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IMMOBILIZED $GABA_A$ RECEPTORS AND THEIR LIGAND BINDING CHARACTERISTICS

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ConA-sepharose and polylysine-agarose beads effectively bound detergent-solubilized $GABA_A$ receptors from rat cerebrocortical membranes. The immobilized receptors showed a single class of high affinity binding sites specific for flunitrazepam or muscimol and displayed GABA-stimulated flunitrazepam binding. Maximal binding capacities of the ConA-immobilized receptor for the ligands were about three times greater than those of the polylysine-immobilized receptors. The relative affinities for each of the ligands were not affected by the method of receptor immobilization. The dissociation constants for muscimol of these immobilized receptors were somewhat dependent on the solubilizing agents used, but were considerably lower than those measured using extensively dialyzed rat cerebrocortical membranes.

Affinity resins have been used extensively for purification of biological macromolecules since the pioneering work by P.Cuatrecasas (1,2). In particular, lectin-sepharose gels have been used in purification of various membrane receptors by taking advantage of the high affinity binding of lectins to specific carbohydrate structures in glycoproteins (3-8). Not much attention, however, has been directed to the ligand binding properties of the lectin gel-receptor complexes, which are potentially useful in studying the properties of solubilized receptors free from tightly-bound endogenous ligands and modulators. We have examined here the immobilization of $GABA_A$ receptors using ConA-sepharose and polylysine-agarose beads, since the receptors are known to contain carbohydrate moieties (9) and also to interact with anion exchangers (10,11).

Abbreviations: GABA, gamma-aminobutyric acid. Kd, dissociation constant. SDS, sodium dodecyl sulfate.

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METHODS AND MATERIALS

Rat cerebrocortical membranes were prepared from brain cortices of male Sprague-Dawley rats (150-180 g) using procedures described elsewhere (12), with the following changes. The sucrose homogenizing buffer contained 300mM sucrose, 10mM Hepes, pH 7.4, 0.1mM EDTA, 1μM pepstatin A, 3 μg/ml leupeptin, 0.2mM diisopropyl fluorophosphate, and 2mM benzamidine. Synaptosomes were lysed by suspending in 20 volumes of 5mM Tris/HCl, pH 8.0, containing the protease inhibitors (diisopropyl fluorophosphate was omitted from this step), and subjecting for 5 min to a circular sonicator (Model G112spit from Lab Supplies, Inc.). The membranes were resuspended in the sucrose homogenization buffer to a final concentration of 10 mg protein/ml. Membranes were dialyzed for 20 hr against 1000 volumes of a medium containing 20 mM potassium phosphate, pH 7.5, 100 mM KCl, and 0.1 mM EDTA for use in binding assays (12). Membranes were diluted with 10 volumes of a medium containing 100 mM NaCl, 20 mM Hepes, pH 7.4, the protease inhibitors, and 0.3% polidocanol (polyoxyethylene 9 lauryl ether) or 2.5% CHAPS for solubilization of receptors. This suspension was incubated for 30 min on ice, and then centrifuged at 100,000 x g for 80 min in a Beckman 60 Ti rotor. The supernatant containing solubilized membrane components was incubated with a 1/6 volume of packed ConA-sepharose or polylysine-agarose beads for 90 min at 4^{0} while mixing gently. The beads had been pre-equilibrated with a solution containing 10 mM Tris/HCl, pH 7.4, 120 mM NaCl, 5 mM KCl, 0.05 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.15% polidocanol (Buffer A). Following incubation with the membrane extract, the beads were washed three times with Buffer B containing 0.015% polidocanol and the same inorganic ingredients as Buffer A. For ligand binding studies, a 50 μl aliquot of a 30% suspension of the beads was diluted with 50 μl of Buffer B and then mixed with 100 μl of 50 mM Tris/HCl, pH 7.4, with the ligands of interest at various concentrations in 1.5 ml polyethylene tubes. The mixtures were incubated on a Nutator (Adams) at 23^{0} for 40 min, and filtered over a Whatman GF/B glass fiber filter. Transfer of the beads from tubes to filters was facilitated by washing the polyethylene tubes twice with 1 ml of 50mM Tris/HCl, pH 7.4. Filters were washed three times with 5 ml of the Tris buffer. When protein was measured, 100 μl aliquots of 30% bead suspensions were centrifuged and after removal of the supernatant, 200 μl of a solution containing 2% SDS and 2M NaCl were mixed with the beads. The mixtures were incubated for 1 hr at 37^{0} and protein in the supernatant was determined by use of the Lowry method using bovine serum albumin for calibration (14). The total volume of the supernatant (including the fluid trapped in beads) was estimated by dilution of the $^{86}Rb^{+}$ specific activity of the SDS-NaCl solution. The amount of protein adsorbed to the beads was calculated by the difference between that measured before and after incubation of the beads with the detergent-extracts of membranes. Analysis of binding data was carried out using computer programs of linear and nonlinear regressions for one or multiple classes of binding sites. The reported values for binding capacities and dissociation constants in the study were obtained using a model for a single class of sites, which best fit the data in this study.

RESULTS

Binding of [^{3}H]flunitrazepam and [^{3}H]muscimol to ConA-sepharose or polylysine-agarose alone was low (less than 12 fmol/100 μl of 30% gel suspension) and non-specific as assessed by its non-displacement by excess non-radiolabeled diazepam and muscimol.

Table 1

Acquisition of [^{3}H]flunitrazepam binding activity by ConA-sepharose upon incubation with polidocanol extracts of rat brain cortical membranes

	[^{3}H]FLUNITRAZEPAM BINDING cpm/100 μl of 30% suspension
ConA-sepharose	745
ConA-sepharose after equilibration with membrane extracts	15,300
After treatment with α-methyl-mannoside (500 mM) overnight	4,460

Suspensions of ConA-sepharose (30%) were prepared before and after equilibration with polidocanol extracts of the cortical membranes, and after treatment of the equilibrated beads with 0.5 M α-methylmannoside overnight at 4°C. Binding activity of [^{3}H]flunitrazepam was measured by mixing 100 μl of the bead suspension with 100 μl of 50 mM Tris/HCl buffer, pH 7.4, containing [^{3}H]flunitrazepam, at a final concentration of 6 nM. Other experimental details were described under "Methods and Materials."

Beads that had been incubated with the polidocanol solubilized membrane fraction exhibited benzodiazepine binding that was nearly 20 times greater than background. This bound radioactive ligand was displaced by excess non-radiolabeled diazepam. Treatment of the beads with 0.5M α-methylmannoside released almost 80% of the binding activity, indicating that adsorption of $GABA_A$ receptors (as monitored here by benzodiazepine binding) to ConA-sepharose was likely via the mannosyl groups of the receptor glycoprotein (Table 1). The rate of the receptor adsorption to ConA-sepharose was slower ($t_{1/2}$ = 12 min) than to polylysine-agarose ($t_{1/2}$ = 3 min). In both cases, nearly 90% of the benzodiazepine binding activity in the solubilized fraction was transferred to the solid supports within 90 min. $GABA_A$ receptors solubilized with CHAPS, a zwitterionic detergent, similarly interacted with the solid-phase supports, displaying the same extents and rates of adsorption.

Equilibrium binding characteristics for flunitrazepam and muscimol of the immobilized receptors were examined using Scatchard analysis (Table 2). The B_{max} values for the two ligands were about three times greater in the ConA-sepharose than the polylysine-agarose preparations. The ligand affinities, on the other hand, were marginally and variably affected by the solid phase supports. The K_d value for muscimol of the receptor ConA-sepharose complexes was 10-30% lower, depending on solubilization conditions, but for

Table 2

Comparison of binding characteristics of muscimol and flunitrazepam to $GABA_A$ receptors immobilized with ConA-sepharose, polylysine-agarose, and in rat brain cortical membranes

	Muscimol		Flunitrazepam	
	Kd[a]	Bmax[b]	Kd	Bmax
Receptor-ConA-Sepharose				
1 Polidocanol solubilized receptors	2.96±0.19	25.4 ±0.98	5.56±0.52	10.32±0.92
2 CHAPS solubilized receptors	4.92±0.57	24.5 ±1.25	4.97±0.50	10.5 ±0.53
Receptor-Polylysine-Agarose				
1 Polidocanol solubilized receptors	3.33±0.17	9.11±0.28	4.35±0.21	3.91±0.11
2 CHAPS solubilized receptors	6.69±0.48	8.56±0.39	3.97±0.35	3.83±0.57
Native Membrane After Extensive Dialysis	9.14±0.85	2.52±0.31	0.76±0.02	1.08±0.02

[a] nM.
[b] pmole/mg protein.

Rat cortical membranes were extracted with 0.3% polidocanol or 2.5% CHAPS. ConA-sepharose and polylysine-agarose beads were equilibrated with the membrane extracts, and then washed three times with Buffer B containing 0.015% polidocanol. Binding activity was measured in the presence of 50 µl of 30% bead suspension and 150 µl 50 mM Tris/HCl buffer, pH 7.5, containing the ligands at various concentrations (0.5 to 60 nM) as described under the "Methods and Materials" section. Non-specific binding was obtained in the presence of 4 µM unlabelled muscimol or diazepam and used to calculate specific binding activity. Binding constants with standard error were computed from Scatchard analysis of the data using a computer program of linear regression and represent the average of two experiments.

flunitrazepam about 20% greater than the corresponding values of the polylysine-agarose immobilized receptors (Table 2). Interestingly, CHAPS selectively increased the K_d value for muscimol of the immobilized receptors relative to that observed when polidocanol was used for solubilization. The CHAPS solubilized receptors, when immobilized with polylysine-agarose beads, showed a K_d for muscimol of 6.69±0.48nM, which was twice that using the polidocanol solubilized receptors (3.33±0.17nM). Similarly, the K_d values for muscimol of the CHAPS- and polidocanol-solubilized receptors, upon immobilization with ConA-sepharose beads, were 4.92±0.57 and 2.96±0.19nM, respectively. The detergent effects on the K_d for flunitrazepam were not marked; i.e. 4.97±0.50 and 5.56±0.52nM for the CHAPS- and polidocanol-solubilized receptors, respectively, with the ConA-sepharose support, and 3.97±0.35 and 4.35±0.21nM, respectively, with the polylysine-agarose support. It should be noted that the

Table 3

Stimulation by GABA of flunitrazepam binding activity to immobilized $GABA_A$ receptors

	[^{3}H]Flunitrazepam Binding cpm/50 µl 30% Suspension	
	Control	+GABA (100µM)
Receptor-ConA-Sepharose		
1 Polidocanol solubilized receptors	4883±184	6394± 78
2 CHAPS solubilized receptors	4324±100	6091±206
Receptor-Polylysine-Agarose		
1 Polidocanol solubilized receptors	5768±258	7599± 78
2 CHAPS solubilized receptors	5499±337	7147±325

$GABA_A$ receptors solubilized from rat brain cortical membranes were immobilized with ConA-sepharose or polylysine-agarose as described in Table 2. Binding activity was measured in triplicate in the presence of 50 µl of the equilibrated bead suspension and 150 µl of 50 mM Tris/HCl buffer, pH 7.4, containing 6 nM [^{3}H]flunitrazepam in the presence or absence of 100 µM GABA.

immobilized receptors were extensively washed and equilibrated with Buffer B containing 0.015% polidocanol regardless of whether the receptors were solubilized with CHAPS or polidocanol. The CHAPS effect on the affinity of muscimol, therefore, could be attributed either to detergent very tightly bound to the immobilized complex and/or to irreversible changes in the receptors during detergent solubilization. In rat cerebrocortical membranes that had been freeze-thawed and dialyzed extensively, the K_d for flunitrazepam was 0.76±0.02nM but was 4-5nM for the immobilized receptors (see Table 2). In contrast, the K_d for muscimol in the membranes was 9.14±0.85nM, which is 2-3 times greater than that of the immobilized receptors. The B_{max} ratio of muscimol to flunitrazepam was 2.3 in the dialyzed membranes and in all the immobilized receptor preparations. This may indicate the presence of tightly bound endogenous ligands or modulators even after extensive dialysis of the membranes. Finally, binding of [^{3}H]flunitrazepam to the immobilized receptors was stimulated by GABA (100µM) as observed in native membranes (15,16) (Table 3). The degree of stimulation ranged from 30-40% and was unaffected by either type of detergent used for solubilization or immobilizing support.

DISCUSSION

The solid phase supports, ConA-sepharose or polylysine agarose, did not markedly influence affinities of the immobilized GABA receptors for muscimol or flunitrazepam. This suggests minimal

involvement of the carbohydrate moiety of $GABA_A$ receptors in the ligand binding process. By contrast, the affinity of the immobilized receptors for muscimol was affected by the choice of solubilizing agent. CHAPS, an ionic detergent, increased the K_d value for muscimol over that measured using polidocanol for solubilization, but the K_d value for flunitrazepam was unaffected. One may postulate that non-ionic detergents would be preferable to ionic ones in preserving the binding of membrane receptors for ionic ligands, such as GABA and other amino acids. Generally, the muscimol K_d value of the immobilized receptors depended somewhat on the choice of solubilizing agent, but was considerably lower than that observed in extensively dialyzed rat cerebrocortical membranes. It appears that the radioligand binding in native membranes, even after extensive dialysis, was hindered by the presence of endogenous ligands tightly bound to the receptors or released during prolonged equilibration periods. Other difficulties associated with ligand binding studies in native membranes involve probable changes in ligand concentrations due to neurotransmitter scavenging activities inherent in various neuronal membranes, and possible variations in ligand affinities induced by intracellular modulators (17). These difficulties may be circumvented by use of lectin-sepharose immobilized receptors. Furthermore, the immobilized receptors would be useful in studying properties of solubilized membrane receptors without interference of bulk detergents, the level of which could be readily manipulated, and in monitoring the degrees of adsorption and elution of solubilized receptors during receptor purification. It is likely that other membrane receptors could be examined in the same manner, since membrane receptors for glycine, acetylcholine (muscarinic), dopamine, and 5-hydroxytryptamine are known to contain potential N-linked carbohydrate sites (Asn-x-thr or-ser) in their extracellular domains (18-21).

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The polypeptide of mol wt 60,000 was immunoprecipitated by polyclonal Ab 42-43 to vWF and MAb 418 (Fig. 2B). No band was observed following immunoprecipitation with non-immune rabbit IgG or with an unrelated MAb to vWF (MAb 345) (Fig. 2B).

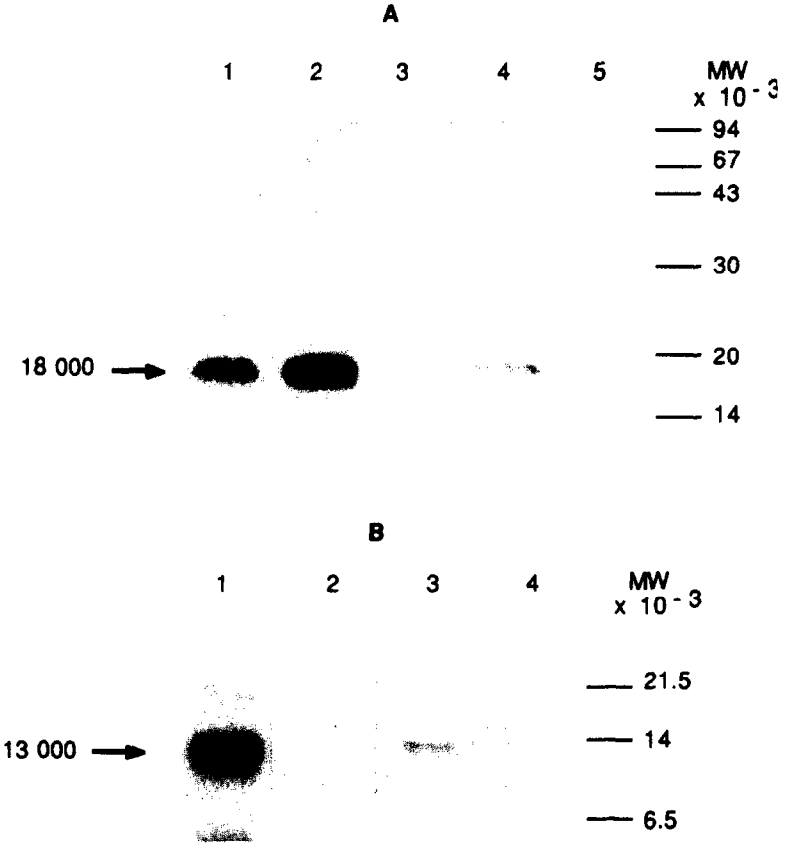

Figure 3 :

A - **Electrophoresis (0.1% SDS-15% polyacrylamide) of <u>in vitro</u> translation products of pBS-TG3522 linearized by BamHI.**
The restriction site BamHI is localized in the vWF cDNA insert 637 bp after the translation initiation site (ATG) (Fig. 1C).
Lane 1 : Translation products labeled with ^{3}H-leucine (150 000 cpm).
Lane 2 : Translation products immunoprecipitated with Ab 42-43 (IgG).
Lane 3 : Translation products treated with non-immune rabbit IgG.
Lane 4 : Translation products immunoprecipitated by MAb 418 (IgG).
Lane 5 : Translation products treated with MAb 345 (IgG).
MW : Molecular weight markers.

B - **Electrophoresis (0.1% SDS-18% polyacrylamide) of <u>in vitro</u> translation products of mRNA transcribed <u>in vitro</u> from pBS-TG3522 linearized by PvuI.**
The restriction site PvuI is localized in the vWF cDNA insert 538 bp after the translation initiation site (ATG) (Fig. 1C).
Lane 1 : Translation products immunoprecipitated with Ab 42-43 (IgG).
Lane 2 : Translation products treated with non-immune rabbit IgG.
Lane 3 : Translation products immunoprecipitated with MAb 418 (IgG).
Lane 4 : Translation products treated with MAb 345 (IgG).
MW : Molecular weight markers.