamma2$ subtypes share many common ligands, but benzodiazepines interact only with the latter (11). Recently, Zn^{2+} has been shown to influence only the $\alpha1\beta2$ subtype (12, 13). It appears that the presence of the $\gamma2$ subunit not only produces benzodiazepine sites but also induces other conformational changes in the receptors.

Recently, we established a high efficiency expression system for cDNAs of GABA$_A$ receptor subunits, using insect SF-9 cells infected with baculovirus, and we have shown that the properties of the receptors expressed in the insect cells are similar to those in mammalian cells (14). Using the system here, we produced a group of cloned GABA$_A$ receptors with variations in the ratios of the virions containing cDNAs for the $\gamma2$ subunit to those containing the $\alpha1$ and the $\beta2$ cDNAs. With these cloned receptors, we examined the appearance of benzodiazepine receptors and investigated whether the presence of the $\gamma2$ subunit affects the interactions of GABA and TBPS with the receptors. In addition, we have confirmed the selective inter-

ABBREVIATIONS: GABA, γ-aminobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; TBPS, *t*-butylbicyclophosphorothionate; PFU, plaque-forming units.

action of Zn^{2+} with the $\alpha1\beta2$ subtype, as detected by TBPS binding, and used it to monitor changes in the population of the $\alpha1\beta2$ subtype; benzodiazepine binding was used as a marker for the $\alpha1\beta2\gamma2$ subtype.

Materials and Methods

The growth of SF-9 cells, as well as that of the baculoviruses that produced the GABA receptors, has been described previously (14). Briefly, SF-9 cells were grown in serum-free Grace medium (GIBCO) to a cell density of 1×10^6 cells/ml and were infected with baculovirus constructs AcNPV-$\alpha1$, AcNPV-$\beta2$, and AcNPV-$\gamma2$, which express $\alpha1$ (16), $\beta2$ (17), and $\gamma2$ subunits (18), respectively. The original titer of each virus was 1×10^8 PFU/ml. Various ratios of the $\alpha1/\beta2/\gamma2$-expressing baculoviruses were mixed and coinfected into SF-9 cells. For example, in the experiments where the viruses were mixed at a ratio of 1:1:1 ($\alpha1$ to $\beta2$ to $\gamma2$), 10 μl of each virus (1×10^6 virus particles) were mixed together and used to infect 1×10^6 cells. For different infectivity ratios, we manipulated the amount of the virus stocks containing the $\gamma2$ subunit in relation to those containing the $\alpha1$ and $\beta2$ subunits.

SF-9 cells infected with baculovirus carrying cDNAs for the $\alpha1$ and the $\beta2$ subunits, with or without the $\gamma2$ subunit, were harvested in 2-liter batches 60 hr after infection. The cells were homogenized in a solution containing 118 mM NaCl, 5 mM KCl, and 20 mM HEPES-Tris, pH 7.3, with a Polytron PT 3000 (Brinkman), for 4 min. Unbroken cells and large nuclei aggregates were removed by centrifugation at $1000 \times g$ for 10 min. The membranes were then recovered with a second centrifugation of the supernatant at $40,000 \times g$ for 50 min, resuspended to a final concentration of 5 mg/ml in a solution containing 300 mM sucrose, 5 mM Tris·HCl, pH 7.5, and glycerol (final concentration of 20%), and stored at −80°.

Binding of [^{3}H]flunitrazepam was measured in medium containing varying concentrations of the radioactive ligand (ranging from 0.5 to 60 nM), 118 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 20 mM HEPES-Tris (pH 7.3), and 30 μg of membrane proteins, in a total volume of 500 μl. The reaction was carried out at 4° for 60 min. The procedures for [^{3}H]muscimol binding were the same except that the concentrations of [^{3}H]muscimol varied from 2 to 160 nM. Binding of [^{35}S]TBPS was measured in medium containing [^{35}S]TBPS (varying from 2 to 160 nM), 50 μg of membrane proteins, 1 M NaCl, and 10 mM Tris·HCl (pH 7.4), in a total volume of 500 μl. The mixtures were incubated for 120 min at 24°. In all binding assays, the reaction mixtures were filtered over Whatman GF/B filters under vacuum. The filters were washed three times with 4 ml of the respective reaction buffer without radioisotope and were counted for radioactivity. Nonspecific binding was estimated in the presence of 200 μM diazepam, 100 μM muscimol, or 1 mM picrotoxin and was subtracted to compute specific binding. The amount of protein in the membranes was determined by the method of Lowry *et al.* (19).

Dissociation constants (K_d) and maximal binding site values (B_{max}) for [^{3}H]muscimol, [^{3}H]flunitrazepam, and [^{35}S]TBPS were obtained from Scatchard analysis of the binding data and are presented as the means ± standard errors from three to six experiments, each consisting of triplicate measurements. In the range of ligand concentrations used here, Scatchard plots for muscimol, TBPS, and flunitrazepam were linear and fit a one-site model. We did not explore low affinity sites for GABA in this study. Dose-response profiles for GABA action on TBPS binding were analyzed by the following logistic equations (20):

$$E = E_{max}[\text{GABA}]^n/([K_{0.5}]^n + [\text{GABA}]^n) \quad (1)$$

$$E = ((E_{max_1}[\text{GABA}]^n/([K_{0.5_1}]^n + [\text{GABA}]^n)) + 100) - ((E_{max_1} + 100)[\text{GABA}]^n/([K_{0.5_2}]^n + [\text{GABA}]^n)) \quad (2)$$

where E is GABA-induced response (percent of control), E_{max} is the maximal response, $K_{0.5}$ is the GABA concentration producing half-maximal response, and n is the degree of cooperativity. Eq. 1 was used for a monophasic response and eq. 2 for a biphasic response to GABA (see Fig. 3). The analysis was carried out using least-squares fitting methods (SigmaPlot).

Results

Among all combinations of the $\alpha1$, $\beta2$, and $\gamma2$ subunits, including homomeric sets, only those containing the $\alpha1\beta2$ subunits or all three subunits produced detectable levels of [^{3}H] muscimol and [^{35}S]TBPS binding in the SF-9 cell-baculovirus expression system under our experimental conditions. [^{3}H] Flunitrazepam binding was detected only with the $\alpha1\beta2\gamma2$ subtype. With [^{3}H]muscimol, for instance, background levels of binding were about 50 fmol/mg of protein. This indicates that expression levels of $\alpha1$, $\beta2$, and $\gamma2$ homomeric receptors and $\alpha1\gamma2$ and $\beta2\gamma2$ heteromeric receptors are below 50 fmol/mg of protein. All these receptors, including homomeric ones, however, are known to produce GABA-mediated Cl^- currents, from electrophysiological measurements (4, 5). It appears that our binding studies are capable of monitoring only those receptors of high stable expression, namely $\alpha1\beta2$ and $\alpha1\beta2\gamma2$.

Binding parameters. The cell membranes were prepared from SF-9 cells infected with varying ratios (0 to 3) of the virions containing cDNAs for the $\gamma2$ subunit to the virions containing cDNAs for the $\alpha1$ and the $\beta2$ subunits. With these membranes we examined binding parameters at equilibrium for [^{3}H]flunitrazepam, [^{3}H]muscimol, and [^{35}S]TBPS (Table 1). Scatchard analysis showed that the K_d value for [^{3}H]flunitrazepam binding was fairly constant, ranging from 3.2 to 5.2 nM, with little dependence on the multiplicity ratios of the $\gamma2$ to the $\alpha1\beta2$ subunits (the variations between batches were not significant). Similarly, the K_d values for [^{3}H]muscimol and [^{35}S]TBPS ranged from 12.2 to 19.1 nM and from 30 to 44 nM, respectively, with no apparent dependence on the relative amount of the virions containing the $\gamma2$ cDNA. These results indicate that the presence of the $\gamma2$ subunit in the receptor complex does not noticeably disturb binding sites for GABA and TBPS.

The B_{max} values for muscimol were quite variable between batches (from 1.9 to 7.9 pmol/mg of protein) but were independent of the relative amount of the $\gamma2$ virions; the plot of B_{max} and the level of the $\gamma2$ virions showed no correlation (data not shown). Because the dissociation constant for muscimol was largely independent of the B_{max} values and fell within a reasonably small range from batch to batch, we assume that the interbatch variability in the B_{max} values for muscimol reflected differences in the levels of stably expressed receptors. These variations might originate from combined differences in culture conditions, the age of SF-9 cells used for each infection, and virus stocks, despite our reasonable efforts to control these factors in a uniform manner. Fig. 1 shows the plot of the B_{max} values for muscimol versus TBPS. The two values displayed a correlation coefficient of 0.95, with a slope of 0.86. This indicates one TBPS binding per GABA binding site of high affinity. The matching B_{max} values for the two independent ligands are consistent with our assumption that the B_{max} for high affinity muscimol binding represents the total number of stably expressed receptors in the cell membranes.

To evaluate the appearance of benzodiazepine receptors, we computed the ratio of the B_{max} value for flunitrazepam to that for muscimol and plotted the ratio against the relative ratio of the virions containing cDNA for the $\gamma2$ subunit to those for

TABLE 1

Equilibrium binding parameters

A comparison is shown of equilibrium binding parameters for [^{3}H]flunitrazepam, [^{3}H]muscimol, and [^{35}S]TBPS in cell membranes from SF-9 cells infected with varying ratios of the virions containing rat cDNA for the γ2 subunit to those containing cDNAs for the α1 and β2 subunits. Experimental conditions for binding were described in detail in Materials and Methods. Binding data were analyzed using Scatchard plots. The data represent typical batches of SF-9 cells infected with the given ratio of the virions containing the cDNAs for the three GABA$_A$ receptor subunits. The values represent the means ± standard errors from three to six separate experiments, each consisting of triplicate measurements.

Ratio of infecting virions (α1:β2:γ2)	[^{3}H]Flunitrazepam		[^{3}H]Muscimol		[^{35}S]TBPS	
	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}
	nM	pmol/mg of protein	nM	pmol/mg of protein	nM	pmol/mg of protein
1:1:0			14.5 ± 4.9	7.8 ± 0.5	30 ± 3.2	6.9 ± 0.3
1:1:0.03	4.8 ± 0.6	0.5 ± 0.1	19.1 ± 3.2	3.8 ± 0.2	42 ± 2.8	3.8 ± 0.2
1:1:0.1	3.2 ± 0.1	2.8 ± 0.1	15.6 ± 0.7	5.2 ± 0.3	43 ± 2.7	5.4 ± 0.2
1:1:0.3	5.2 ± 0.3	3.2 ± 0.1	12.1 ± 1.2	3.8 ± 0.2	44 ± 2	2.9 ± 0.1
1:1:1	3.4 ± 0.2	3.7 ± 0.1	10.2 ± 1.0	4.4 ± 0.2	40 ± 1.2	3.2 ± 0.1

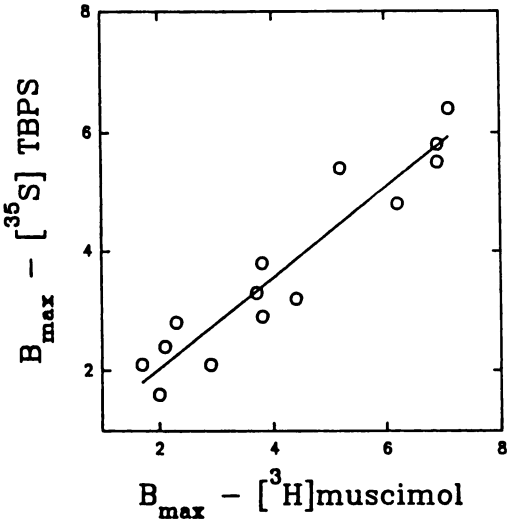

Fig. 1. Plot of the B_{max} values for [^{3}H]muscimol versus those for [^{35}S] TBPS in various batches of SF-9 cell membranes infected with α1 and β2 GABA$_A$ subunits, with or without γ2. The values for B_{max} were obtained from Scatchard analysis of the binding data. Each *point* represents the mean determined from three to six experiments with a given batch. The correlation coefficient between the two groups was 0.95, with a slope of 0.86.

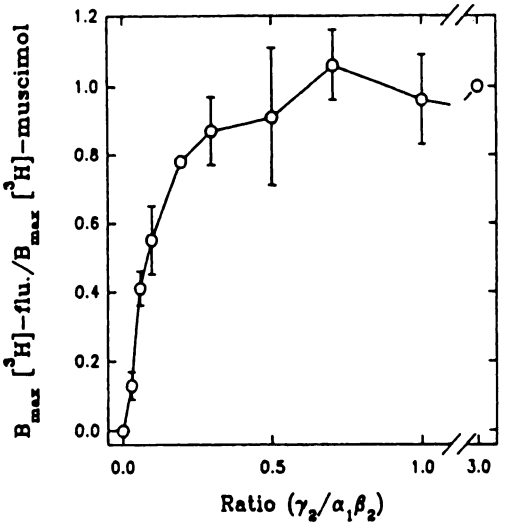

Fig. 2. Plot showing progressive increases in the number of benzodiazepine sites as a function of the virions containing cDNA for the γ2 subunit. SF-9 cells were infected with constant amounts of the virions carrying cDNAs for the α1 and β2 subunits (3 PFU/ml) plus varying amounts of those carrying cDNA for the γ2 subunits, ranging from 0 to 9 PFU/ml. The data represent the means with standard errors between batches (two to four batches).

the α1 and β2 subunits (Fig. 2). The ratio increased from 0 to nearly 1, as the relative ratio of the γ2 to α1β2 virions rose from 0 to 0.5 or above. This confirmed that the formation of benzodiazepine receptors is dependent on the γ2 subunit (11).

Characterization of TBPS binding. TBPS is a ligand with high affinity for the picrotoxin site that is presumably near the mouth of chloride channels (15), and its binding has been shown to be highly sensitive to GABA and various allosteric modulators that affect the chloride channel conformation in rat brain membranes (15, 21–25). We examined the effects of GABA on TBPS binding to the α1β2 and α1β2γ2 receptors (Fig. 3). For the latter subtype, we used membranes from cells infected with the same multiplicity ratio of γ2 to α1β2 virions (1:1) and displaying the number of benzodiazepine binding sites equal to 90% or more of the muscimol binding sites. GABA produced a biphasic effect on TBPS binding to the α1β2 receptors, and its dose-response profile was fitted by eq. 2 (Fig. 3). The stimulatory phase was observed with GABA at concentrations below 1 μM, with an $K_{0.5}$ of 94 ± 10 nM, a maximal net stimulation of 153 ± 10%, and a Hill coefficient of 1.1. The inhibitory phase was observed with GABA at concentrations above 1 μM, with an $K_{0.5}$ of 2.7 ± 0.4 μM and a Hill coefficient of 1.4 ± 0.1. The $K_{0.5}$ value for GABA-induced stimulation of TBPS binding to the α1β2 receptors (94 ± 10 nM) corresponded to the high affinity site for GABA (K_d = 83 nM).

On the other hand, the effect of GABA on TBPS binding in membrane preparations representing the α1β2γ2 subtype was considerably different from that with the α1β2 subtype. Although GABA stimulated TBPS binding in these preparations, the degree of stimulation was much less than that observed with the α1β2 receptors; the maximal stimulation by GABA of TBPS binding to the α1β2γ2 receptors ranged from 10 to 20% above the control (TBPS binding without GABA). This marginal stimulation of TBPS binding may arise from a small fraction of α1β2 receptors in these preparations. Otherwise, GABA was largely inhibitory on TBPS binding to the α1β2γ2 receptors, with a $K_{0.5}$ of 7.1 ± 1.4 μM and a Hill coefficient of 1.6 ± 0.2. These parameters were obtained by fitting the data to eq. 1.

Sensitivity of TBPS binding to Zn^{2+}. Zn^{2+} is known to block GABA-mediated Cl^- currents in the α1β2 subtype but not in the α1β2γ2 subtype (12, 13). We examined here the effect of Zn^{2+} (50 μM) on TBPS binding in the presence of

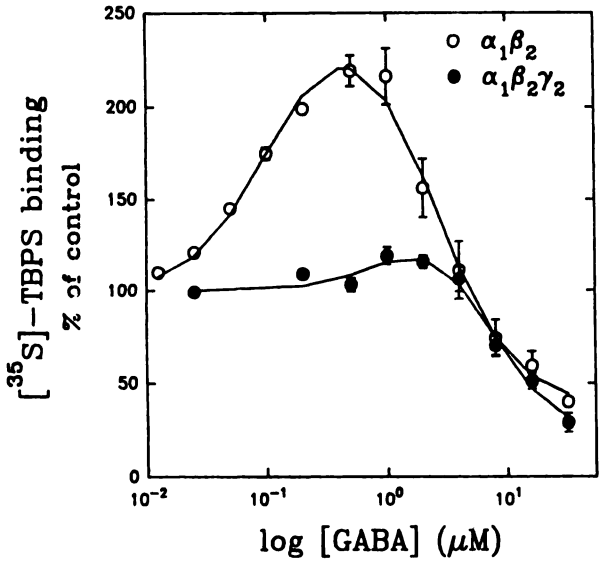

Fig. 3. Comparison of the effects of GABA on [^{35}S]TBPS binding in the $\alpha1\beta2$ and $\alpha1\beta2\gamma2$ subtypes of $GABA_A$ receptors. [^{35}S]TBPS binding at the concentration of 3 nM was measured with GABA at various concentrations in membranes prepared from SF-9 cells infected with virions containing cDNAs for the $\alpha1$ and $\beta2$ (1:1) or the $\alpha1$, $\beta2$, and $\gamma2$ (1:1:1) subunits. We used membranes with the $\alpha1\beta2\gamma2$ subtype that showed a B_{max} value for [^{3}H]flunitrazepam nearly equal to that for [^{3}H]muscimol. The logistic equation for two sites was used to fit the data for the $\alpha1\beta2$ subtype and that for one site for the $\alpha1\beta2\gamma2$ subtype. The data represent the means ± standard errors from three separate experiments, each consisting of triplicate measurements.

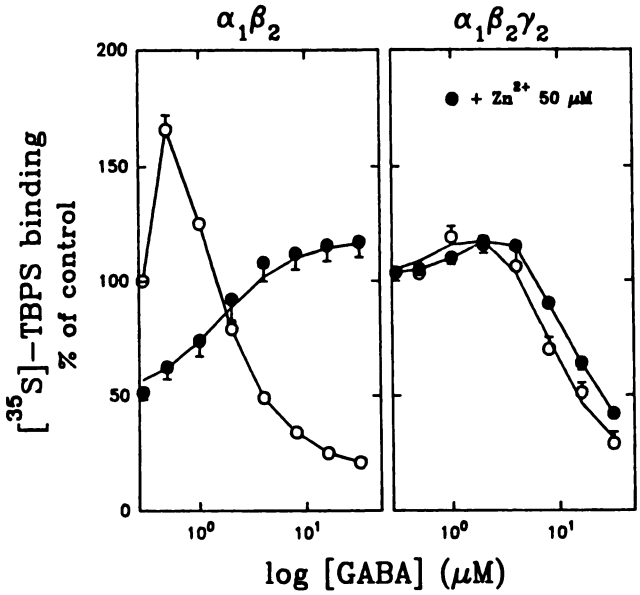

Fig. 4. Differential effects of Zn^{2+} on [^{35}S]TBPS binding to the $\alpha1\beta2$ and $\alpha1\beta2\gamma2$ subtypes of $GABA_A$ receptors. [^{35}S]TBPS binding was measured in the presence of GABA at various concentrations, with (●) or without (○) Zn^{2+} (50 μM), and otherwise under the same conditions as described in the legend to Fig. 3. Zn^{2+} blocked the biphasic effect of GABA only with the $\alpha1\beta2$ subtype and not with the $\alpha1\beta2\gamma2$ subtype. The data represent the mean ± standard error from three measurements.

GABA at varying concentrations (Fig. 4). Zn^{2+} reduced TBPS binding by 50% without GABA in the $\alpha1\beta2$ receptors but did not reduce binding noticeably in the $\alpha1\beta2\gamma2$ receptors. As the concentration of GABA was raised to the levels (>5 μM) at which it inhibited TBPS binding, Zn^{2+} enhanced TBPS binding to the $\alpha1\beta2$ subtype, apparently due to its reversal of the inhibitory effect of high GABA concentrations on TBPS binding. Interestingly, the higher the concentration of GABA, the greater the Zn^{2+}-induced increase in TBPS binding. For instance, GABA at 40 μM blocked TBPS binding by >90% at the $\alpha1\beta2$ subtype but Zn^{2+} restored TBPS binding up to 120% of the control (TBPS binding without GABA). On the other hand, with the $\alpha1\beta2\gamma2$ subtype, Zn^{2+} (50 μM) did not noticeably reverse the inhibitory effect of GABA on TBPS binding; GABA at high concentrations (5 μM or more) marginally enhanced TBPS binding in the preparations representing the $\alpha1\beta2\gamma2$ subtype, probably due to the presence of a small fraction of the $\alpha1\beta2$ subtype in these preparations.

Fig. 5 shows the dose-response curve for Zn^{2+} action in the presence of GABA at 40 μM; Zn^{2+} enhanced TBPS binding with a $K_{0.5}$ of 5.4 ± 1.4 μM, a Hill coefficient of 1.2 ± 0.25, and a maximal recovery of 150 ± 15% of the control (TBPS binding without GABA). The $K_{0.5}$ value for Zn^{2+} obtained here was quite close to values reported for Zn^{2+} inhibition of Cl^- currents at the $\alpha1\beta2$ subtype (12). We compared the changes in Zn^{2+} sensitivity with the appearance of benzodiazepine binding sites. Fig. 6 shows a plot of percentage of recovery of TBPS binding in the presence of Zn^{2+} (50 μM) and GABA (40 μM) versus the ratio of the B_{max} for flunitrazepam to that for muscimol. Despite substantial scatter, the data are inversely correlated, with a correlation coefficient of 0.94. Another unique property of the $\alpha1\beta2$ subtype was the stimulation of TBPS binding by GABA at low concentrations (<1 μM). The degree of GABA (0.5 μM)-induced stimulation of TBPS binding, normalized to that without GABA, was also inversely related to the ratio of the B_{max} for flunitrazepam to that for muscimol (Fig. 6).

Discussion

In various expression systems for $GABA_A$ receptor cDNAs, the combination of the $\alpha1$ and $\beta2$ subunit cDNAs produces functional $GABA_A$ receptors that respond to GABA, bicuculline, barbiturates, and neurosteroids, but an additional cDNA for the $\gamma2$ subunit is required to express benzodiazepine recep-

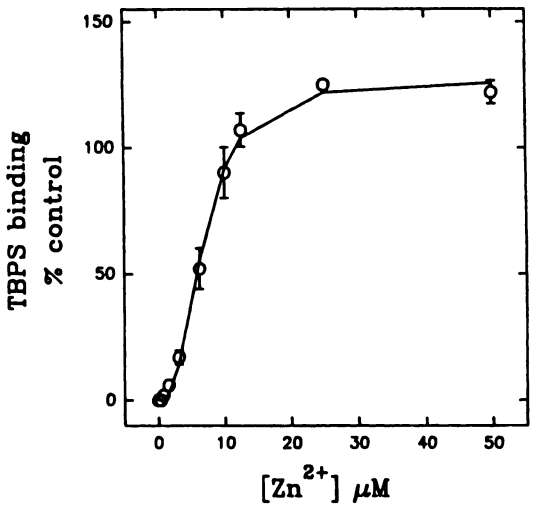

Fig. 5. Dose-response profile for Zn^{2+} effect on [^{35}S]TBPS binding to the $\alpha1\beta2$ subtype. [^{35}S]TBPS binding was measured with GABA at 40 μM in the presence of Zn^{2+}, with its concentration varying from 0.1 to 50 μM. The data were fitted with the logistic equation for one site and represent the means ± standard errors from three separate measurements.

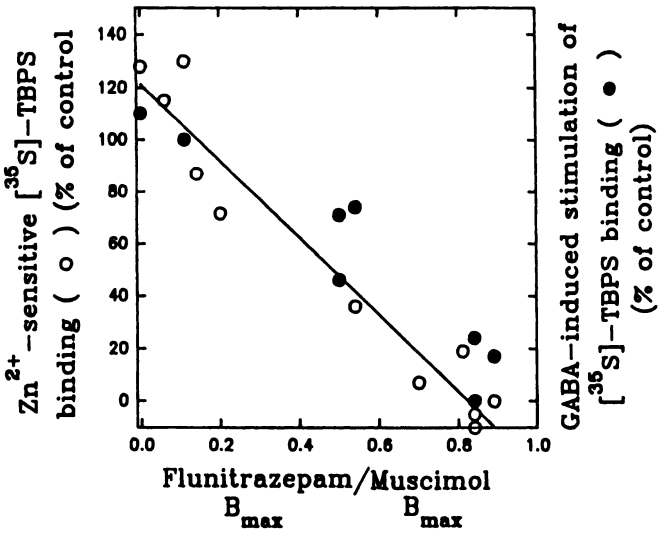

Fig. 6. Plot showing decreases in Zn^{2+}-sensitive TBPS binding and stimulation of TBPS binding with low GABA concentrations as a function of the ratio of the B_{max} value for flunitrazepam to that for muscimol. Zn^{2+}-sensitive TBPS binding was measured in the presence of GABA at 40 μM, in membranes of SF-9 cells infected with various ratios of the virions containing the cDNA for the γ2 subunit. The degree of stimulation of TBPS binding was measured in the presence of 0.5 μM GABA. The values for both Zn^{2+} sensitivity and GABA stimulation were normalized with respect to the TBPS binding observed without GABA.

tors (6, 10, 11). Here we have attempted to study how the presence of the γ2 subunit affects the binding properties and composition of the cloned receptors produced using a high-level expression system with SF-9 cells and baculovirus. Several generalizations have emerged from the current studies with a group of cloned receptors expressed by varying the relative ratio of the virion carrying the cDNA for the γ2 subunit to those carrying the cDNAs for the α1 and β2 subunits.

First, the formation of benzodiazepine receptors, as expected, was dependent on the level of the γ2. The number of benzodiazepine receptors increased proportionately as the virions carrying the γ2 cDNA increased from 0 to 50% of those carrying the α1 and the β2 subunits and then reached that of the high affinity GABA sites as the level of the γ2 virions exceeded 50%. In the latter situation, all the stably expressed receptors are likely to contain the γ2 subunit and benzodiazepine sites, assuming that the number of high affinity GABA sites represents the total number of stably expressed GABA$_A$ receptors. One may further postulate from the apparent absence of the α1β2 subtype with moderate levels of the γ2 virions (compared with the α1 and β2 virions) that the putative pentameric structure of GABA$_A$ receptors containing α1β2γ2 subunits is more stable than those formed without the γ2 subunit. One reservation, however, should be stated; the ratio of the virions may not predict the ratio of the corresponding polypeptides expressed in the cells, because of unpredictable expression efficiency of the virions carrying different cDNAs and because of their potential superinfectivity (because they replicate every 12 hr).

Second, the presence of the γ2 subunit in the GABA$_A$ receptors induced no noticeable changes in the binding parameters (K_d and B_{max}) for GABA at its high affinity site or for TBPS. This implies that these binding sites are primarily associated with the α1 and β2 subunits and perhaps immune to changes in the quaternary structures induced by the γ2 subunit. This contrasts with the situation with Zn^{2+}, the binding site for which disappears with inclusion of the γ2 subunit in the receptors (12, 13).

A rather unexpected finding is that binding of TBPS, a highly sensitive probe for allosteric modulation of the chloride channel of GABA$_A$ receptors (15), was differentially affected by GABA in the α1β2γ2 and α1β2 subtypes, although the binding parameters (K_d and B_{max}) for TBPS were independent of the subtypes. With the α1β2 subtype GABA at low concentrations (<1 μM) stimulated and at high concentrations inhibited TBPS binding, whereas with the α1β2γ2 subtype GABA inhibited TBPS binding without the early stimulatory phase. It appears that the high affinity GABA site on the α1β2 subtype is coupled to the chloride ionophore of the receptor differently, compared with that on the α1β2γ2 subtype. Also, the biphasic response for GABA with the α1β2 subtype shown in Fig. 3 is the first demonstration that the high affinity and low affinity GABA sites may exert differential effects on the chloride ionophore of the receptors. At present, not much is known about what the changes in TBPS binding mean in terms of conformational changes in the chloride ionophore. Most agonistic allosteric ligands have been shown to potentiate the inhibition by GABA of TBPS binding in rat brain membranes, implying that lower TBPS binding is related to the ligand-activated channels (15, 21, 23, 24). However, the opposite effects have also been reported, i.e., stimulation of TBPS binding by agonistic allosteric ligands in well washed rat brain membranes (low endogenous GABA) (22, 25). Clearly more information is needed about the correlation between TBPS binding and functional changes in the chloride ionophore of GABA$_A$ receptors, and further studies on TBPS binding with the α1β2 and α1β2γ2 subtypes will be useful in this regard.

Another interesting aspect of TBPS binding comes from earlier reports that the ratio of the B_{max} for TBPS to that for benzodiazepine was usually >2 in rat brain membranes (15). (The ratio of the B_{max} for TBPS to that for GABA or muscimol was not reported in rat brain membranes, probably because of the presence of high levels of endogenous GABA.) This indicates that >50% of GABA$_A$ receptors in the brain are without benzodiazepine receptors (on the basis of our current observation of one TBPS site per one high affinity GABA site; see Fig. 1). So far, the receptors with no affinity for classical benzodiazepines include those without the γ subunit and the α4β2γ2 and α6β2γ2 subtypes (6, 10, 11, 26). The α4-containing subtypes appear substantially in thalamic nuclei, where low levels of [^{3}H]flunitrazepam binding and high levels of [^{3}H]muscimol binding were observed (27). The receptors containing the α6 subunit are concentrated only in cerebellar granule cells and are likely to be a minor component of the total GABA$_A$ receptors in the brain. Therefore, we would like to note the possibility that the α1β2 subtype may account for a considerable portion of GABA$_A$ receptors without benzodiazepine sites in the brain.

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