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Effects of GABA and various allosteric ligands on TBPS binding to cloned rat $GABA_A$ receptor subtypes

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1 [^{35}S]*t*-butylbicyclophosphorothionate (TBPS) is a high affinity ligand for the picrotoxin site of $GABA_A$ receptors. Here we examined TBPS binding to the cloned receptors made of α1, α3 or α6 in combination with β2 or β2 and γ2 subunits, in the presence of GABA and several allosteric ligands (diazepam, methyl 6,7-dimethoxy-4-methyl-β-carboline-3-carboxylate (DMCM), 3α,21-dihydroxy-5α-pregnan-20-one (5α-THDOC), pentobarbitone and Zn). The cloned receptors were transiently expressed in SF-9 insect cells by infecting with recombinant baculoviruses.

2 In αβ subtypes, GABA at nanomolar concentrations enhanced TBPS binding but inhibited binding at micromolar concentrations. Half maximal GABA concentrations for enhancement or inhibition of TBPS binding were correlated with high and low affinity GABA binding sites, respectively, in individual subtypes. The maximal enhancement of binding also varied according to the α isoform (α3β2>>α1β2>α6β2). In αβγ subtypes, TBPS binding was unaffected by GABA at nanomolar concentrations, but was inhibited by GABA at micromolar concentrations. Addition of γ2 thus appeared to abolish conformational coupling between high affinity GABA sites and TBPS sites, and also altered low affinity GABA sites; in particular, the half maximal GABA concentration for inhibition of TBPS binding changed from >100 (α6β2) to 1 μM (α6β2γ2).

3 Allosteric ligands also altered TBPS binding to sensitive $GABA_A$ receptor subtypes. For instance, diazepam only in the α1β2γ2 and α3β2γ2 subtypes, and 5α-THDOC in all the subtypes enhanced TBPS binding in the absence of GABA, and intensified the inhibitory effect of GABA. Pentobarbitone exhibited only the latter effect in all the subtypes we examined.

4 DMCM and Zn, inhibitors of GABA-induced Cl currents in αβγ and αβ subtypes, respectively, produced opposite effects to agonists, decreasing TBPS binding in the absence of GABA and attenuating (or eliminating in the case of Zn) the inhibitory effect of GABA on TBPS binding.

5 These results show that GABA binding sites and their conformational coupling with TBPS sites are differentially affected by the α isoform (particularly α6 as compared to α1 or α3) and by quaternary interactions involving the γ2 subunit. Moreover, changes in TBPS binding by allosteric ligands include not only direct (allosteric) effects on TBPS sites but also indirect effects via GABA sites, and are consistent with their known subtype selectivity and functionality from previous studies.

Keywords: GABA; $GABA_A$ receptors; $GABA_A$ receptor subunits; GABA ligands; allosteric [^{3}H]TBPS binding; cloned receptors

Introduction

[^{35}S]*t*-butylbicyclophosphorothionate (TBPS) is a high affinity ligand to the picrotoxin site on $GABA_A$ receptors (Squires *et al.*, 1983), which is presumably located near the mouth of the chloride channel of the receptor complex. TBPS binding has received much attention because of its sensitivity to GABA and various allosteric ligands (Trifiletti *et al.*, 1984; Gee *et al.*, 1986; Lloyd *et al.*, 1990; Im & Blakeman, 1991; Sieghart, 1992; Squires & Seaderap, 1993). It has been shown in rat brain membranes that GABA inhibits TBPS binding, and its action is potentiated by agonists, such as benzodiazepines, neurosteroids and barbiturates, and partially reversed by inverse agonists, such as β-carbolines (Trifiletti *et al.*, 1984; Gee *et al.*, 1986; Lloyd *et al.*, 1990; Im & Blakeman, 1991; Sieghart, 1992; Squires & Seaderap, 1993). These results appear to be consistent with TBPS being a probe sensitive to the conformational changes in the chloride channel of the receptor complex. However, recent cloning studies have revealed that $GABA_A$ receptors are made of various subunits which exist in several isoforms in mammalian brains, and that there is functional diversity of cloned receptors depending on their subunit compositions (Barnard *et al.*, 1987; Schofield, 1989; Olsen & Tobin, 1990; Sigel *et al.*, 1990; Verdoorn *et al.*, 1990). This physical and functional multiplicity of $GABA_A$ receptors in brain membranes makes it difficult to study mechanisms of actions for GABA and allosteric ligands on TBPS binding. It would be useful to study TBPS binding in individual cloned receptors. Earlier we carried out an initial study on TBPS binding to α1 containing receptors (Pregenzer *et al.*, 1993), expressed in SF-9 insect cells infected with baculovirus carrying cDNAs for $GABA_A$ subunits (Carter *et al.*, 1992). This current study represents an expanded investigation on the effects of GABA and several allosteric ligands on TBPS binding to three major classes of $GABA_A$ receptor subtypes, Type 1 (α1β2γ2), Type 2 (α3β2γ2) and the third receptor type unique to cerebellar granule cells (α6β2γ2), and their counterparts without γ2. We found that GABA-sensitivity of TBPS binding varied widely among the subtypes, and that changes in TBPS binding by allosteric ligands correlate with their functional characteristics deduced from electrophysiological studies of the various $GABA_A$ receptor subtypes.

Methods

The growth of SF-9 cells as well as that of the baculoviruses for cloning of GABA receptors have been described previously (Carter *et al.*, 1992). Briefly, SF-9 cells were grown in serum-free Grace medium (Gibco) to a cell density of 1×10^6 cells ml^{-1} and then infected with baculovirus constructs (AcNPV) carrying rat cDNAs for $GABA_A$ receptor subunits (Levitan *et al.*, 1988; Khrestchatisky *et al.*, 1989; Shivers *et al.*, 1989; Ymer *et al.*, 1989; Luddens *et al.*, 1990). The original titer of each virus was 1×10^8 plaque fermenting units (PFU) ml^{-1}. Baculoviruses carrying desired cDNAs

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were mixed in equal ratio (1×10^6 virus particles for each) and co-infected into SF-9 cells. The cells were grown in 2 l batches, harvested 60 h after infection, and homogenized in a solution containing NaCl 118 mM, KCl 5 mM and HEPES/Tris 20 mM pH 7.3, with Polytron PT 3000 (Brinkman) for 4 min. Unbroken cells and large nuclei aggregates were removed by centrifugation at 1000 g for 10 min. The membranes were recovered with a second centrifugation of the supernatant at 40,000 g for 50 min, were resuspended to a final concentration of 5 mg ml^{-1} in a solution containing sucrose 300 mM, Tris/HCl 5 mM, pH 7.5, and glycerol (a final concentration of 20%), and stored at −80°C.

Binding of radioactive ligands was measured by filtration techniques as described elsewhere (Pregenzer *et al.*, 1993). Briefly, for binding of [^{3}H]-flunitrazepam and [^{3}H]-muscimol we used a medium containing mM: NaCl 118, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, HEPES/Tris (pH 7.3) 20, varying concentrations of the radioactive ligand (0.5 to 60 nM for flunitrazepam, and 2 to 160 nM for muscimol), and 30 μg membrane proteins in a total volume of 500 μl. The reaction was carried out at 4°C for 60 min. For TBPS binding, we used a medium containing NaCl 1 M, and Tris/HCl (pH 7.4) 10 mM, [^{35}S]-TBPS ranging from 2 to 160 nM, and 50 μg membrane proteins in a total volume of 500 μl. For experiments to examine the effects of GABA or allosteric ligands, the level of [^{35}S]-TBPS was fixed at 3 nM. The mixtures were incubated for 120 min at 24°C. At the end of the reaction, the membranes were separated by filtration over a Whatman GF/B filter under vacuum, and used to determine membrane bound radioactivity. Non-specific binding was estimated in the presence of 200 μM diazepam for [^{3}H]-flunitrazepam, 100 μM muscimol for [^{3}H]-muscimol, or 1 mM picrotoxin for [^{35}S]-TBPS, and was subtracted to compute specific binding.

Dissociation constants (K_d) and maximal binding sites (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 mean ± standard errors (s.e.) from three 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 one site models. Dose-response profiles for GABA action on TBPS binding were analysed by the following logistic equations (Black & Leff, 1983):

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

$$E = ((Emax1[GABA]^n/([K1_{0.5}]^n + [GABA]^n + 100) - ((Emax1 + 100)[GABA]^n/([K2_{0.5}]^n + [GABA]^n) \quad \text{Equation 2}$$

where E is GABA-induced response (% of control), Emax is the maximal response, $K_{0.5}$ (equivalent to EC_{50} or IC_{50}) is GABA concentration for the half maximal response, and n is the degree of cooperativity. Equation 1 was used for a monophasic response and Equation 2 for a biphasic response for GABA (see the α1β2 profile Figure 1). The analysis was carried out using least-squares fitting methods (Sigma plot).

GABA-mediated Cl^- currents in SF-9 cells were measured by the whole cell configuration of the patch clamp technique (Hamill *et al.*, 1981; Draguhn *et al.*, 1990). Briefly, patch pipettes prepared from borosilicate glass tubes were filled with a solution containing (mM): CsCl 140, EGTA 11, $MgCl_2$ 4, ATP 2 and HEPES 10, pH 7.3. The external solution contained (mM): NaCl 135, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.8 and HEPES 5 pH 7.2. GABA and drugs were dissolved in the external solution to a final concentration of 5 μM, unless indicated otherwise, and were applied through a U-tube placed within 100 μm of the target cell. The current was recorded with an Axopatch 1D amplifier and a CV-4 headstage (Axon Instrument Co.). GABA currents were measured at the holding potential of −60 mV at room temperature (21–24°C).

[^{3}H]-flunitrazepam, [^{3}H]-Ro 15-4513, [^{3}H]-muscimol and [^{35}S]-TBPS were obtained from NEN Research Products, Du Pont Company. GABA, DMCM and TBPS were purchased from Research Biochemical Incorporated. BaculoGold transfection kit and SF-9 cells were obtained from PharMingen. Pentobarbitone and 5α-THDOC were from the Upjohn Company.

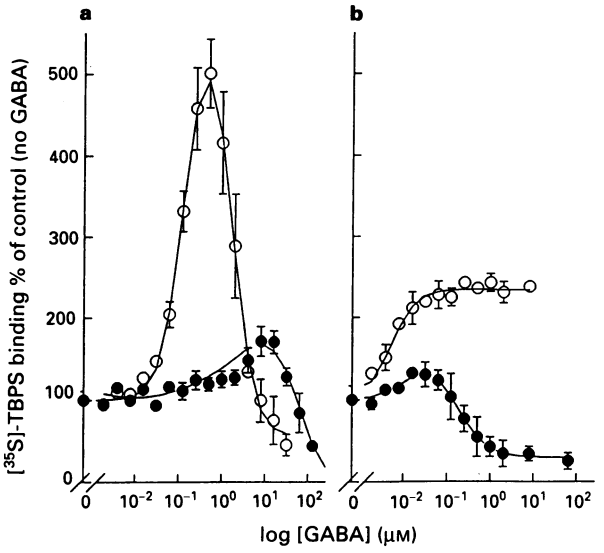

Figure 1 Sensitivity of TBPS binding to GABA concentrations in subtypes of cloned $GABA_A$ receptors. Binding of [^{35}S]-TBPS (3 nM) was measured in the presence of various GABA concentrations, using filtration techniques, with the membranes of SF-9 cells infected for 60 h with baculovirus carrying indicated cDNAs for $GABA_A$ receptors. Non-specific binding of the ligand was estimated in the presence of 1 mM picrotoxin and used to compute the specific binding. In (a) (○) α3β2; (●) α3β2γ2; (b) (○) α6β2; (●) α6β2γ2. The binding data were normalized to that observed without GABA, and represent the mean ± s.e. from three experiments. The other experimental details were as described in Methods.

Results

Ligand binding parameters in cloned $GABA_A$ receptor subtypes

We compared equilibrium binding parameters for [^{3}H]-muscimol, [^{3}H]-flunitrazepam (or [^{3}H]-Ro 15-4513 for the α6β2γ2 subtype) and [^{35}S]-TBPS in the α3β2, α3β2γ2, α6β2 and α6β2γ2 subtypes of cloned $GABA_A$ receptors in membranes of SF-9 cells which had been infected with corresponding recombinant baculoviruses (Table 1). The data confirmed several trends we had already observed with the α1β2 and α1β2γ2 subtypes in an earlier study (Pregenzer *et al.*, 1993). (1) Addition of γ2 to the α3β2 or α6β2 subtype had no appreciable effect on the dissociation constants (K_d) for muscimol and TBPS. (2) Benzodiazepine binding (Ro 15-4513 or flunitrazepam) was observed only in the receptors containing γ2. (3) The maximal binding site (B_{max}) for muscimol which represents the high affinity GABA site closely matched with that for TBPS, and may represent the total number of $GABA_A$ receptors. Besides these trends, we noted that the K_d values for muscimol and TBPS in the receptors containing α6 (an average value of 4 nM for muscimol and 47 nM for TBPS) were considerably lower than those observed in the receptors containing α3 (an average value of 13 nM for muscimol and 128 nM for TBPS). Furthermore, the B_{max} for Ro 15-4513 in the α3β2γ2 subtype was nearly equal to those for muscimol and TBPS, but about 30% greater than that for flunitrazepam. Both ligands were expected to interact with the same site (benzodiazepine site). Further study may be needed to clarify this discrepancy.

Table 1 Binding parameters for [^{3}H]-muscimol, [^{3}H]-Ro 15-4513, [^{3}H]-flunitrazepam and [^{35}S]-TBPS at equilibrium

	Muscimol		*Ro 15-4513*		*Flunitrazepam*		*TBPS*	
	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}
α3β2	14.2 ± 4.8	5.1 ± 0.4	ND	ND	ND	ND	122 ± 17	4.4 ± 3
α3β2γ2	9.1 ± 2.2	4.2 ± 0.4	8.7 ± 2	4.5 ± 0.3	4.7 ± 0.4	3.2 ± 0.4	135 ± 47	4.5 ± 0.6
α6β2	3.1 ± 0.5	5.2 ± 0.5	ND	ND	ND	ND	48 ± 7	5.8 ± 0.6
α6β2γ2	4.8 ± 0.3	9.5 ± 1.3	11.6 ± 2.1	11.4 ± 1.2	ND	ND	45 ± 7	8.8 ± 0.5

Binding studies were carried out in the cell membranes of SF-9 cells infected for 60 h with baculoviruses carrying indicated cDNAs for rat $GABA_A$ receptor subunits. Experimental conditions were described in detail in Methods. The parameters were obtained from Scatchard analysis of binding data, and represent the mean ± standard errors from three experiments, each consisting of triplicate measurements. The unit for K_d values is nM, and that for B_{max} values is pmol mg^{-1} protein. ND: No detectable binding with the radioactive ligands at 10 nM.

GABA-sensitivity of TBPS binding

We examined the effects of GABA at various concentrations on TBPS (3 nM) binding to the α3β2, α3β2γ2, α6β2 and α6β2γ2 subtypes (Figure 1). In the α3β2 subtype, GABA at low concentrations (<0.5 μM) enhanced TBPS binding by more than 400%, but at high concentrations (>1 μM) inhibited TBPS binding (nearly abolished it at 50 μM). The data were analysed with Equation 2. For the enhancement phase, we found $EC_{50} = 110 \pm 10$ nM for GABA, $n = 1.4 \pm 0.2$, and $E_{max} = 438 \pm 16\%$, and for the inhibition phase, $IC_{50} = 2$ μM, and $n = 1.7 \pm 0.4$. The increased TBPS binding in the presence of low GABA (0.25 μM) was due to a decrease in the K_d for TBPS, from 111 ± 6 to 45 ± 1 nM, with little change in B_{max} (3 pmol mg^{-1} protein for this particular preparation). In preparations for the α3β2γ2 subtype, on the other hand, GABA at low concentrations (<1 μM) had no effect, but at high concentrations (>10 μM) inhibited TBPS binding, except for a minor enhancement (about 60% above the control) which peaked at 10 μM GABA. The receptor subtype responsible for the peak is not certain. The peak could not be due to traces of the α3β2 subtype because of the difference in GABA sensitivity (10 μM vs 0.5 μM GABA for α3β2), and also could not be attributed to the α3β2γ2 subtype, because of its disappearance in the presence of Zn (50 μM), which has been shown to interact with the αβ subtypes, but not with the αβγ subtypes (Draguhn *et al.*, 1990) (see below).

In the α6β2 subtype, TBPS binding profile was quite different from that in the α3β2 subtype. GABA monophasically enhanced TBPS binding with no sign of inhibition even at 100 μM. Analysis of the data with Equation 1 yielded an $EC_{50} = 5.2 \pm 0.5$ nM, $n = 1.3 \pm 0.2$ and $E_{max} = 134 \pm 29\%$. In the α6β2γ2 subtype, again GABA at low concentrations had no effect, but at high concentrations inhibited TBPS binding with a minor Zn-sensitive enhancement of about 25% at 50 nM GABA, probably representing a minor receptor subtype of unknown subunit composition.

Differential modes of modulation of TBPS binding by allosteric ligands

We examined the effects of several allosteric ligands (diazepam, methyl 6,7-dimethoxy-4-methyl-β-carboline-3-carboxylate (DMCM), 3α,21-dihydroxy-5α-pregnan-20-one (5α-THDOC), pentobarbitone and Zn) on TBPS binding to the various subtypes of cloned $GABA_A$ receptors. Two parameters were used to characterize the drug effect on TBPS binding: (1) the drug-induced change in TBPS binding in the absence of GABA (%) and (2) the change in the GABA concentration to reduce TBPS binding to 50% of the control level (IC_{50}, without GABA and drugs) (Table 2).

Diazepam, 5α-THDOC and pentobarbitone are agonists for $GABA_A$ receptors, and interact with three major modulatory sites on $GABA_A$ receptors, namely the benzodiazepine, neurosteroid and barbiturate sites, respectively. In the absence of GABA, diazepam and 5α-THDOC enhanced TBPS binding, but pentobarbitone did not (Figure 2). Diazepam (0.2 μM) increased TBPS binding by 29 ± 6 and 54 ± 7% in the α1β2γ2 and α3β2γ2 subtypes, respectively, but not in the α6β2γ2 and the subtypes without γ2. 5α-THDOC (0.8 μM) increased TBPS binding by 42 ± 5, 44 ± 11, 10 ± 22, 51 ± 45, 228 ± 15 and 14 ± 8% in the α1β2γ2, α3β2γ2, α6β2γ2, α1β2, α3β2 and α6β2 subtype, respectively. Pentobarbitone (20 μM) failed to enhance TBPS binding in all these subtypes. In the presence of GABA, however, all these agonists potentiated the inhibitory effect of GABA on TBPS binding, as shown by changes in the IC_{50} value for GABA (Figure 2 and Table 2). Diazepam reduced the IC_{50} value from 8.2 ± 0.6 to 4.5 ± 0.8 μM in the α1β2γ2 subtype, and from 116 ± 12 to 54 ± 8 μM in the α3β2γ2 subtype, but produced no changes in the other subtypes. 5α-THDOC reduced

Table 2 Differential effects of allosteric ligands on TBPS binding in the subtypes of cloned $GABA_A$ receptors

	Control		*Diazepam*		*DMCM*		*Pentobarbitone*		*5α-THDOC*	
	% change (no GABA)	IC_{50} (μM)	% change (no GABA)	IC_{50} (μM)	% change (no GABA)	IC_{50} (μM)	% change (no GABA)	IC_{50} (μM)	% change (no GABA)	IC_{50} (μM)
α1β2γ2	100	8.2 ± 0.6	129 ± 6	4.5 ± 0.8	47 ± 8	20 ± 3	100 ± 3	4.7 ± 0.5	142 ± 5	1.4 ± 0.4
α3β2γ2	100	116 ± 12	154 ± 7	54 ± 8.3	86 ± 8	265 ± 60	98 ± 12	47 ± 22	144 ± 11	42 ± 12
α6β2γ2	100	1 ± 0.4	–	–	–	–	97 ± 3	0.1 ± 0.03	110 ± 22	0.01 ± 0.005
α1β2	100	1.3 ± 0.3	–	–	–	–	105 ± 5	0.4 ± 0.1	151 ± 45	0.17 ± 0.05
α3β2	100	9.0 ± 1.1	–	–	–	–	97 ± 3	3.9 ± 0.2	328 ± 15	1.3 ± 0.3
α6β2	100	>100	–	–	–	–	87 ± 5	3.7 ± 2	114 ± 8	0.1 ± 0.05

Binding of TBPS (6 nM) in the subtypes of $GABA_A$ receptors was examined as a function of GABA concentration (0.01 to 100 μM) with or without diazepam (0.2 μM), DMCM (0.1 μM), 5α-THDOC (0.8 μM) or pentobarbitone (20 μM). We monitored drug effects with two parameters: (1) % change (no GABA) in the level of TBPS binding by the ligand in the absence of GABA as normalized to the value observed without the ligand, and (2) the value of IC_{50}, representing the GABA concentration with or without the drug, which reduces TBPS binding to the 50% level of that observed in the absence of GABA and drugs. The values represent the means ± standard errors from at least three experiments, each consisting of triplicate measurements.

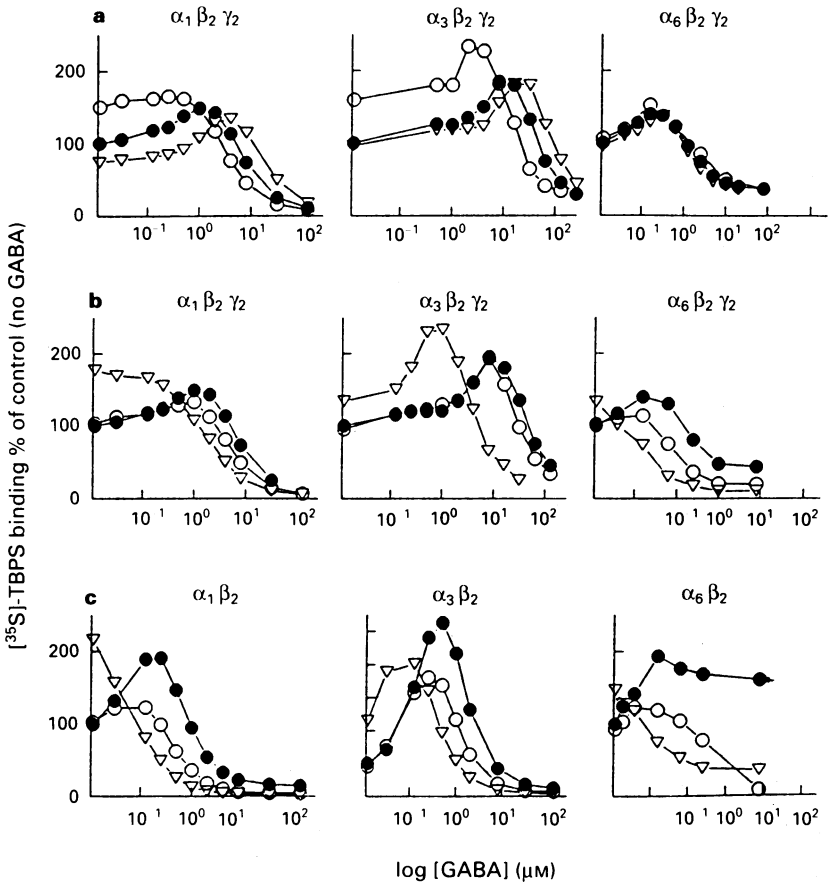

Figure 2 Effects of diazepam, DMCM, 5α-THDOC and pentobarbitone on GABA-sensitive TBPS binding in various subtypes of cloned $GABA_A$ receptors. TBPS binding (in the presence of GABA from 0 to 100 μM) was measured without or with diazepam (0.2 μM), DMCM (0.1 μM), 5α-THDOC (0.8 μM) or pentobarbitone (20 μM) in membranes of SF-9 cells infected with baculovirus carrying indicated cDNAs for $GABA_A$ receptor subunits. In (a) (●) control; (○) diazepam; (▽) DMCM. In (b) (●) control; (○) pentobarbitone; (▽) 5α-THDOC. In (c), (●) control; (○) pentobarbitone; (▽) 5α-THDOC. The data were normalized to the level of TBPS binding observed without GABA, and represent the mean from triplicate measurements. Two parameters were obtained from the data: the % changes in TBPS binding without GABA (control), and the changes in the GABA concentration to reduce the level of TBPS binding to 50% of the control (IC_{50}) by the drugs. The results are listed in Table 2. For abbreviations, see text.

the IC_{50} value from the control (shown in parentheses for each subtype) to 1.4 ± 0.4 (8.2 ± 0.6, α1β2γ2), 42 ± 12 (116 ± 12, α3β2γ2), 0.01 ± 0.005 (1 ± 0.4, α6β2γ2), 0.17 ± 0.05 (1.3 ± 0.3, α1β2), 1.3 ± 0.3 (9 ± 1.1, α3β2), and 0.1 ± 0.05 μM (<100, α6β2). Pentobarbitone also reduced the IC_{50} value in all the subtypes, to 4.7 ± 0.5 (8.2 ± 0.6, α1β2γ2), 47 ± 22 (116 ± 12, α3β2γ2), 0.1 ± 0.03 (1 ± 0.4, α6β2γ2), 0.4 ± 0.1 (1.3 ± 0.3, α1β2), 3.9 ± 0.2 (9 ± 1.1, α3β2), and 3.7 ± 2 μM (<100, α6β2). These results reflect two types of drug actions, allosteric modulation of GABA sites (changes in the IC_{50} value) and direct (allosteric) action on TBPS site (enhancement of TBPS binding in the absence of GABA). Pentobarbitone was unique among agonists in its inability to influence TBPS sites in the absence of GABA.

DMCM, a benzodiazepine inverse agonist, produced effects that were the opposite to those of agonists. In the absence of GABA, DMCM (0.1 μM) inhibited TBPS binding by −53 ± 8 and −14 ± 8% in the α1β2γ2 and α3β2γ2 subtype, respectively, and increased the IC_{50} value to 20 ± 3 (8.2 ± 0.6, α1β2γ2) and 265 ± 60 μM (116 ± 12, α3β2γ2), respectively. DMCM produced no effect in the other subtypes, displaying the same subtype selectivity as diazepam (Pritchett *et al.*, 1989a,b; Luddens *et al.*, 1990).

Zn represents another class of inhibitors of GABA-induced Cl^- currents, selective for the α1β2 subtype (not effective in α1β2γ2) (Draguhn *et al.*, 1990). Here we also confirmed similar Zn-sensitivity for the α3β2 subtype (Figure 3). Zn at 25 μM nearly abolished GABA-induced Cl^- currents in the α3β2, but reduced Cl^- currents by only 30 ± 9% in the α3β2γ2 subtype. As expected, Zn affected TBPS binding only in αβ subtypes in the manner opposite to the agonists. In the absence of GABA, Zn (50 μM) decreased TBPS binding (−37 ± 11%) to the α3β2 subtype, but produced no appreciable effect in the α3β2γ2 subtype. In the presence of GABA, Zn abolished GABA-mediated inhibition of TBPS binding to the α3β2 subtype; for instance, GABA (50 μM) alone reduced TBPS binding by 80%, but with Zn (50 μM) enhanced it to over 200% as normalized to the control level (no GABA). In the membrane preparations for the α3β2γ2 subtype, however, Zn (50 μM) shifted the GABA-dose response profile for TBPS binding to the left, largely because of disappearance of a minor Zn-sensitive enhancement in the preparations, as mentioned above; for instance, in the presence of Zn (50 μM), GABA monophasically blocked TBPS binding with an IC_{50} = 10 μM (compare with IC_{50} = 116 ± 12 μM without Zn) and n = 1.8. The profile for Zn-sensitive TBPS binding in the preparations for the α3β2γ2 subtype was obtained by the differences between the profiles with and without Zn (Figure 4, inset). An earlier study reported similar selective effects of Zn on TBPS binding to the α1β2 subtype (Pregenzer *et al.*, 1993).

Discussion

In this study we examined the effects of GABA and several allosteric ligands on TBPS binding to three major classes of $GABA_A$ receptor subtypes, Type 1 (α1β2γ2), Type 2 (α3β2γ2),

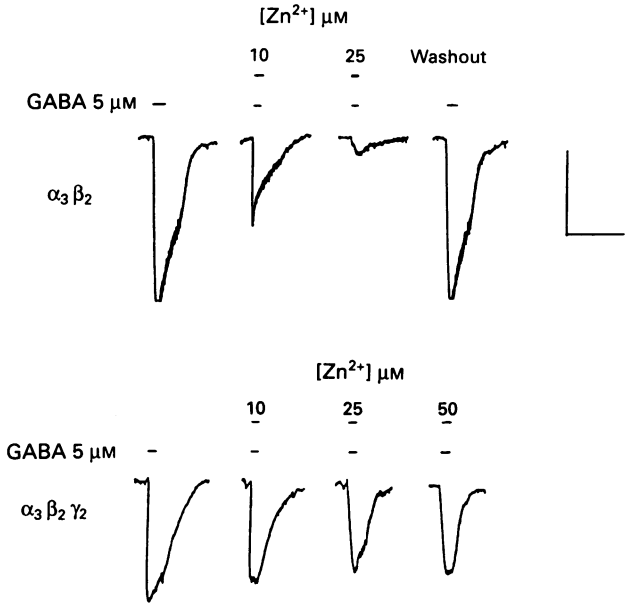

Figure 3 Representative current traces showing inhibition of GABA-induced Cl^- currents by Zn in the $\alpha3\beta2$, but not in the $\alpha3\beta2\gamma2$ subtype. GABA-induced Cl^- currents were measured in the SF-9 cells transiently expressing the $\alpha3\beta2$ or $\alpha3\beta2\gamma2$ subtypes using the whole cell configuration of patch clamp techniques. The currents were induced at the holding potential of -60 mV (under a symmetrical Cl^- gradient) by application of 5 μM GABA without or with Zn (10, 25 or 50 μM). Zn at 25 μM nearly abolished GABA-induced Cl^- in the $\alpha3\beta2$ subtype in a reversible manner, but was largely ineffective in blocking the Cl^- currents in the $\alpha3\beta2\gamma2$ subtype. Only $30 \pm 9\%$ ($n = 3$) current reduction was observed in the presence of Zn at 50 μM. The horizontal calibration bar represents 30 s, and the vertical bar 200 pA for the upper panel, and 500 pA for the lower panel.

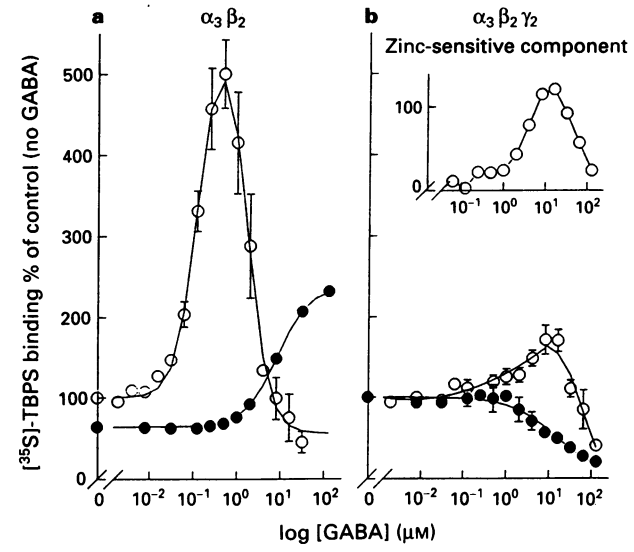

Figure 4 Effect of Zn on TBPS binding in (a) the $\alpha3\beta2$ and (b) $\alpha3\beta2\gamma2$ subtypes of cloned GABA$_A$ receptors. TBPS binding in the presence of GABA (0 to 100 μM) was measured with (●) or without Zn (○) at 50 μM. The binding data were normalized to that observed without GABA, and represent the mean ± s.e. from three experiments. In the preparations for the $\alpha3\beta2\gamma2$ subtype, we noted a Zn-sensitive component, shown in the inset.

the receptor subtype unique to cerebellar granule cells ($\alpha6\beta2\gamma2$), and their counterparts without γ2. Several findings are noteworthy. In αβ subtypes, the biphasic effect of GABA on TBPS binding (Figure 1) was differentially affected by the α isoform. Various α isoforms induced changes in the half maximal GABA concentration for enhancement of TBPS binding with EC_{50} values of 94 ± 10 nM, 110 ± 10 and 5.2 ± 0.5 nM for $\alpha1\beta2$, $\alpha3\beta2$ and $\alpha6\beta2$, respectively, and for inhibition of TBPS binding with IC_{50} values for GABA of 2, 2.7 and >100 μM for $\alpha1\beta2$, $\alpha3\beta2$ and $\alpha6\beta2$, respectively. The maximal enhancement of TBPS binding by GABA also varied with the α isoform (153 ± 10, 438 ± 16 and $139 \pm 29\%$ of TBPS binding for $\alpha1\beta2$, $\alpha3\beta2$ and $\alpha6\beta2$, respectively). All the values for α1 containing receptors were from an earlier study (Pregenzer *et al.*, 1993). Interestingly, the EC_{50} value in each subtype matched well with its K_d value for the high affinity GABA site (83, 71 and 12 nM for $\alpha1\beta2$, $\alpha3\beta2$ and $\alpha6\beta2$, respectively). Stimulation of TBPS binding thus seems to result from interaction of GABA with its high affinity site. Inhibition of TBPS binding, on the other hand, may reflect interaction of GABA with its low affinity site, and could be related to channel openings, which were observed only in the presence of GABA at micromolar concentrations in patch-clamp studies with various subtypes of cloned GABA$_A$ receptors (Puia *et al.*, 1991). We propose that the IC_{50} values approximate the K_d values for low affinity GABA sites. According to this proposal, the $\alpha6\beta2$ subtype possesses low affinity GABA sites ($IC_{50} > 100$ μM) much different from those on the $\alpha1\beta2$ (2 μM) and $\alpha3\beta2$ subtype (2.7 μM), suggesting the α isoform as the major determinant for low affinity GABA sites. Also the α isoform influences the degree of coupling between GABA (high affinity) and TBPS sites, as reflected by the α isoform-dependent change in the maximal enhancement of TBPS binding by GABA.

Addition of γ2 to αβ subtypes brought about two changes; the disappearance of enhancement of TBPS binding by GABA at nanomolar concentrations and changes in the IC_{50} values for inhibition of TBPS binding by GABA (8.2, 10 and 1 μM for the $\alpha1\beta2\gamma2$, $\alpha3\beta2\gamma2$ and $\alpha6\beta2\gamma2$ subtype, respectively). Note that the IC_{50} for the $\alpha3\beta2\gamma2$ subtype was obtained in the presence of Zn to eliminate the minor Zn-sensitive component as discussed in the Results section. It appears that the quaternary interactions involving the γ2 subunit abolished conformational coupling between high affinity GABA sites and TBPS sites, and altered low affinity GABA sites, particularly for the $\alpha6\beta2\gamma2$ (IC_{50} change from >100 to 1 μM). It should be noted that addition of γ2 produced no appreciable change in high affinity GABA site as monitored with [^{3}H]-muscimol binding (see Table 1 and Pregenzer *et al.*, 1993). These results emphasize functional and physical distinctions between high and low affinity GABA sites in various GABA$_A$ receptors.

Our current study with various allosteric ligands generally confirmed earlier observations in rat brain membranes that agonists potentiate, and inverse agonists attenuate the inhibitory effect of GABA on TBPS binding (Gee *et al.*, 1986; Trifiletti *et al.*, 1986; Lloyd *et al.*, 1990; Im & Blakeman, 1991; Sieghart, 1992; Squires & Seaderap, 1993). Subtype selectivity for individual ligands observed with TBPS binding also matched well with that observed with ligand binding studies. For instance, diazepam and DMCM (0.1 μM) affected TBPS binding in the $\alpha1\beta2\gamma2$ and $\alpha3\beta2\gamma2$ subtypes to which the drugs bind with high affinity, but produced no effect on TBPS binding in the $\alpha6\beta2\gamma2$ and αβ subtypes where the drugs at the concentrations we used here showed no binding activity (Pritchett *et al.*, 1989a,b; Luddens *et al.*, 1990). The $\alpha6\beta2\gamma2$ subtype appears to represent the diazepam-insensitive population of GABA$_A$ receptors in bovine cerebellar membranes (Turner *et al.*, 1991). Several other interesting points are: (1) in the $\alpha6\beta2$ subtype 5α-THDOC (0.8 μM) and pentobarbitone (20 μM) reduced IC_{50} value from <100 μM to 0.1 and 3.9 μM, respectively. This suggests a marked allosteric modulation of low affinity GABA sites by the drugs. (2) Diazepam and 5α-THDOC enhanced TBPS binding in the absence of GABA, but pentobarbitone did not. This differential property among agonists would be useful for classifying novel ligands for GABA$_A$ receptors. (3) We detected a minor Zn-sensitive

subtype in the preparations of the $\alpha 3\beta 2\gamma 2$ subtype. This subtype shares the same Zn-sensitivity with the $\alpha 3\beta 2$, but differs in GABA concentrations required to induce a maximal enhancement of TBPS binding (10 μM as compared to 0.5 μM for $\alpha 3\beta 2$). The subunit composition of this subtype would be of future research interest.

In summary, differential modulation of TBPS binding by GABA at various concentrations in cloned $GABA_A$ receptors (particularly $\alpha\beta$ subtypes) provided important information on both high and low affinity GABA binding sites, and was useful for understanding how a subunit or isoform contributes to GABA binding sites and their coupling with TBPS sites. The α isoform seems to contribute much to GABA binding sites, since the presence of $\alpha 6$ produced drastic changes in high and low affinity GABA sites as compared to those observed with $\alpha 1$ or $\alpha 3$. Addition of $\gamma 2$ to $\alpha\beta$ subtypes or presence of allosteric ligands, on the other hand, seems to induce marked changes in low affinity GABA sites. In addition, the α isoform and the $\gamma 2$ subunit influence conformational coupling between GABA sites and TBPS sites.

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