Reconstitution of Purified $GABA_A$ Receptors: Ligand Binding and Chloride Transporting Properties†

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ABSTRACT: $GABA_A$ receptors have been solubilized from bovine brain membranes and, following purification by benzodiazepine affinity chromatography, have been reconstituted into phospholipid vesicles. Reconstituted vesicles were about 120 nm in diameter, and, on average, each vesicle contained fewer than one $GABA_A$ receptor which was reconstituted in an outside-out orientation. These preparations have been used in parallel studies of radiolabeled ligand binding and chloride flux, the latter being measured by following the fluorescence changes of a chloride-sensitive probe which was trapped within the vesicles at the time of reconstitution. The benzodiazepine [^{3}H]flunitrazepam binds to an apparently homogeneous population of sites in these preparations (K_d of 5 nM) whereas the GABA analogue [^{3}H]muscimol binds to both high- and low-affinity sites (K_ds of 10 nM and 0.27 μM). Muscimol stimulated chloride flux with an EC_{50} of 0.2 μM and, at similar concentrations (EC_{50} = 0.16 μM), potentiated [^{3}H]flunitrazepam binding, suggesting that occupancy of the low-affinity sites may be important for these effects. Diazepam shifted the dose–response curve for muscimol-stimulated flux to about 4-fold lower concentrations without affecting the maximum response. Diazepam did not, however, alter the equilibrium binding of [^{3}H]muscimol. The purified receptor showed densensitization since flux responses were abolished by prior exposure to muscimol. The competitive antagonist bicuculline and the channel blocker picrotoxin completely inhibited ion flux mediated by 3 μM muscimol with EC_{50} values of 5.3 and 2.5 μM, respectively. These results are discussed in terms of possible mechanisms for activation, inhibition, and modulation of $GABA_A$ receptors. The results further demonstrate the usefulness of reconstituted preparations for studying the biochemical and functional properties of purified, native $GABA_A$ receptors.

$GABA_A$ receptors are major inhibitory neurotransmitter receptors in the central nervous system where they mediate rapid chloride fluxes in response to the binding of γ-aminobutyric acid (GABA).[1] They belong to the family of ligand-gated ion channels that includes nicotinic, glycine, and $5HT_3$ receptors [reviewed by Barnard (1992)]. These receptors are multisubunit proteins, probably pentamers, composed of homologous subunits that form a pseudosymmetric complex assembled around a central pore [reviewed by Stroud et al. (1990) and Olsen and Tobin (1990)]. In the case of $GABA_A$ receptors, it is now clear that multiple subtypes exist but the subunit complement of native receptors remains to be established. Protein purification studies originally identified an α and a β subunit as major components of the $GABA_A$ receptor [reviewed by Stephenson (1988)], but molecular cloning has since revealed a much greater degree of heterogeneity with the identification of several isoforms of several different subunit types [α, β, γ, δ, and ρ; reviewed by DeLorey and Olsen (1992) and Wisden and Seeburg (1992)]. In theory, a very large number of different subunit compositions may exist (Burt & Kamachi, 1991), but it appears likely that some combinations predominate and, for example, a good candidate for a major receptor subtype is the $\alpha_1\beta_2\gamma_2$ combination [see Wisden and Seeburg (1992)].

In the past few years, considerable information has been accumulating from the results of heterologous expression studies in which $GABA_A$ receptors with different subunit compositions have been expressed in transient and permanent expression systems (Levitan et al., 1988; Sigel et al., 1990; Verdoorn et al., 1990; Smart et al., 1991; Wafford et al., 1992; Angelotti & Macdonald, 1993). The functional properties of the $GABA_A$ receptor have been shown to depend on the subunit composition, and this approach is providing valuable information on the structural determinants necessary for the action of the many classes of $GABA_A$ receptor modulators, including benzodiazepines, barbiturates, and neurosteroids. It is obvious that the interpretation of these experiments would benefit greatly from a detailed knowledge of the properties of native receptors, but such information is presently limited.

Although there have been many reports of the purification of $GABA_A$ receptors [see Stephenson (1988)], only recently were such preparations shown to retain their ability to mediate chloride flux responses. Solubilized (Hirouchi et al., 1987; Dunn et al., 1989) and purified (Schoch et al., 1984; Sigel, et al., 1985; Hirouchi et al., 1987; Bristow & Martin, 1990) $GABA_A$ receptors have been successfully reconstituted, but only in a few cases have chloride flux measurements been described. Hirouchi et al. (1987) reported that, after reconstitution of impure or purified receptors, there was a significant $^{36}Cl^-$ uptake in response to GABA and, in the case of the impure preparation, this response was modulated by flunitrazepam. In a more comprehensive study of $^{36}Cl^-$

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[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GABA, γ-aminobutyric acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; MQAE, *N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide; β-octylglucoside, *n*-octyl β-D-glucopyranoside; MSQ, 6-methoxy-*N*-(sulfopropyl)quinolinium; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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responses by affinity-purified and reconstituted $GABA_A$ receptors, Bristow and Martin (1990) showed that GABA-stimulated flux was partially inhibited by bicuculline and picrotoxin and potentiated by flunitrazepam, by pentobarbital, and by the pyrazolopyridine cartazolate. However, the magnitude of the measured responses was small, and this precluded quantitative analysis.

We previously demonstrated that $GABA_A$ receptors could be solubilized from bovine cerebral cortex by β-octylglucoside and reconstituted into phospholipid vesicles with retention of many of their functional characteristics (Dunn et al., 1989a). A stopped-flow assay based on the quenching of the fluorescence of a chloride-sensitive probe trapped within the vesicles was developed (Dunn et al., 1989b) and used to measure chloride flux responses of reconstituted $GABA_A$ receptors. In this report, we extend these studies to investigation of the ligand binding and chloride transporting properties of $GABA_A$ receptors purified by benzodiazepine affinity chromatography. A preliminary account of this work has appeared (Thuynsma & Dunn, 1991).

MATERIALS AND METHODS

Materials. 1012-S was a kind gift from Dr. K. Hirai (Shionogi Research Laboratories, Osaka, Japan). [^{3}H]-Muscimol and [^{3}H]flunitrazepam were from DuPont Canada, Mississauga, Ontario. CHAPS, clorazepate, diazepam, muscimol, and picrotoxin were from Sigma Chemical Co., St. Louis, MO, and bicuculline methochloride was from Cambridge Research Biochemicals, Cambridge, England. Asolectin was from Associated Concentrates, Woodside, NY, and MQAE was from Molecular Probes, Inc., Eugene, OR.

Preparation of 1012-S–Agarose Affinity Resin. 1012S-acetamide–adipic hydrazide agarose was prepared starting from adipic acid dihydrazide agarose (Sigma) as previously described (Taguchi & Kuriyama, 1984). Alternatively, 1012-S was coupled to Affigel 202 (BioRad) to give a resin with a less hydrophobic side arm similar to that described by Stauber et al. (1987). Several different affinity resins of each type were synthesized and used in the experiments described below with no significant differences in the results.

Preparation and Solubilization of Bovine Brain Membranes. Bovine brains were obtained from a local slaughterhouse and were immediately frozen on dry ice and stored at −86 °C until required. Membranes were prepared from thawed bovine cerebral cortex (Agey & Dunn, 1989) and were finally resuspended in 20 mM Tris–citrate, pH 7.5, containing the following as protease inhibitors: 1 mM EDTA, 0.5 mM DTT, 1 mM benzamidine, 0.3 mM PMSF, 20 μg/mL bacitracin, 10 μg/mL soybean trypsin inhibitor, 10 μg/mL chick egg white inhibitor, and 0.02% NaN_3. Prior to each purification, 60-mL membranes (15 mg of protein/mL) were stirred on ice and solubilized (Thuynsma & Dunn, 1991) by dropwise addition of an equal volume of ice-cold solubilization buffer (20 mM Tris–citrate, pH 7.5, 0.5 M KCl, 3% CHAPS, 0.3% asolectin, and the same protease inhibitors as above). After being stirred for 30 min at 4 °C, the mixture was centrifuged at 100000*g* for 75 min at 4 °C, and the resulting supernatant containing solubilized receptor was carefully decanted.

Purification of $GABA_A$ Receptor. The solubilized receptor (approximately 110 mL) was divided into two equal volumes, and purification of $GABA_A$ receptors from each sample proceeded in parallel. Each sample was applied to a 20-mL column of 1012-S–agarose equilibrated in 50 mM Tris–citrate, pH 7.5, 0.1 M KCl, 1 mM EDTA, 0.6% CHAPS, 0.06% asolectin, and 0.02% NaN_3, and each column was washed, at a flow rate of 40 mL/h, with 200 mL of the same buffer. The resins were washed with 200 mL of buffer A (50 mM Tris–citrate, pH 7.4, 0.6% CHAPS, 0.06% asolectin, 0.1 mM EDTA, and 0.02% NaN_3), and the receptor was then eluted with 40 mL of the same buffer containing 10 mM clorazepate which was allowed to recycle through the column overnight. The clorazepate eluates were applied to columns (2 mL) of DEAE-Sephacel (Sigma) equilibrated in buffer A which were then washed with the same buffer for 3 h at a flow rate of 40 mL/h to remove the clorazepate. The columns were eluted with linear gradients formed from 20 mL each of 0.6 M and 1.2 M KCl in buffer A, and fractions of 2 mL were collected. Fractions were measured for [^{3}H]muscimol binding activity as described below, and those fractions containing activity (6–12 mL per column) were pooled and dialyzed overnight against 1 L of buffer A. Prior to reconstitution, each sample was concentrated to about 0.8 mL using Amicon Centricon-100 microconcentrators.

Regeneration of 1012-S–Agarose Affinity Columns. After each purification procedure, resins were regenerated by washing at a flow rate of 40 mL/h with 100 mL each of (a) 20 mM Tris–citrate, pH 7.5, 0.5 M NaCl, 1 mM EDTA, 0.6% CHAPS, and 0.02% NaN_3; (b) 50 mM sodium acetate, pH 5.5, 6 M urea, 0.6% CHAPS, and 0.02% NaN_3; and (c) equilibration buffer as described above. The resins were replaced if there was a decrease in $GABA_A$ receptor binding activity, but some columns have now been in use for several years without significant deterioration.

Estimation of Protein Concentration. Protein concentration was usually estimated by the BioRad procedure except for the purified $GABA_A$ receptor in which case the bicinchoninic protein assay was used (Smith et al., 1985).

Reconstitution of Purified $GABA_A$ Receptor and Loading of Vesicles with MQAE. Purified $GABA_A$ receptors were reconstituted into phospholipid vesicles using a 4:1 ratio of asolectin to natural bovine brain lipids (Dunn et al., 1989a). For flux studies, the chloride-sensitive dye MQAE was loaded into the vesicles at the time of reconstitution (Thuynsma & Dunn, 1991). Bovine brain lipids were extracted by homogenization of bovine cerebral cortex in 2:1 chloroform/methanol and were partially purified by silicic acid chromatography (Tamkun & Catterall, 1984; Dunn et al., 1989a). A solution of 40 mg/mL asolectin and 10 mg/mL bovine brain lipids in chloroform was dried under a stream of nitrogen at 30 °C and further dried *in vacuo*. Dried lipids were resuspended, by vortexing and sonication, at 50 mg/mL in 50 mM Tris–citrate, pH 7.4, 0.1 mM EDTA, 1.5% CHAPS, and 0.02% NaN_3. For reconstitution, 0.8 mL of concentrated $GABA_A$ receptor preparation was added to 0.2 mL of the lipid mixture and 0.2 mL of 60 mM MQAE prepared in the same buffer. The mixture was rotated for 20 min at 4 °C after which detergent, in addition to extravesicular MQAE, was removed by chromatography on a 1.5 × 90 cm column of Sephadex G-50 equilibrated in 10 mM Hepes–Tris, pH 7.4, and 150 mM potassium gluconate. During reconstitution, MQAE was trapped within the vesicles, and the dye-loaded vesicles eluted in the void volume.

Sucrose Gradient Centrifugation. Linear gradients of 2–20% (w/v) sucrose (10 mL) in 10 mM Hepes–Tris, pH 7.4, and 150 mM potassium gluconate were prepared on top of a 1-mL cushion of 60% sucrose in Beckman SW41 centrifuge tubes. Aliquots of 200 μL of concentrated reconstituted receptor preparations were layered on top, and the gradients were centrifuged at 275000*g* for 21 h at 4 °C. Fractions

(approximately 0.3 mL) were collected from the bottom of the tube using a Hoefer fractionator and were assayed for [^{3}H]muscimol binding.

Estimation of Size of Reconstituted Vesicles. Vesicle size was estimated by laser light scattering using a Brookhaven BI-90 particle size analyzer.

Phosphate Analysis. Total phosphate in the reconstituted membranes was measured by a modification of the Fiske–Subbarow method (Dittmer & Wells, 1969).

Radiolabeled Ligand Binding to Solubilized and/or Purified $GABA_A$ Receptors. The binding of [^{3}H]muscimol or [^{3}H]flunitrazepam to solubilized preparations was measured by filtration assay using a Hoefer filtration manifold. Receptor aliquots were incubated with the radiolabeled ligand for 30 min at 4 °C in 20 mM potassium phosphate buffer, pH 7.4, containing 200 mM KCl, 0.1 mM EDTA, and 0.1% (v/v) Triton X-100. Nonspecific binding was estimated from parallel samples that contained excess unlabeled ligand. Aliquots were filtered under vacuum through Whatman GF/C filters that had been soaked overnight in 0.3% poly(ethylenimine) (Bruns et al., 1983), and the filters were immediately washed with 2 5-mL volumes of ice-cold 20 mM potassium phosphate, pH 7.4, and 0.1 mM EDTA. After the filters were dried, they were counted for ^{3}H in 5 mL of ACS (Amersham Canada) scintillation fluid.

Radiolabeled Ligand Binding to Reconstituted $GABA_A$ Receptors. The binding of [^{3}H]muscimol and [^{3}H]flunitrazepam was measured using an automated rapid filtration technique (Dupont, 1984) to minimize dissociation from low-affinity binding sites (Agey & Dunn, 1989). Briefly, aliquots of reconstituted membranes were incubated on ice in 10 mM Hepes–Tris, pH 7.4, 125 mM potassium gluconate, and 25 mM KCl with increasing concentrations of labeled ligand in a final volume of 1 mL After 60-min incubation, duplicate aliquots of 0.35 mL were applied under vacuum to GF/C filters [presoaked in 0.3% poly(ethylenimine)] mounted in the holder of a Biologic rapid filtration system (Biologic, Meylan, France). Unbound ligand was removed by force-filtration with the same buffer for 0.5 s at a flow rate of 5 mL/s. The filters were dried and counted for ^{3}H as described above. Duplicate 50-μL aliquots of each sample were also counted to estimate the total ligand concentration, and nonspecific binding was estimated in the presence of 100 μM unlabeled ligand.

Chloride Flux Responses of Reconstituted $GABA_A$ Receptors. Chloride flux responses were measured in stopped-flow experiments similar to those previously described (Dunn et al., 1989b). MQAE-loaded vesicles in chloride-free buffer (10 mM Hepes–Tris, pH 7.4, and 150 mM potassium gluconate) were rapidly mixed with an equal volume of buffer containing ligands, as indicated, and KCl (10 mM Hepes–Tris, 100 mM potassium gluconate, and 50 mM KCl) in an Applied Photophysics Ltd. (Leatherhead, Surrey, England) SF17.MV stopped-flow fluorometer. The influx of Cl^- was measured by monitoring changes in the fluorescence of the entrapped dye. The sample compartment was thermostated at 25 °C, the excitation wavelength was 355 nm, and fluorescence emission was measured using a GG420 Schott glass filter (Melles Griot, Irvine, CA). Where indicated, the appropriate drug was incubated with the receptor preparation for 10 min in the stopped-flow compartment before mixing with buffer containing the same concentration of the preincubated drug.

Data Analysis. Simple equilibrium binding isotherms and competition curves were analyzed by nonlinear regression using Inplot Version 4.0 (GraphPad Software, San Diego, CA) and the equations given in the figure legends. [^{3}H]Muscimol binding data are represented as Scatchard plots, and the equation used for curve-fitting (Marquardt, 1961) was the two-site model described by Rodbard and Feldman (1975) which assumes the presence of two distinct independent sites:

$$B/F = {}^1/_2\{[(R_1 - B)/K_{d1} + (R_2 - B)/K_{d2}] + \mathrm{SQR}([(R_1 - B)/K_{d1} - (R_2 - B)/K_{d2}]^2 + 4R_1R_2/K_{d1}K_{d2})\}$$

in which B and F are the concentrations of bound and free ligand, respectively, R_1 and R_2 are the concentrations of the two classes of binding sites, and K_{d1} and K_{d2} are the corresponding dissociation constants.

Chloride flux data were analyzed using the Archimedes kinetic package from Applied Photophysics Ltd. and the equation:

$$F(t) = A_0 + A_1/\{1 + KC_\infty[1 - \exp(-k_1 t)]\}$$

in which $F(t)$ is the fluorescence at time t, A_0 is the remaining fluorescence after the initial phase of quench, A_1 is the amplitude of the rapid quench, and k_1 is the apparent rate of this quench. The term KC_∞ was fixed at 1.65 using a Stern–Volmer constant (K) of 66 M^{-1} for quenching of MQAE by Cl^- in the presence of 150 mM potassium gluconate [measured as described by Dunn et al. (1989b)] and the known final concentration of Cl^- ions (C_∞ = 25 mM).

RESULTS

Purification of $GABA_A$ Receptors. The $GABA_A$ receptor was purified by benzodiazepine affinity chromatography and ion-exchange chromatography using modifications of procedures described by others [see Stephenson and Barnard (1986)]. Solubilization by 1.5% CHAPS in the presence of 0.15% asolectin resulted in solubilization of 50 ± 10% protein and 55 ± 11% [^{3}H]flunitrazepam binding sites. Following adsorption to immobilized 1012-S, elution with clorazepate, and DEAE-Sephacel chromatography, the overall recovery of [^{3}H]flunitrazepam binding sites was estimated to be 2.6 ± 0.6% (n = 8) of the sites present in the original membrane fraction. Analysis of such purified $GABA_A$ receptors by SDS–gel electrophoresis has revealed two major protein bands with apparent molecular weights of 53 000 and 57 000 [see Thuynsma and Dunn (1991)]. However, additional components were clearly apparent, as would be expected in view of the known heterogeneity of $GABA_A$ receptor subunits, previously revealed by both photoaffinity labeling and immunoblotting techniques [see Sieghart (1991)].

Reconstitution of Purified $GABA_A$ Receptors. $GABA_A$ receptors were reconstituted using a 4:1 mixture of asolectin and natural brain lipids as we have used previously for impure receptor preparations (Dunn et al., 1989a). For flux studies, the fluorescent dye MQAE was loaded at the time of vesicle formation during detergent removal by Sephadex G-50 chromatography. The vesicles eluted in the void volume, and more than 80% of the high-affinity binding sites for [^{3}H]-muscimol and [^{3}H]flunitrazepam were recovered in this fraction. Upon sucrose density gradient centrifugation, all of the specific [^{3}H]muscimol binding sites migrated to a density of 12–15%. This coincided with the position of the membrane fraction on the gradient which could be directly identified by its turbidity. Thus, $GABA_A$ receptors appear to have been efficiently incorporated into liposomes. For flux studies, the vesicles were used immediately after reconstitution. The binding sites for both [^{3}H]flunitrazepam and [^{3}H]muscimol,

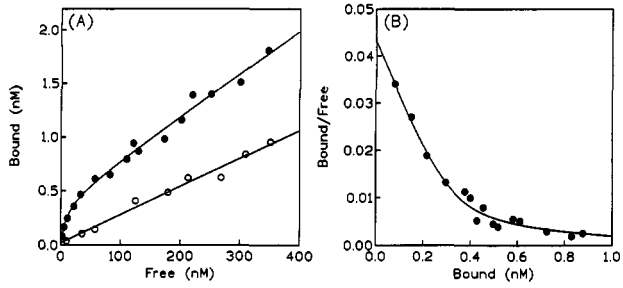

FIGURE 1: [^{3}H]Muscimol binding to purified and reconstituted $GABA_A$ receptor. (A) Binding was measured using the automated filtration technique described under Materials and Methods in the absence (●) or presence (○) of unlabeled muscimol. (B) Scatchard plot of the data in (A). The solid line is fit by the two-site model given in the text using the following best-fit parameters: K_{d1} = 7.9 nM; R_1 = 0.32 nM; K_{d2} = 0.4 μM; R_2 = 1.3 nM. Data are representative of four similar experiments using different reconstituted preparations.

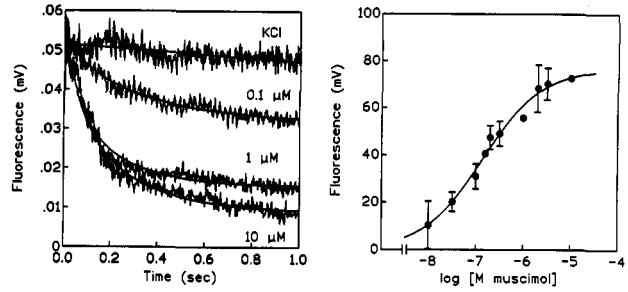

FIGURE 2: Chloride flux responses of purified and reconstituted $GABA_A$ receptors. Panel A shows representative stopped-flow traces upon rapid mixing of MQAE-loaded vesicles (in 10 mM Hepes–Tris, pH 7.4, and 150 mM potassium gluconate) with an equal volume of buffer containing 50 mM KCl (isoosmotic replacement of potassium gluconate) plus 0.1 μM, 1 μM, or 10 μM muscimol as indicated. Each fluorescence trace (recorded as an electrical signal in millivolts) is the average of at least four determinations, and solid lines are best-fit to the equation given in the text, giving values of amp_1 = 35 mV and k_1 = 1.9 s^{-1} (0.1 μM); amp_1 = 61 mV and k_1 = 3.72 s^{-1} (1 μM); and amp_1 = 72 mV and k_1 = 3.5 s^{-1} (10 μM). Panel B shows the dependence of the amplitude of the observed fluorescence change (amp_1) on muscimol concentration. Data are pooled from four separate experiments using different reconstituted preparations. The solid line is fit by a simple sigmoid curve: fluorescence = $A + (B - A)/[1 + \log(EC_{50})^n$ where A = 2.8 mV, B = 76.5 mV, EC_{50} = 0.2 μM, and n = 0.75.

although labile in detergent solution [see Sigel and Barnard (1984) and Bristow and Martin (1990)], were very stable after reconstitution, and preparations could be stored at –4 °C for several days without detectable loss of activity and were stable after prolonged storage at –86 °C.

Characterization of Reconstituted $GABA_A$ Receptor Preparations. Laser light scattering measurements using four different preparations have shown that reconstituted vesicles have an average diameter of 120 ± 25 nm. The sidedness of reconstituted $GABA_A$ receptors has been estimated by the effects of detergent on the binding of [^{3}H]muscimol, a charged ligand which is membrane-impermeant (Sigel et al., 1985). In several preparations, the binding of [^{3}H]muscimol was identical in the absence and presence of 0.1% Triton X-100, which was added to expose the interior of the vesicles (data not shown). Thus, as reported in a previous study (Sigel et al., 1985), the [^{3}H]muscimol binding sites are exposed to the outside of the vesicles, showing that the $GABA_A$ receptors are reconstituted in a predominantly, if not exclusively, outside-out orientation. The approximate number of receptors per vesicle has been estimated as previously described (Wu et al., 1981; Dunn et al., 1989a) by measuring the relative abundance of phospholipids and [^{3}H]flunitrazepam binding sites in the preparations. The reconstituted preparations contain approximately 2.1 μmol of phospholipid/pmol of [^{3}H]flunitrazepam binding sites. Assuming that each $GABA_A$ receptor contains one high-affinity site for [^{3}H]flunitrazepam, the phospholipid:$GABA_A$ receptor mole ratio is thus (2.1×10^6): 1. Since a vesicle that is 120 nm in diameter can be estimated to contain 1.5×10^5 phospholipid molecules [see Wu et al. (1981)], it may be calculated that the number of receptors per vesicle is about 0.071. The reconstituted vesicles are thus approximately 120 nm in diameter; the majority do not contain receptors, but in those that do (about 7%, assuming random reconstitution), the majority of the receptors are reconstituted in a rightside-out orientation.

Binding of [^{3}H]Muscimol to Reconstituted $GABA_A$ Receptors. Figure 1 shows a representative experiment of the binding of [^{3}H]muscimol to reconstituted preparations. After subtraction of nonspecific binding, the specific [^{3}H]muscimol binding did not saturate in the range of [^{3}H]muscimol concentrations used. A Scatchard plot of the data was clearly biphasic, suggesting that, as in brain membranes (Agey & Dunn, 1989), there are two populations of binding sites. Estimates of the binding parameters for the low-affinity sites have been facilitated by the use of the rapid filtration technique which reduced the time of washing of the filters to 0.5 s and thus limited the extent of ligand dissociation. The estimates, however, remain subject to large error due to the intrinsic low affinity and practical restrictions on the range of [^{3}H]muscimol concentrations that can be used. In four separate experiments, average values for the two K_d values have been 9.6 ± 3.0 nM and 0.27 ± 0.10 μM. Accurate estimates of the density of low-affinity binding sites are also difficult, but in these experiments, the number of low-affinity sites exceeded the number of high-affinity sites by a factor of 2.6 ± 1.0.

Binding of [^{3}H]Flunitrazepam to Reconstituted $GABA_A$ Receptors. In contrast to the heterogeneity of [^{3}H]muscimol binding sites, the binding of [^{3}H]flunitrazepam was to an apparently homogeneous population of sites with a K_d of 5.6 nM (data not shown). Parallel measurements of [^{3}H]-muscimol binding have shown that there are approximately twice as many high-affinity binding sites for muscimol as flunitrazepam sites.

Stimulation of Chloride Uptake by Muscimol. When MQAE-loaded vesicles were mixed with buffer containing Cl^- and muscimol, there was a rapid quench in fluorescence due to influx of Cl^- ions. As shown in Figure 2, the magnitude of the fluorescence quench was dependent upon the muscimol concentration and reached a maximum above about 1 μM. As discussed previously (Dunn et al., 1989b), this increase in amplitude is likely to be due to the low density of receptors (<1 $GABA_A$ receptor per vesicle) such that the effect of increasing occupancy of the binding sites is the recruitment of increasing numbers of vesicles that contribute to the flux response. The rate of the observed quench also increased with increasing muscimol concentration, reaching a maximum at about 4.5 s^{-1}. However, since the rate increased only about 2-fold over the concentration range used, there are large errors in quantitation. Above 3–10 μM muscimol, the magnitude of the fluorescence quench often decreased. As discussed below, this is possibly a consequence of rapid receptor desensitization at the higher agonist concentrations.

Modulation of Agonist-Mediated Chloride Flux. Muscimol-stimulated flux responses were abolished if the receptor was preincubated with muscimol, suggesting that the receptor desensitizes upon exposure to agonist (Figure 3A). Flux was

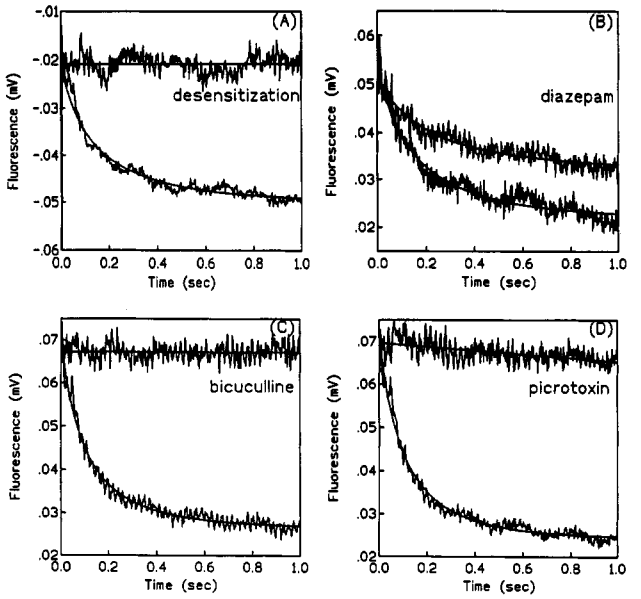

FIGURE 3: Modulation of muscimol-stimulated chloride flux responses. (A) Effect of desensitization. The lower trace is the control flux response to 10 μM muscimol measured as described in the legend to Figure 4. The upper trace was obtained after preincubation of reconstituted $GABA_A$ receptor with 10 μM muscimol for 10 min prior to mixing with buffer containing 50 mM KCl and 10 μM GABA. (B) Effect of diazepam. The upper trace is the control response to 0.1 μM muscimol, and the lower trace was obtained when reconstituted $GABA_A$ receptor was preincubated with 10 μM diazepam before mixing with 10 μM diazepam and muscimol (0.1 μM final concentration). (C) Effect of bicuculline and (D) effect of picrotoxin. In each case, the lower curves are control responses to 3 μM muscimol, and the upper traces were obtained after preincubation for 10 min with 100 μM bicuculline or 30 μM picrotoxin before mixing with KCl buffer and the same concentration of blocker and muscimol (3 μM final).

also inhibited by preincubation with the competitive receptor antagonist bicuculline methochloride (Figure 3C), and by picrotoxin (Figure 3D) which, in single-channel recordings of mouse spinal neurons, has been shown to reduce the mean channel open time and the frequency of opening (Twyman et al., 1989a). Diazepam alone had no effect on the fluorescence of entrapped MQAE, but potentiated agonist-induced flux (Figure 3B).

Potentiation of Muscimol-Stimulated Chloride Flux by Diazepam. Detailed studies of the effects of a saturating concentration of diazepam (10 μM) on agonist-mediated flux responses have shown that this benzodiazepine shifts the dose-dependence of flux to lower concentrations but does not affect the maximum response (Figure 4). In some experiments, diazepam did appear to potentiate chloride flux induced by 10 μM muscimol, but, on detailed analysis, this was found to be a consequence of a decrease in the control response (i.e., in the absence of diazepam) at this muscimol concentration (see above).

Allosteric Interactions between Muscimol and Benzodiazepine Binding Sites. As discussed in more detail below, it has frequently been suggested that benzodiazepines potentiate $GABA_A$ receptor responses by increasing the affinity of the receptor for agonists. However, under equilibrium conditions, we have detected no effect of 10 μM diazepam on the parameters for [^{3}H]muscimol binding to either its high-affinity or its low-affinity binding sites as shown by the data in Figure 5A. In the purified and reconstituted preparations, allosteric interactions between the agonist and benzodiazepine sites are, however, maintained as shown by the ability of muscimol to

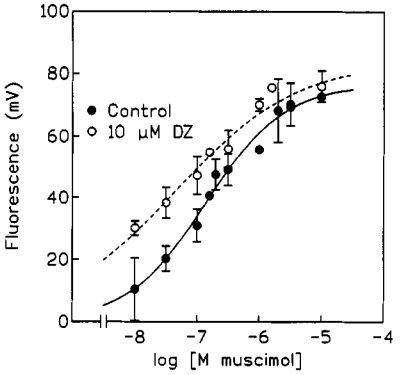

FIGURE 4: Effect of diazepam on the concentration dependence of muscimol-stimulated flux. Data were obtained in four separate experiments. The data for muscimol-stimulated flux (●) are the same as those shown in Figure 2. In parallel experiments, reconstituted $GABA_A$ receptor was preincubated with 10 μM diazepam before mixing with 10 μM diazepam and increasing concentrations of muscimol. Data in the presence of diazepam (○) were obtained and analyzed as described in the legend to Figure 5, giving the following best-fit parameters: A = 1.8 mV, B = 86 mV, EC_{50} = 46 nM, and n = 0.41.

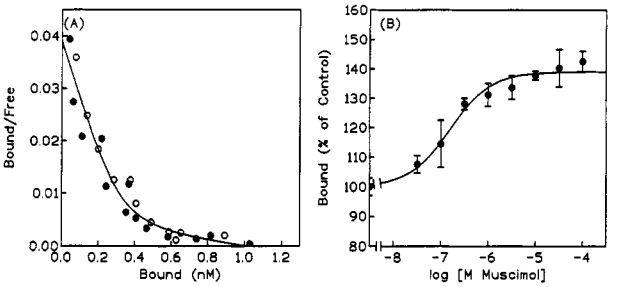

FIGURE 5: (A) Lack of effect of diazepam on equilibrium binding of [^{3}H]muscimol. [^{3}H]Muscimol binding in the absence (●) or presence (○) of 10 μM diazepam was measured as described in the legend to Figure 3 and are represented as a Scatchard plot. Data were fit by a two-site model, giving K_{d1} = 8.4 nM, R_1 = 0.31 nM, K_{d2} = 0.28 μM, and R_2 = 0.71 nM. (B) Stimulation of [^{3}H]-flunitrazepam binding by muscimol. Reconstituted $GABA_A$ receptor was incubated with 1 nM [^{3}H]flunitrazepam and increasing concentrations of muscimol for 60 min on ice prior to separation of bound and free ligand by filtration assay as described under Materials and Methods. The solid line is fit to the simple sigmoidal model: [bound] = $A + (B-A)/[1 + \log(EC_{50})]$ where A = 100, B = 139.1, and EC_{50} = 0.16 μM.

potentiate the binding of [^{3}H]flunitrazepam (Figure 5B), an effect that occurs with an EC_{50} of 0.16 μM.

Block of Muscimol-Stimulated Chloride Flux by Bicuculline and Picrotoxin. The concentration dependence of the inhibition of the flux response by bicuculline and picrotoxin is shown in Figure 6. Both ligands reduced both the amplitude and the rate of the flux response in a dose-dependent manner, but as described above, the small range of apparent rates results in large errors. The effects on the amplitude of the flux response to 3 μM muscimol were, however, readily measured, and apparent EC_{50} values were 5.3 μM for bicuculline and 2.5 μM for picrotoxin.

DISCUSSION

The results of this study demonstrate that $GABA_A$ receptors, purified by benzodiazepine affinity chromatography, can be reconstituted into phospholipid vesicles in a functionally active form. This provides the opportunity to study the properties of native receptor proteins in a controlled and reproducible environment and to examine the molecular mechanisms that underlie the gating of the $GABA_A$ receptor chloride channel

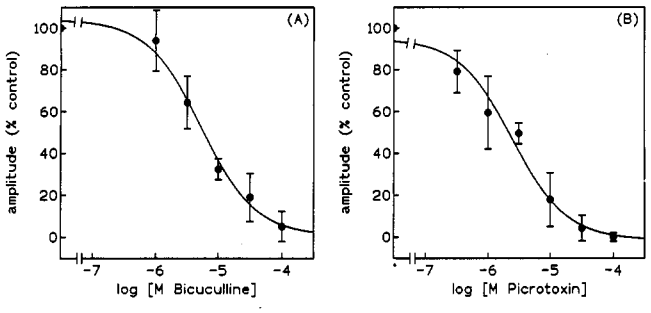

FIGURE 6: Concentration dependence of the inhibition of muscimol-stimulated flux by bicuculline (A) and picrotoxin (B). The flux response to 3 μM muscimol (final concentration) was measured in experiments similar to those shown in Figure 5. In each case, bicuculline or picrotoxin was incubated with reconstituted preparations for 10 min prior to mixing with buffer containing KCl, muscimol, and the blocker at the same concentration as in the preincubation. Data were obtained and analyzed as in the legend to Figure 4. Data fitting by the simple sigmoidal model: amplitude = $A + (B - A)/[1 + \log(EC_{50})]$ gave for bicuculline $A = 104.2$, $B = 0.7$, and $EC_{50} = 5.3$ μM and for picrotoxin $A = 95$, $B = -1.4$, and $EC_{50} = 2.5$ μM.

and its modulation by the diverse array of drugs and endogenous ligands that specifically target this receptor.

Although the extent of heterogeneity of the $GABA_A$ receptor population in bovine cerebral cortex remains to be established, it is likely that the reconstituted preparations contain more than a single receptor subtype. Purification by benzodiazepine affinity chromatography selects only for those receptors that carry a high-affinity benzodiazepine binding site, for which the minimum structural requirements appear to be the presence of an α and a γ subunit (Pritchett et al., 1989). Using subunit-specific antibodies to probe the subunit complement of similarly purified cortical preparations, α_1, α_2, α_3, β_3, and γ_2 subunits have been detected [see Stephenson (1992)], with α_1 being the most abundant of the α subunits. Receptors containing only a single α-subunit isoform in combination with a β and a γ subunit appear to predominate (Duggan & Stephenson, 1990; McKernan et al., 1991), but a minor population may contain $\alpha_1\alpha_2$, $\alpha_1\alpha_3$, and $\alpha_2\alpha_3$ pairs [see Pollard et al. (1993)]. The extent to which the differences in subunit composition affect ligand binding and functional properties of native receptors is presently unknown. Within the limitations of the techniques used in the present investigation, no major heterogeneity has been observed, and the results obtained from one preparation to another have been remarkably consistent. It is thus likely that determination of the nature and extent of functional heterogeneity will require detailed biophysical analysis of single $GABA_A$ receptors after purification and reconstitution in lipid bilayers.

When analyzed by gel electrophoresis in the presence of SDS, purified receptor preparations have been shown to contain two major protein components of apparent molecular weights 53 000 and 57 000, in addition to other components that are likely to reflect receptor heterogeneity (Thuynsma & Dunn, 1991). This pattern is similar to profiles reported previously, and our overall recoveries of about 2.6% of binding activity are also similar to previous reports [see Stephenson and Barnard (1986) and Stephenson (1988)]. The methods used for reconstitution were modified from those that were developed for successful functional reconstitution of impure solubilized preparations (Dunn et al., 1989a,b). One important change was the use of CHAPS rather than β-octylglucoside for solubilization. This change was made mainly on the basis of economy but also because the use of CHAPS throughout purification has previously been shown to preserve most of the expected receptor modulatory sites (Sigel & Barnard, 1984).

Reconstitution by gel filtration was very efficient, with more than 80% of the $GABA_A$ receptors being incorporated into liposomes. The average size of the vesicles (120 ± 25 nm) is similar to previous reports in which similar techniques have been used (Gould et al., 1980; Hirouchi et al., 1987; Bristow & Martin, 1990). Most, if not all, $GABA_A$ receptors were reconstituted in an outside-out orientation with the muscimol binding site(s) accessible from the extravesicular space, as has been previously reported for reconstituted $GABA_A$ receptors (Sigel et al., 1985) and nicotinic acetylcholine receptors [see McNamee et al. (1986)]. In agreement with our previous reports (Dunn et al., 1989a,b) and with the study of Bristow and Martin (1987), the inclusion of native brain lipids is crucial if the receptor is to be reconstituted in a functional form. Purified $GABA_A$ receptors reconstituted with asolectin alone retain their ability to bind both [^{3}H]-muscimol and [^{3}H]flunitrazepam, but do not display any agonist-mediated chloride flux. No attempt has yet been made to determine the nature of the native lipids that stabilize receptor function.

There have been many reports describing heterogeneity in the binding of GABAergic agonists to brain membrane preparations, and these have been interpreted in terms of the presence of multiple independent binding sites or interconvertible states of a single site [see Olsen et al. (1981) and Agey and Dunn (1989)]. After purification and reconstitution, the agonist and benzodiazepine binding sites of the $GABA_A$ receptor are very stable, and this facilitates detailed analysis. As in the native membrane-bound state, the binding of [^{3}H]-muscimol to reconstituted $GABA_A$ receptors was heterogeneous, suggesting the presence of both high-affinity (K_d = 10 nM) and low-affinity (K_d = 0.26 μM) sites. The estimated affinities are almost identical to those of 7 nM and 0.3 μM that we have previously reported for the binding of [^{3}H]-muscimol to bovine brain membranes (Agey & Dunn, 1989). The relative abundance of these two populations of sites in the reconstituted preparation (with the low-affinity sites exceeding the density of high-affinity sites by a factor of about 2.6) is also in good agreement with estimates for the receptor in its native environment. The prolonged procedures for purification and reconstitution do not, therefore, appear to have any significant effect on agonist recognition. The possibility that the high- and low-affinity sites reside on different subtypes cannot be excluded, but, as discussed below, there is considerable evidence to favor the existence of multiple binding sites on individual receptor molecules.

In contrast to the binding of [^{3}H]muscimol, the binding of [^{3}H]flunitrazepam was to an apparently homogeneous population of high-affinity sites. The estimated affinity of these sites (K_d = 5.6 nM) is similar to previous reports for binding to native membranes and purified and reconstituted receptors (Sigel et al., 1985; Bristow & Martin, 1990). Measurement of the relative number of binding sites suggests that there are about twice as many high-affinity sites for muscimol as for flunitrazepam. This is in agreement with our estimates of site densities in bovine brain membranes and in impure reconstituted preparations (Dunn et al., 1989a) although reports for the muscimol:flunitrazepam site ratio in purified receptors have been variable, ranging from 0.35 to 3.8 [reviewed by Stephenson (1988)]. Taken with the [^{3}H]-muscimol binding data above, the present results suggest that, for each benzodiazepine binding site, there are two high-affinity muscimol sites and approximately five low-affinity sites (although, as noted under Results, the latter estimate is

subject to large error). It seems unlikely, though it is not impossible, that there are seven muscimol binding sites on each receptor. On the basis of a pentameric receptor structure and the evidence that, in the homologous nicotinic acetylcholine receptor, agonist binding sites occur at subunit–subunit interfaces [see Dunn and Raftery (1993) and references cited therein], it is possible that a binding site occurs at each of the five homologous interfaces. However, since different subunit combinations are undoubtedly present and these may have different binding characteristics, further investigation of the ratio of binding sites will require the use of more homogeneous receptor preparations.

The chloride flux responses of the purified, reconstituted $GABA_A$ receptors were measured using modifications of the rapid fluorescence method that was previously developed to measure functional responses of impure preparations (Dunn et al., 1989b). One change was the choice of fluorescent probe (from MSQ in the earlier study to MQAE here). MQAE, although an uncharged ester, has been reported to leak less readily from liposomes and cells and to have a higher Stern–Volmer constant for fluorscence quenching by Cl^- (Verkman et al., 1989). We have found no particular advantage of one dye over the other, which may be due to the partial quenching of MQAE fluorescence by potassium gluconate used during reconstitution (see Materials and Methods). A second change was to load MQAE into the vesicles during reconstitution. Previously the dye was loaded by freeze–thaw cycles after reconstitution. However, as has previously been reported for nicotinic acetylcholine receptors reconstituted using asolectin (McNamee et al., 1986), freezing and thawing appear to have an adverse effect on the magnitude of the flux responses, although the internal volume of the vesicles is increased (unpublished observations).

Muscimol stimulated chloride influx in a dose-dependent manner with an EC_{50} of about 0.2 μM, which is similar to earlier estimates of 0.2–1.0 μM obtained using impure preparations (Dunn et al., 1989b). The results of electrophysiological studies have indicated that the opening of the $GABA_A$ receptor channel requires the binding of two agonist molecules (Sakmann et al., 1983). The dose-dependence of fluxes mediated by reconstituted $GABA_A$ receptors did not display such cooperativity, and the estimated Hill coefficient was less than 1. However, it should be noted that in the fluorescence experiments, it is not possible to correct for receptor desensitization which may occur more rapidly at the higher concentrations, resulting in an apparent flattening of the dose-dependence. Evidence supporting interference from desensitization is that, in some preparations, the flux response actually decreased above about 3 μM (see Results). Since this effect was somewhat variable, it has not yet been studied in detail. The $GABA_A$ receptor has been shown to desensitize in electrophysiological experiments (Mathers, 1987), and Cash and Subbaroa (1987) have reported rapid desensitization of $^{36}Cl^-$ flux responses in synaptoneurosome preparations. Since the purified $GABA_A$ receptor also densensitizes, this phenomenon must be an intrinsic property of the $GABA_A$ receptor and does not depend on, for example, phosphorylation which has been proposed to play some role in desensitization of the nicotinic acetylcholine receptor [see Stroud et al. (1990)]. One important consequence of desensitization is that, in flux studies, the apparent EC_{50} values may be considerably lower than the actual concentrations of muscimol that induce a half-maximal effect.

The agreement between the muscimol concentrations that stimulate flux and the affinity of the lower affinity sites makes it tempting to speculate that, as has been suggested previously [see Fischer and Olsen (1986)], occupancy of the low-affinity sites leads to channel activation. However, since the concentrations of muscimol that mediate half-maximal flux responses may be higher than measured EC_{50} values (see above), flux responses may involve sites of even lower affinity (>1 μM) that have been reported previously (Fischer & Olsen, 1986). It should also be noted that the binding studies have been carried out under equilibrium conditions, when the receptor is desensitized, and the affinities of the binding sites may be different from their affinities in the resting state of the receptor. High-affinity sites (K_ds in the nanomolar range) have been measured in most binding studies, and although no function has been directly ascribed to these sites, it is usually assumed that they represent a desensitized state of the receptor [see Fischer and Olsen (1986)]. From the results of their studies of the kinetics of $^{36}Cl^-$ fluxes and the rates of desensitization of $GABA_A$ receptors in rat brain membranes, Cash and Subbarao (1987) have concluded that channel opening and receptor desensitization are independent processes that are mediated by agonist binding to different binding sites. We previously proposed a similar model for the nicotinic acetylcholine receptor in which activation and desensitization are parallel pathways mediated by different binding sites (Dunn & Raftery, 1982a,b, 1993; Raftery et al., 1983). An intriguing possibility is, therefore, that this is a characteristic of all members of the ligand-gated ion channel family. In the case of $GABA_A$ receptors, all subunits must be capable of binding GABA since individual subunits have been reported to form functional GABA-gated chloride channels when expressed from their encoding mRNAs in *Xenopus* oocytes (Blair et al., 1988).

Diazepam shifted the dose-dependence of muscimol-stimulated flux to the left, without affecting the maximal response. This is consistent with previous electrophysiological results (Choi et al., 1981) and with the effects of benzodiazepines on $^{36}Cl^-$ flux responses in synaptoneurosomes (Schwartz et al., 1986; Morrow & Paul, 1988; Yu et al., 1988; Edgar & Schwartz, 1992). As noted above, and reported by us previously (Thuynsma & Dunn, 1991), diazepam does sometimes appear to potentiate the response even at saturating muscimol concentrations. On more detailed analysis, however, this seems to be a consequence of a decrease of the control responses recorded in the absence of diazepam, perhaps due to the competing effect of receptor desensitization (see above).

In an early study in which noise analysis techniques were used to study $GABA_A$ receptor currents in spinal cord and sensory neurons, it was suggested that the main effect of diazepam is to increase the frequency of channel opening (Study & Barker, 1981). More recently, single-channel analysis has indicated that diazepam does not affect single-channel events but rather increases the frequency of bursting activity (Twyman et al., 1989b). These effects were similar to the effects of increasing GABA concentration which would be consistent with a mechanism by which diazepam increases the affinity of the receptor for GABA. In the present study, however, we have found that diazepam has no effect on the equilibrium binding of [^{3}H]muscimol to reconstituted preparations. These results are in agreement with a recent report in which it was shown that, in rat brain synaptoneurosomes, diazepam (10 μM) shifted the EC_{50} for muscimol-stimulated $^{36}Cl^-$ flux from 11.2 to 4.6 μM but did not affect the number or affinity of the binding sites for [^{3}H]muscimol (Edgar & Schwartz, 1992). In most previous studies, benzodiazepines have been reported to have little effect on the binding of GABA

analogues [reviewed by Fischer and Olsen (1986) and Dunn et al. (1993)]. Thus, the molecular mechanisms that underlie the effects of diazepam on flux remain to be established. The potentiation of benzodiazepine binding by GABA analogues is, however, a robust phenomenon [see Fischer and Olsen (1986)], and in the present study, we have found that muscimol (EC_{50} = 0.16 μM) potentiates the binding of [^{3}H]flunitrazepam to purified, reconstituted receptors.

Flux responses of the reconstituted $GABA_A$ receptor were completely inhibited by the competitive antagonist bicuculline and the noncompetitive blocker picrotoxin. Bicuculline inhibited the flux response stimulated by 3 μM muscimol with an EC_{50} of 5.3 μM. Micromolar concentrations of bicuculline have also been reported to inhibit physiological responses to GABA (Frere et al., 1982) and to inhibit muscimol-stimulated flux responses in synaptoneurosomes (Yu & Ho, 1990). Bicuculline also inhibits the binding of [^{3}H]GABA (Olsen & Snowman, 1983), and it appears to be a more potent inhibitor of the low-affinity sites ($IC_{50} \approx 0.8$ μM) than of the high-affinity sites ($IC_{50} \approx 6$ μM). The EC_{50} for inhibition of muscimol-stimulated flux by picrotoxin was 2.5 μM, which is similar to the concentrations that inhibit chloride currents in intact cells [see Newland and Cull-Candy (1992)]. Thus, after purification and reconstitution, $GABA_A$ receptors appear to retain their sensitivity to both a competitive and a noncompetitive antagonist.

The purified $GABA_A$ receptor retains many of the properties of a native receptor. Unfortunately, however, as we reported earlier for an impure preparation (Dunn et al., 1989), one aspect of receptor function that has not been successfully reconstituted is the expected barbiturate modulation. We have been unable to detect pentobarbital stimulation of chloride flux, potentiation of agonist-induced flux, or potentiation of [^{3}H]flunitrazepam binding. It has previously been reported that the barbiturate sites are particularly labile during solubilization and are critically dependent on the presence of exogenous lipids during purification [see Stephenson et al. (1986)]. However, Bristow and Martin (1990) have reported successful reconstitution of barbiturate responses of a purified receptor preparation that had been solubilized in the presence of natural brain lipids and cholesterol hemisuccinate. It may be possible, therefore, to preserve barbiturate responses by altering the conditions used for solubilization.

In conclusion, $GABA_A$ receptors have been purified by benzodiazepine affinity chromatography and reconstituted into phospholipid vesicles with retention of many, though not all, of their native properties. Further analysis of ligand binding and ion flux responses will provide further information of the mechanisms that underlie activation and modulation of this important receptor. In the future, it may be possible to use similar techniques to study the properties of single subtypes of $GABA_A$ receptors purified, for example, by immunoaffinity chromatography [see Stephenson (1992)]. This will permit analysis of possible differences in the ligand binding and functional properties of native $GABA_A$ receptors having different subunit compositions.

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