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# Monoclonal Antibodies and Conventional Antisera to the $GABA_A$ Receptor/Benzodiazepine Receptor/$Cl^-$ Channel Complex

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**Monoclonal antibodies (mAbs) and conventional antisera were raised to the affinity-purified $GABA_A$ receptor/benzodiazepine receptor/$Cl^-$ channel complex. The antibodies immunoprecipitated the affinity-purified complex in Triton X-100 and also reacted with the complex in a solid-phase radioimmunoassay. Immunoblots indicated that the mAb 62-3G1 reacted with the 57,000 $M_r$ peptide subunit of the affinity-purified complex, while the antisera mainly reacted with the 51,000 $M_r$ peptide subunit.**

**The mAbs and the antisera also immunoprecipitated the $GABA_A$ receptor/benzodiazepine receptor/$Cl^-$ channel complex after being solubilized from cerebral cortex membranes by the zwitterionic detergent CHAPS. The immunoprecipitated complex bound $^3$H-muscimol, $^3$H-flunitrazepam (FNZ) and $^{35}$S-*t*-butylbicyclophosphorothionate (TBPS). The $^3$H-FNZ binding was stimulated by GABA, indicating that the functional interactions among the immunoprecipitated components of the complex were preserved. The mAb 62-3G1 also recognized the 57,000 $M_r$ peptide in immunoblots with crude brain membranes. Immunocytochemistry experiments showed that the binding of both the mAb 62-3G1 and $^3$H-muscimol colocalized throughout the brain. The results suggest that (1) the 57,000 $M_r$ peptide is the muscimol ($GABA_A$ receptor agonist) binding peptide of the complex, and (2) in the cerebral cortex, most of the $GABA_A$ receptors (GABARs), benzodiazepine receptors (BDZRs), and $Cl^-$ channels are physically coupled to one another.**

The benzodiazepine receptor (BZDR) is a membrane protein functionally associated to the $GABA_A$ receptor (GABAR), the $Cl^-$ channel, and a receptor for barbiturates (for reviews, see Ticku, 1983; Turner and Whittle, 1983; Richards and Möhler, 1984; Squires, 1984; Tallman and Gallager, 1985; Olsen et al., 1986). Each component of the complex is characterized by high-affinity binding for specific ligands. Thus, muscimol (agonist), and bicuculline (antagonist) bind to the GABAR, while benzodiazepines and $\beta$-carbolines bind to the BZDR. Picrotoxinin and *t*-butylbicyclophosphorothionate (TBPS) bind to or very near to the $Cl^-$ channel. The proteins of the complex interact in a functional way with one another, probably by allosteric mechanisms, such that the binding of a ligand to its receptor alters the binding properties of the other receptor proteins for their respective ligands.

The GABAR/BZDR/$Cl^-$ channel complex has been purified from bovine (Sigel et al., 1983; Sigel and Barnard, 1984), rat (Stephenson et al., 1984), and pig brain (Kirkness and Turner, 1986) by affinity chromatography on the immobilized benzodiazepine Ro7-1986/1. The purified receptor complex in Triton X-100 has both $^3$H-muscimol- and $^3$H-flunitrazepam (FNZ)-binding activities, indicating that the GABAR and the BZDR copurify (Sigel et al., 1983). In addition, when the purification of the complex is done in the zwitterionic detergent CHAPS, the $^{35}$S-TBPS binding activity of the $Cl^-$ channel is also preserved (Sigel and Barnard, 1984). The purified GABAR/BZDR/$Cl^-$ channel complex shows, in SDS–PAGE, 2 bands corresponding to peptides of $M_r$ 51,000 and 57,000 (Sigel et al., 1983; Sigel and Barnard, 1984).

Photoaffinity labeling of the receptor complex with $^3$H-FNZ, using crude membranes, solubilized receptor, or affinity-purified receptor (Möhler et al., 1980; Sieghart and Karobath, 1980; Sigel and Barnard, 1984; De Blas et al., 1987), indicated that the benzodiazepine binding site is localized in the 51 kDa peptide. In contrast, the 57 kDa peptide was specifically photoaffinity-labeled with $^3$H-muscimol, indicating that the GABAR activity is localized in this peptide (Casalotti et al., 1986; Deng et al., 1986).

In this paper, we describe the making of monoclonal antibodies (mAbs) and conventional antisera to the GABAR/BZDR/$Cl^-$ channel complex. Some of these results have been discussed in a preliminary report (Vitorica et al., 1987).

## Materials and Methods

*Purification of the GABAR/BZDR complex.* The complex was purified from bovine cerebral cortex by the procedure of Sigel et al. (1983). Bovine cerebral cortex (100 gm) was homogenized in 10 volumes of homogenization medium [10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 300 mM sucrose, 0.5 mM dithiothreitol (DTT), 1 mM benzamidine/HCl, 10 mg/liter of each trypsin inhibitor (types II and I-S; Sigma), and 0.3 mM phenylmethylsulfonyl fluoride (PMSF)]. All operations were carried out at 4°C. The homogenate was centrifuged at 1000 × *g* for 12 min, and the resulting supernatant was centrifuged at 27,000 × *g* for 35 min. The pellet was resuspended in homogenization medium (without sucrose and PMSF) and centrifuged at 27,000 × *g* for 35 min. The pellet (crude synaptosomal fraction) was resuspended in 200 ml of the latter medium (15 mg protein/ml), containing 150 mM KCl, 18 mg bacitracin, and 10% (wt/vol) Na-deoxycholate. The membranes were solubilized for 10 min at 4° C with agitation and centrifuged at 100,000 × *g* for 1 hr. The supernatant contained the solubilized receptor. The latter was applied to a 30 ml column of Ro7-1986/1 bound to agarose. The column with the retained complex was washed overnight at 4°C with 600 ml of 10 mM K phosphate, pH 7.4, 200 mM KCl, 2 mM Mg acetate, 0.1 mM EGTA, 10% sucrose, and 0.2% (wt/vol) Triton X-100. The receptor complex was eluted with 10 mM chlorazepate in 10 mM K phosphate,

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pH 7.4, 2 mM Mg acetate, 10% sucrose, and 0.05% (wt/vol) Triton X-100. Finally, the chlorazepate was removed by retaining the complex in a DEAE–Sephacel column. The receptor complex was eluted with 0.8 M KCl in 20 mM phosphate, pH 7.4, 2 mM Mg acetate, 10% sucrose, and 0.05% (wt/vol) Triton X-100. The receptor preparations had approximately 100 μg of protein at a concentration of 10–20 μg/ml.

*Binding assays.* The binding of 20 nM $^3$H-FNZ or $^3$H-muscimol to the solubilized or purified receptor complex was done in buffer 1 [20 mM K phosphate, pH 7.4, 0.1 mM EDTA, 200 mM KCl, and 0.1% (wt/vol) Triton X-100 or 2.5 mM CHAPS] or in buffer 2 (50 mM Tris-HCl, pH 7.4, 50 mM KCl, 0.1 mM EDTA, and 2.5 mM CHAPS). The mixture was incubated at 4°C for 30 or 45 min for $^3$H-muscimol or $^3$H-FNZ binding, respectively. The receptor–ligand complexes were precipitated by polyethylene glycol and recovered by filtration, as described elsewhere (Mernoff et al., 1983). The nonspecific binding was determined with 20 μM FNZ or 40 μM muscimol. The binding of $^{35}$S-TBPS to the CHAPS-solubilized Cl⁻ channel was measured with 40 nM $^{35}$S-TBPS in buffer 1 with 2.5 mM CHAPS for 90 min at 30°C. Nonspecific binding was determined by adding 600 μM picrotoxinin.

*Immunizations.* The purified GABAR/BZDR complex was dialyzed for 4 hr at 4°C in 20 mM K phosphate, pH 7.4, 0.1 mM EDTA, and 0.05% (wt/vol) Triton X-100. For mouse immunizations, the dialyzed receptor (15–20 μg/ml) was concentrated 7–10× by ultrafiltration through an Amicon membrane (PM10). Each of 2 BALB/c mice (8-week-old females) was immunized intraperitoneally at day 0 with 60 μg of receptor complex in complete Freund's adjuvant, followed by 60 μg in incomplete Freund's adjuvant at day 21 and by 80 μg without adjuvant at day 49.

For conventional antisera, 2 rabbits were immunized intradermally with 50 μg of dialyzed receptor in complete Freund's adjuvant at day 0. Fifty to sixty micrograms of antigen in incomplete Freund's adjuvant were also injected at day 21 to each rabbit, and at days 49 and 77 to rabbit B only. Anti-receptor antibodies could be detected 10 d after the second immunization.

*Production of mAbs.* The hybridoma lines were generated 4 d after the last injection, following the procedure of De Blas et al. (1981), using the myeloma line P3X63Ag8.6.5.3. The hybridoma lines that secreted mAbs to the receptor complex were identified by both solid-phase radioimmunoassay and immunoprecipitation, using in both assays the affinity-purified GABAR/BZDR complex.

*Solid-phase radioimmunoassay.* Each well of the 96-well polyvinyl chloride plates was incubated overnight at 4°C with 0.2 μg of affinity-purified and dialyzed GABAR/BZDR complex in 50 μl of PBS (10 mM Na phosphate, pH 7.4, 140 mM NaCl). The plates were then incubated with 3% BSA (in PBS) for 2 hr at room temperature (RT), followed by an incubation with 50 μl of hybridoma culture supernatants (overnight at 4°C). All the subsequent steps were carried out in the presence of 0.05% (wt/vol) Tween-20. The mAbs that were bound to the immobilized receptor complex were detected after incubation with $^{125}$I-sheep anti-mouse antibodies (4 hr at RT).

*Immunoprecipitation of the affinity-purified receptor complex.* For conventional antibodies, the immunoprecipitation was performed in a final volume of 100 μl by incubating 0.4 μg of purified and dialyzed receptor with 1 μl of various dilutions of immune serum for 3 hr at 4°C. Then 20 μl of a suspension of 10% (wt/vol) protein A–Sepharose [in 20 mM K phosphate, pH 7.4, 0.1 mM EDTA, 150 mM KCl, and 0.1% (wt/vol) Triton X-100] was added, followed by 40 nM of either $^3$H-muscimol or $^3$H-FNZ. The mixture was further incubated for 1 hr (with agitation and at 4°C) and the immunoprecipitate was collected by centrifugation. The pellet (resuspended in 100 μl of the initial buffer) and the supernatant were separately precipitated with polyethylene glycol and filtered (Mernoff et al., 1983). The nonspecific immunoprecipitation was determined by using preimmune serum. Over 97% of the receptor-binding activity for both radioligands in the immunoprecipitate and of the remaining supernatant used in the assays was recovered.

For mAbs, the immunopreciptation was done by incubating, for 4 hr at 4°C, 100 μl (0.4 μg) of purified and dialyzed GABAR/BZDR complex with 100 μl of hybridoma supernatant. Triton X-100, 10 μl (in 20 mM K phosphate, pH 7.4, 150 mM KCl, 0.1 mM EDTA), was added to obtain a final concentration of 0.1% (wt/vol). A 100 μl mixture (50:50) of goat anti-mouse IgG and IgM bound to agarose [50% suspension in 20 mM K phosphate, pH 7.4, 0.1 mM EDTA, 150 mM KCl, 0.1% (wt/vol) Triton X-100, and 1% BSA] was added, incubated for 2 hr at 4°C with agitation, and centrifuged. The supernatant and washed pellet were diluted to a final volume of 0.5 ml with buffer 1–Triton X-100 and incubated with 10 nM $^3$H-FNZ or 40 nM $^3$H-muscimol for 45 or 30 min, respectively, at 4°C. The agarose-bound receptor complex was recovered by filtration through Whatman GF/B filters. The supernatant's soluble receptor complex with bound ligand was precipitated by polyethylene glycol and recovered by filtration, as indicated above.

*Immunoprecipitation of the CHAPS-solubilized receptor complex.* The GABAR/BZDR/Cl⁻ channel complex was solubilized with CHAPS using a modification of the procedures of Stephenson and Olsen (1982) and Mernoff et al. (1983). Rat or bovine membranes (prepared as described elsewhere; Mernoff et al., 1983) were solubilized with 20 mM CHAPS (in 50 mM Tris-HCl, pH 7.4, 50 mM KCl, 0.1 mM EDTA, and 0.5 mM DTT) for 30 min at 4°C with agitation. The extract was centrifuged at 100,000 × *g* for 1 hr at 4°C and the supernatant was subjected to gel filtration on a Pharmacia PD-10 column. The column was equilibrated with 20 mM K phosphate, pH 7.4, 150 mM KCl, and 5 mM CHAPS, and the soluble receptor was eluted with the same buffer. This step was performed to reduce the concentration of CHAPS in the extract. Of the solubilized receptor preparation, 50 μl (75 μg protein) was mixed with 50 μl of the latter buffer and with 100 μl of hybridoma supernatant and incubated for 4 hr at 4°C. Then 100 μl of a suspension of goat anti-mouse IgG (or IgM) bound to agarose was added, incubated for 2 hr at 4°C with agitation and centrifuged. The pellets (washed once) were resuspended in 500 μl of either buffer 2 with 2.5 mM CHAPS for both the 10 nM $^3$H-FNZ and the 60 nM $^3$H-muscimol binding, or in buffer 1 with 2.5 mM CHAPS for the 40 nM $^{35}$S-TBPS binding.

A similar protocol was followed with the rabbit antisera. The solubilized membranes (50 μl) were mixed with 50 μl of 20 mM K phosphate, pH 7.4, 150 mM KCl (without CHAPS), and 2 μl of either the antiserum or the preimmune serum, and incubated for 4 hr at 4°C. The receptor–antibody complex was precipitated by adding 50 μl of a 15% suspension of protein A–Sepharose. The radioligand binding was performed as for the mAbs. In a representative experiment, the specific binding activities of 50 μl of a CHAPS-solubilized membrane extract were 4000, 1500, and 10,000 cpm for $^3$H-FNZ, $^3$H-muscimol, and $^{35}$S-TBPS, respectively.

*Immunocytochemistry.* This was based on our standard procedure (De Blas, 1984; De Blas et al., 1984). The fixative was periodate/lysine/4% paraformaldehyde and the assay was an avidin–biotin procedure (ABC; previous paper: De Blas et al., 1988).

*Other methods.* Protein was assayed by the method of Lowry et al. (1951) using BSA as standard. SDS–PAGE and immunoblots were carried out according to Laemmli (1970) and De Blas and Cherwinski (1983), respectively. The mAb isotype was determinated both by SDS–PAGE and by an immunodot assay using anti-class- and anti-chain-specific antibodies.

## Results

### *Purification of the GABAR/BZDR complex*

The GABAR/BZDR complex from bovine cerebral cortex membranes was solubilized with Na-deoxycholate and purified by affinity- and ion-exchange chromatography. Both $^3$H-muscimol- and $^3$H-FNZ-binding activities were eluted in the same fractions, indicating that the GABAR and the BZDR were physically associated with each other, forming a complex. Table 1 shows the results of a representative purification experiment. The specific activity of the purified receptor complex for $^3$H-FNZ binding was 1650 times that of the homogenate, and the final yield was 1%. Similar high purification and low yield values have been reported by others (Sigel et al., 1983; Kirkness and Turner, 1986). Scatchard analysis (Fig. 1) indicated that the $B_{max}$ for $^3$H-muscimol binding to the complex was approximately 2 times the $B_{max}$ of $^3$H-FNZ binding. This result suggests that the complex has twice as many $^3$H-muscimol as $^3$H-FNZ binding sites. Nevertheless, the immunoprecipitation of the radioligand-binding activities of the CHAPS-solubilized receptor complex (using receptor-saturating or near-saturating concentrations of radioligands) suggests that the ratio of $^3$H-muscimol:$^3$H-FNZ:$^{35}$S-TBPS binding sites in the complex is 1.4:1:1.1 (calculated from Table 3). However, preferential inactivation of specific sites might have occurred during the purification and/or immunoprecipitation procedures. Therefore, these results might

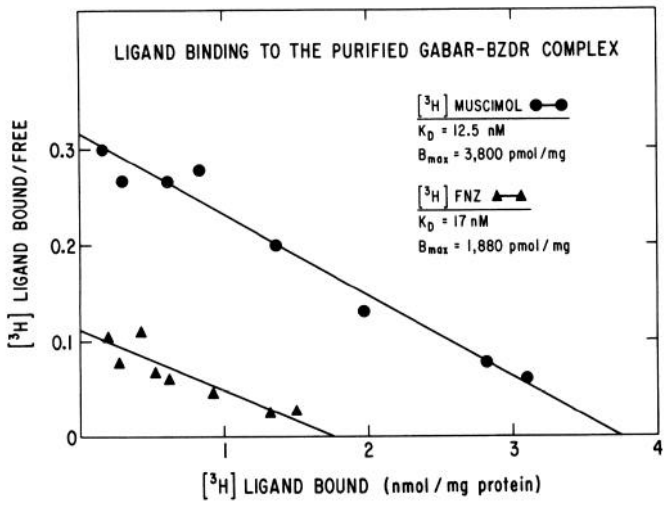


*Figure 1.* Scatchard analysis of the $^3$H-FNZ and $^3$H-muscimol binding to the purified GABAR/BZDR complex.

not indicate the true stoichiometry of the binding sites in the intact receptor complex.

In SDS–PAGE, the purified receptor complex showed 2 main peptides, with $M_r$ values of 51,000 and 57,000 Da, respectively (Fig. 2). Less abundant peptides of 250, 125, 45, 30, and 20 kDa, as well as other minor peptides, were also found. We do not know whether the 250 kDa peptide corresponds to the non-dissociated receptor complex. A $M_r$ of 250 kDa has been proposed for the receptor complex in Triton X-100 (Stephenson et al., 1982). The low-$M_r$ peptides could arise from the proteolytic degradation of the main peptides, even though we included protease inhibitors during the purification of the complex.

### *Conventional antisera*

The presence of antibodies to the receptor complex in the rabbit sera was demonstrated by both ELISA and immunoprecipitation assays using affinity-purified receptor complex (not shown). Several control experiments indicated that the immunoprecipitation reaction was specific for the receptor complex: (1) The radioactive ligands were not precipitated by the immune sera in assays where the receptor was omitted, (2) the immunoprecipitated $^3$H-FNZ- or $^3$H-muscimol-binding activities were blocked by 10 μM clonazepam or 60 μM muscimol, respectively, and (3) the preimmune rabbit sera did not immunoprecipitate the receptor complex.


*Figure 2.* SDS–PAGE of the GABAR/BZDR complex purified by affinity and ion-exchange chromatography. The SDS–PAGE of 1 μg of purified receptor complex was carried out in a 5–20% polyacrylamide gradient. The calibration was done with both the high- and low-molecular-weight marker kits from Pharmacia. The gel was stained with a silver method (Merril et al., 1981) aimed to show most of the peptide components of the preparation. With either shorter staining times or a smaller amount of protein or Coomassie brilliant blue staining, only the 57 and 51 kDa peptides were revealed (not shown).

**Table 1. Recoveries of the GABAR/BZDR purification**

| Fraction | Total protein from 100 gm of tissue (mg) | Binding ($^3$H-muscimol (pmol/mg protein)) | Binding ($^3$H-FNZ (pmol/mg protein)) | Recovery of $^3$H-FNZ (%) |
|---|---|---|---|---|
| Homogenate | 15,000 | ND[a] | 0.54 | 100 |
| Membranes | 2672 | ND[a] | 1.18 | 39 |
| Solubilized receptor | 1422 | 1[b] | 0.90 | 16 |
| Affinity chromatography | 0.22 | 2581 | ND[c] | ND[c] |
| Ion exchange | 0.09 | 2600 | 875 | 1 |

[a] Not determined owing to the interference of endogenous GABA.

[b] The endogenous GABA was removed by dialysis [1 ml of sample in 1000 ml of 20 mM potassium phosphate, pH 7.4; 0.1 mM EDTA; 0.5% (wt/vol) Triton X-100; 0.2% (wt/vol) $NaN_3$; for 12 hr at 4°C].

[c] Not determined owing to the presence of chlorazepate.

The antibodies were not directed to the ligand-binding sites because they interfered neither with the binding of $^3$H-FNZ nor of $^3$H-muscimol to either the membrane bound or the solubilized receptor complex (not shown). In addition to the precipitation of the bovine receptor complex purified in Triton X-100, the antisera also precipitated the GABAR/BZDR/$Cl^-$ channel complex from a crude extract of rat cerebral cortex membranes that were solubilized with 20 mM CHAPS (Table 3). The immunoprecipitated receptor complex retained both the GABA stimulation of $^3$H-FNZ-binding and the $^{35}$S-TBPS-binding activities. These experiments indicated that the antisera im-

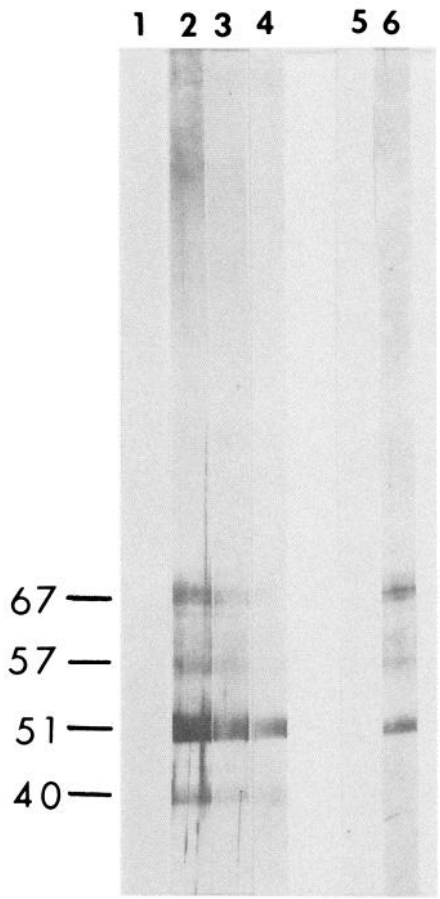


*Figure 3.* Immunoblots of the affinity-purified GABAR/BZDR complex with rabbit antisera. Lanes 1–4 are for rabbit A. Lane *1* is the preimmune serum at 1/2000 dilution and lanes *2–4* are the immune serum at 1/2000, 1/5000, and 1/10,000 dilution, respectively. Lanes *5* and *6* are the preimmune and immune sera of rabbit B, respectively (both at 1/1000 dilution). Each lane had 0.6 μg of purified GABAR/BZDR complex. The SDS–PAGE was in 10% acrylamide.

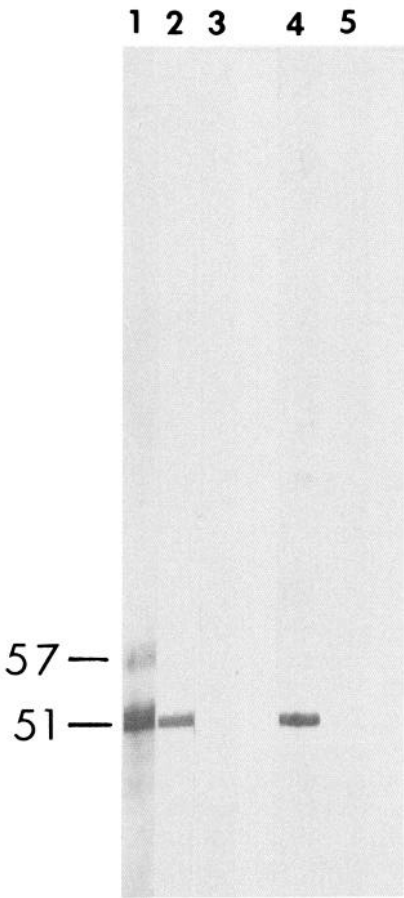


*Figure 4.* Immunoblots of the affinity-purified GABAR/BZDR complex with mouse antisera. Lane *1* is the silver-staining of the SDS–PAGE (10% acrylamide). Lanes *2–5* are immunoblots. Lanes *2* and *4* show the antisera of mice 1 and 2, respectively, and lanes *3* and *5* are their respective preimmune sera. The sera were used at 1/1000 dilution. At lower antisera dilutions (i.e., 1/100) a weak reactivity of the mouse sera with the 57,000 $M_r$ peptide is also seen. Each lane contained 0.6 μg of affinity-purified receptor complex.

munoprecipitated all the elements of the solubilized GABAR/BZDR/Cl⁻ channel complex.

Immunoblots using affinity-purified receptor complex (Fig. 3) showed that the conventional antisera preferentially recognized the 51 kDa peptide, although they also recognized, to a lesser extent, the 57 kDa peptide. The antisera also bound to a 67 kDa peptide, which normally is not detected with the silver-staining method. The antiserum from rabbit A also recognized a 40–45 kDa peptide that is frequently seen in the purified receptor preparations after SDS–PAGE and silver-staining.

**Table 2. Immunoprecipitation of the affinity-purified GABAR/BZDR complex**

| Hybridoma supernatant | $^3$H-Muscimol | $^3$H-FNZ |
|---|---|---|
| 62-3G1 | 85 ± 5 (15) | 87 (1) |
| 62-5F6 | 51 ± 4 (7) | 45 (1) |
| 62-2G4 | 40 ± 15 (4) | 45 (1) |
| 62-3F7 | 35 ± 8 (4) | 42 (1) |
| 62-1H3 | 32 ± 6 (4) | 37 (1) |
| 62-7H3 | 30 ± 8 (4) | 25 (1) |
| 62-4E5 | 27 ± 6 (8) | 27 (1) |

The immunoprecipitation assay was indicated in Materials and Methods. The binding of the radioligand to the immunoprecipitates is given as the percentage of the binding to the purified receptor complex used in each precipitation assay. Negative hybridoma supernatants were used as controls and the cpm value (under 15% of the total) was subtracted from the mAb cpm. The values are the mean ± SEM. The number of experiments is indicated in parentheses.

*Monoclonal antibodies*

BALB/c mice were immunized with the affinity-purified GABAR/BZDR complex. After 2 injections, the mice developed antibodies in their sera, as revealed by both the ability to immunoprecipitate the $^3$H-muscimol-binding activity of the purified receptor complex and by a solid-phase radioimmunoassay (not shown). In immunoblots, the antibodies showed reactivity mostly with the 51 kDa peptide (Fig. 4).

The initial screening of the hybridoma supernatants was done by both solid-phase radioimmunoassay and by immunoprecipitation assay, using the affinity-purified receptor complex. Seven positive hybridoma lines (of 180 generated) were selected on the basis of the stability of the hybridomas and the activity of the supernatants. Table 2 shows the immunoprecipitation of the affinity-purified receptor complex by the hybridoma supernatants. Each monoclonal antibody precipitated both the $^3$H-FNZ-

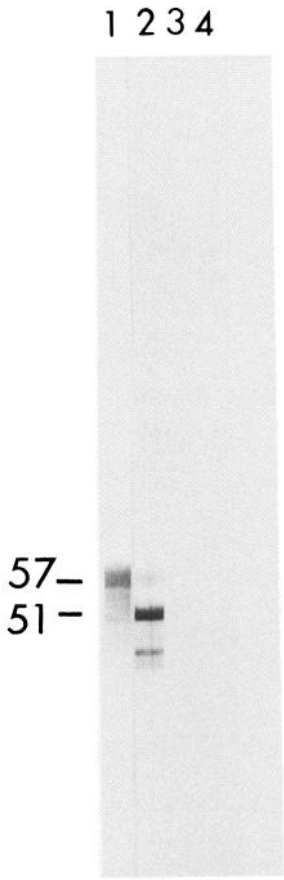


*Figure 5.* Immunoblots of affinity-purified GABAR/BZDR complex with mAbs. Lanes *1–4* are the mAb 62-3G1 (1/5 dilution), the antiserum from mouse 2 (1/100 dilution), the mAb 62-5F6, and the mAb 62-2G4, respectively. The mouse antiserum was used for the identification of the 51,000 $M_r$ peptide (see also Fig. 5). Each lane resulted from the transfer of 0.16 μg of receptor complex.

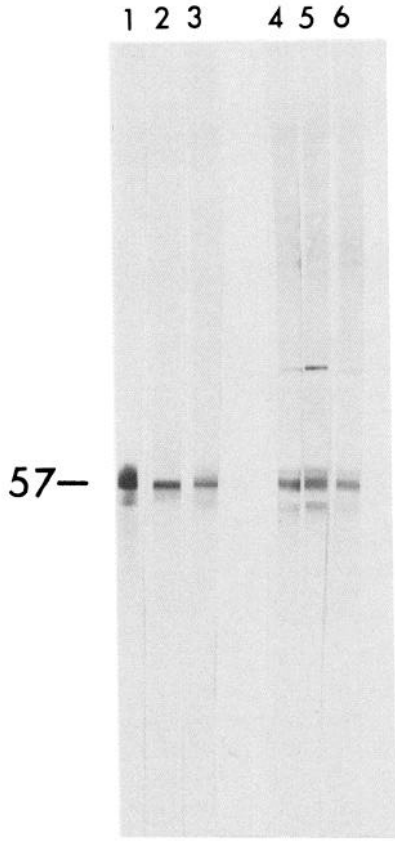


*Figure 6.* Brain membrane immunoblots with the mAb 62-3G1. Lane *1* is an immunoblot with affinity-purified GABAR/BZDR complex (0.11 μg) used as a control. Lane *2* has 5.7 μg of crude membrane protein from bovine cerebral cortex prepared according to Mernoff et al. (1983). Lane *3* has 2.9 μg of synaptosome membrane protein from the rat cerebral cortex, prepared according to the procedure of Jones and Matus (1974) as described by De Blas et al. (1979). Lanes *4–6* are crude membranes (11.4 μg protein/lane) obtained from rat brain cerebral cortex, cerebellum, and hippocampus, respectively. The crude membranes were prepared by the method of Nielsen et al. (1981). In this particular experiment (lanes 4–6), the nuclear fraction was not eliminated from the membranes because the cerebellar glomeruli, which are rich in GABAR (see the preceding paper, De Blas et al., 1988), sediment in this fraction (Tapia et al., 1974). The mAb 62-3G1 culture medium was used at 1/100, 1/2, and 1/10 dilutions, respectively, in lanes 1–3, and at 1/5 dilution in lanes 4–6.

and $^3$H-muscimol-binding activities to the same extent in terms of the percentage of total binding. The immunoprecipitation values varied from 27 to 85%, depending on the hybridoma supernatant. The different values probably reflected both the various antibody titers and/or the affinities of the mAbs for the complex. The mAbs inhibited neither the $^3$H-FNZ nor $^3$H-muscimol binding to the membrane-bound or solubilized receptor complex from either cow or rat brain. The precipitated $^3$H-FNZ- and $^3$H-muscimol-binding activities were blocked by 10 μM clonazepam and 60 μM muscimol, respectively.

In immunoblots, using affinity-purified receptor complex preparations, the mAb 62-3G1 showed a specific reaction with the 57,000 $M_r$ peptide (Fig. 5). However, none of the other antibodies showed reactivity in this assay, which suggests that the epitopes recognized by these mAbs are conformation-dependent and sensitive to the denaturing conditions imposed by the immunoblot assay (probably by SDS).

We also tested the ability of the mAbs 62-3G1 (IgG1, κ) and 62-5F6 (IgM, χ) to precipitate the GABAR/BZDR/$Cl^-$ channel complex from crude preparations of CHAPS-solubilized receptor from rat cerebral cortex membranes. The mAbs immunoprecipitated the $^3$H-FNZ; $^3$H-muscimol- and $^{35}$S-TBPS-binding activities (Table 3). Moreover, the precipitated receptor also showed the functional interaction between the components of the complex, since GABA stimulated the $^3$H-FNZ binding to the immunoprecipitated receptor (Table 3). Similar results were obtained with CHAPS-solubilized receptor from cow brain membranes (not shown).

Figure 6 shows that the mAb 62-3G1 binds to the 57,000 $M_r$ peptide component of the complex even when crude membrane fractions are used in the immunoblots. However, the rabbit antisera showed reactivity with several membrane peptides in addition to with the peptides of the receptor complex. This was observed when brain membranes, instead of affinity-purified receptor, were used in the immunoblots (not shown). Figure 6 also shows that the 62-3G1-immunoreactive 57,000 $M_r$ peptide is present in all brain regions tested, such as cortex, cerebellum, and hippocampus.

Immunocytochemistry experiments indicated that the mAb 62-3G1 was very good for the localization of the $GABA_A$ receptor in the brain (see preceding paper, De Blas et al., 1988). The mAb 62-5F6 showed a strong reaction with the rat brain in the immunocytochemical assay. This mAb reacted with both

**Table 3. Immunoprecipitation of the CHAPS-solubilized GABAR/BZDR/Chloride channel complex**

| Antibody | Radioligand binding to the immunoprecipitate (% of the binding to the CHAPS extract)[a] | | | GABA stimulation of $^3$H-FNZ binding to the immunoprecipitate[b] |
|---|---|---|---|---|
| | $^3$H-muscimol | $^{35}$S-TBPS | $^3$H-FNZ | |
| mAb 62-3G1 | 59 ± 2 | 61 ± 6 | 54 ± 5 | 48 ± 9 |
| mAb 62-5F6 | 38 ± 7 | 36 ± 2 | 33 ± 2 | 35 ± 2 |
| Rabbit serum A | 43 ± 8 | 46 ± 7 | 37 ± 3 | 22 ± 6 |
| Rabbit serum B | 16 ± 2 | 19 ± 1 | 22 ± 4 | 44 ± 8 |

Rat cortex membranes were solubilized with CHAPS and the receptor complex was immunoprecipitated as indicated in Materials and Methods. The binding of the radioligand to the immunoprecipitates is given as a percentage of the binding to the CHAPS extract used in each precipitation assay. The values are the means ± SEM of 3–5 experiments for the mAbs, and 1 experimental assay for the antisera. Triplicate samples were used in each experiment.

[a] The specific binding activities of 50 μl of CHAPS extract were 49.5 ± 5.9, 68.6 ± 7.2 and 54.6 ± 11 fmol for $^3$H-FNZ, $^3$H-muscimol, and $^{35}$S-TBPS, respectively.

[b] The values represent the stimulation (%) by $10^{-4}$ M GABA of the $^3$H-FNZ binding to the immunoprecipitated receptor complex. The GABA stimulation of $^3$H-FNZ binding to the CHAPS extract was 32 ± 9%.

neurons and glia and also with intracellular, as well as surface, antigens. The distribution throughout the brain of the mAb 62-5F6 did not correspond to the distribution of $^3$H-FNZ or $^3$H-muscimol binding. Therefore, in addition to binding to the receptor complex, this mAb probably binds to other antigens that might not be associated with the complex. The mAb 62-2G4 showed low general reactivity, although it bound preferentially to the substantia nigra and the ventral pallidum, which are rich in GABAR and BZDR (Schoch et al., 1985; Sieghart et al., 1987; and the preceding paper, De Blas et al., 1988). The other mAbs did not react with the brain in the immunocytochemistry assay. The conformation-dependent epitopes recognized by these antibodies are also sensitive to the aldehyde fixation.

## Discussion

In this paper, we have described the production of mAbs and conventional antisera to the affinity-purified GABAR/BZDR/Cl⁻ channel complex from cow brain membranes. The antibodies also recognized the rat brain receptor complex. The making of conventional antisera (Stephenson et al., 1986) and monoclonal antibodies to the complex (Haring et al., 1985; Mamalaki et al., 1987) has also been reported by others.

The antibodies immunoprecipitated the binding activities that characterize each component of the GABAR/BZDR/Cl⁻ channel complex. These are $^3$H-FNZ for BZDR, $^3$H-muscimol for $GABA_A$ receptor, and $^{35}$S-TBPS for the Cl⁻ channel. In addition, GABA enhanced the $^3$H-FNZ binding to the precipitated BZDR, which indicated that both the functional and physical interactions among the components of the complex were preserved after immunoprecipitation. The results also support the theory that the GABAR, BZDR, and Cl⁻ channel are part of a membrane protein complex. Neither the rabbit antisera nor the mAbs immunoprecipitated the solubilized glycine receptor ($^3$H-strychnine binding) from the rat spinal cord (not shown). These immunological differences are worth noticing in view of the existing electrophysiological similarities between the GABA- and glycine-gated chloride channels (Barker and McBurney, 1979; Hamill et al., 1983).

The conventional antisera (from rabbits and mice) contained antibodies that reacted with conformation-independent epitopes of the 51 kDa subunit, as the immunoblots show (Figs. 3, 4). These results suggest that either the 51 kDa peptide is more immunogenic or that, after immunization, the 57 kDa peptide is the more rapidly degraded protein component of the complex. The latter interpretation is consistent with the observed easy degradation of this peptide by proteases (Sigel et al., 1983; Sigel and Barnard, 1984). Therefore, it was surprising to us when we found that none of the mAbs of our collection reacted in immunoblots with the denatured 51,000 $M_r$ peptide. The mAb 62-3G1 was the only one that recognized the receptor under the denaturing conditions imposed by the immunoblots. In addition, this mAb recognized the 57 kDa subunit of the complex (Figs. 5, 6). On the other hand, Häring et al. (1985) have obtained mAbs that recognized the denatured 51,000 $M_r$ subunit in immunoblots. We do not know why none of our mAbs reacted with the denatured 51,000 $M_r$ peptide. The reason might be related to the use of a different myeloma cell line in the fusions (we have used the P3X63Ag8.6.5.3 line, while Häring et al. (1985) used the PAI line). More likely, these results are derived from our immunization and screening assays, in which we have used "native" receptor preparations in Triton X-100. These favor the selection of mAbs to conformation-dependent antigens. From the 7 mAbs obtained, we have selected 62-3G1 and 62-5F6 for further characterization. This selection was based on both the high antibody titer and high immunoprecipitation activity of these hybridoma supernatants.

Immunoprecipitation, immunoblotting, and immunocytochemistry experiments showed that the mAb 62-3G1 reacted with the 57,000 $M_r$ peptide of the receptor complex from both rat and bovine brains. In addition, 62-3G1 also reacted with human brain in immunocytochemistry assays. Nevertheless, immunoprecipitation and immunoblot assays have not yet been done with human brain tissue. In immunoblots, no immunoreactivity of the mAb 62-3G1 was detected in either rat kidney or liver, indicating that the receptor complex is either present in very small quantitites or absent from these organs.

Photoaffinity labeling of either the membrane or the affinity-purified BZDR showed that (1) in hippocampus, $^3$H-FNZ binds to 2 peptides of 51 and 57 kDa $M_r$ (Sieghart and Karobath, 1980); (2) in the cerebral cortex, both peptides are photoaffinity-labeled, although most of the binding occurs in the 51 kDa peptide (Casalotti et al., 1986; De Blas et al., 1987); and (3) in cerebellum, only the 51,000 $M_r$ peptide is photoaffinity-labeled with $^3$H-FNZ (Sieghart and Karobath, 1980; Sieghart et al., 1987). These results have been interpreted as supportive evidence for the existence of heterogeneity in the BZDR from various brain regions (Sieghart and Karobath, 1980; Sieghart et al., 1987). Nevertheless, others have not seen these regional differences (Thomas and Tallman, 1981). In addition, the binding of $^3$H-FNZ and $^3$H-muscimol does not colocalize in the cerebellum. The former preferentially binds to the molecular layer, while the latter mostly binds to the granular cell layer (Palacios et al., 1980, 1981; Unnerstall et al., 1981). Immunoblots with 62-3G1 (Fig. 5) showed that the 57,000 $M_r$ peptide is present in the cortex, cerebellum, and hippocampus. Therefore, the differences found among the various regions with respect to the $^3$H-FNZ photoaffinity labeling of the 57,000 $M_r$ peptide do not correspond to major differences in the quantity of the 57,000 Da peptide. These results are similar to the ones obtained by Haring et al. (1985). We do not know yet whether the 57,000 $M_r$ band seen in SDS–PAGE corresponds to one peptide with both a low

affinity for $^3$H-FNZ and a high affinity for the mAb 62-3G1 or to a mixture of 2 or more different peptides, each having a high affinity for either the mAb or for $^3$H-FNZ.

It has been shown that the 57,000 $M_r$ peptide can be photoaffinity-labeled with $^3$H-muscimol (Casalotti et al., 1986; Deng et al., 1986). We have confirmed this result (not shown). This finding, together with the colocalization of the mAb 62-3G1 and the $^3$H-muscimol binding throughout the brain (see preceding paper, De Blas et al., 1988), suggest that the 57,000 $M_r$ peptide recognized by the mAb 62-3G1 is the $^3$H-muscimol-binding subunit of the receptor complex.

Immunoblots have shown that the rabbit antisera preferentially bound to the 51,000 $M_r$ peptide, while the mAb 62-3G1 bound to the 57,000 peptide. In addition, the mAb 62-5F6 binds to a still-unknown epitope of the receptor complex. Nevertheless, for each antibody, no major differences were found between the percentage values of the immunoprecipitated $^3$H-muscimol, $^3$H-FNZ, and $^{35}$S-TBPS binding activities (Table 3). These results, obtained with the CHAPS-solubilized receptor from cerebral cortex membranes, suggest that most of the $GABA_A$ receptors, the benzodiazepine receptors, and the $Cl^-$ channels are molecules that are physically coupled to each other, forming a complex. This interpretation also agrees with the colocalization of $^3$H-FNZ and $^3$H-muscimol binding in the cerebral cortex. Therefore, in the cerebral cortex, few of these receptor proteins are physically uncoupled or separated from the other receptors. We do not yet know whether the mismatch in $^3$H-FNZ and $^3$H-muscimol binding observed in the cerebellum and in some other areas of the brain (Palacios et al., 1980, 1981; Unnerstall et al., 1981) results from the physical separation of the 2 receptor proteins or from the different binding properties of the receptor complex in these brain areas.

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