Vol. 172, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1990 Pages 22-27

SOLUBILIZATION AND PARTIAL PURIFICATION OF $GABA_B$ RECEPTOR FROM BOVINE BRAIN

Yoshiyuki Ohmori and Kinya Kuriyama*

Department of Pharmacology, Kyoto Prefectural University of Medicine,

Kawaramachi-Hirokoji, Kamikyo-Ku, Kyoto 602, Japan

Received August 24, 1990

SUMMARY. γ-Aminobutyric acid $(GABA)_B$ receptor has been solubilized and partially purified by an affinity column chromatography. $GABA_B$ receptor was solubilized by 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) in the presence of asolectin. The solubilized $GABA_B$ receptor was adsorbed on baclofen-coupled epoxy-activated Sepharose 6B. The affinity matrix adsorbed 80 % of the solubilized [^{3}H]GABA binding activity to $GABA_B$ receptor, and approximately 75 % of the adsorbed activity could be eluted with 1 M KCl. $GABA_B$ receptor binding in the fraction eluted from affinity column was displaced by GABA, baclofen and 2-hydroxy saclofen in a dose-dependent manner. Furthermore, the purified $GABA_B$ receptor showed approximately 2800-fold purification as compared with the original solubilized fraction and possessed the specific binding activity of 17.68 p mol/mg of protein. This binding consisted of a single binding site with a dissociation constant of 64.4 nM. The present results indicate that affinity column chromatographic procedures using baclofen-coupled epoxy-activated Sepharose 6B are suitable for the partial purification of $GABA_B$ receptor from cerebral tissues.

$GABA_B$ receptor in the brain is known to mediate the inhibitions of adenylate cyclase activity (1), formation of inositolphosphates (2,3) and calcium influx (4) via the activation of an inhibitory GTP-binding protein. It is not yet clear, however, whether or not these reactions occur sequentially or independently and which component is directly coupled with $GABA_B$ receptor. In addition, molecular pharmacological characterization of $GABA_B$ receptor is not well established. In order to clarify these unknown properties of cerebral $GABA_B$ receptor, the purification of $GABA_B$ receptor must be important. Recently affinity chromatography has proven to be a successful technique for the purification of membrane-bound neurotransmitter receptors such as muscarinic cholinergic (5) and α-adrenergic receptors (6).

Baclofen (β-(4-chloro-3-phenyl)γ-aminobutyric acid) is known as a potent and selective agonist for $GABA_B$ receptor. In this study, we have, therefore, attempted to develop an affinity gel using this compound as an immobilized ligand and to apply the affinity gel to the purification of $GABA_B$ receptor.

* To whom correspondence should be addressed.

Abbreviations : GABA, γ-aminobutyric acid; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate.

0006-291X/90 $1.50

MATERIALS AND METHODS

Materials [2,3-^{3}H]GABA (71.0 Ci/mmol) was purchased from New England Nuclear, Boston, U.S.A. Baclofen was a gift of Ciba-Geigy. CHAPS was obtained from Dojin Chemical, Kumamoto, Japan. Epoxy-activated Sepharose 6B was purchased from Pharmacia Fine Chemicals.

Preparation of baclofen-epoxy-activated Sepharose 6B Epoxy-activated Sepharose 6B (1.0 g) was suspended in 10 ml of distilled water and allowed to stand for 10 min. The moist gel was transferred to a glass filter and washed extensively with 100 ml of distilled water, and it was finally washed with 100 ml of 0.1 M $NaHCO_3$. Baclofen (0.1 g) was dissolved in 5 ml of 0.1 N NaOH, and pH of the solution was adjusted to 13. The epoxy-activated Sepharose 6B gel prepared as described above was transferred to the baclofen solution and shaken overnight at 37 C to react the aliphatic epoxy group of the Sepharose with the amino group of the ligand.

Membrane Solubilization Frozen crude synaptic membrane of the bovine cerebral cortex prepared by the method of Zukin et al.(7) was thawed and centrifuged at 48,000 x g for 20 min at 4 C. The pellet was suspended in 10 volumes of 50 mM Tris-HCl buffer (pH 7.4) containing 1.2 % CHAPS, 0.2 % asolectin and 40 μg/ml bacitracin. The suspension was incubated at 2 C for 90 min and centrifuged for 120 min at 105,000 x g. The clear supernatant was then saved as the solubilized preparation.

Affinity Column Chromatography Baclofen-epoxy-activated Sepharose 6B (30 ml) was packed into a 1 x 40 cm glass column and equilibrated with 50 mM Tris-HCl buffer containing 0.2 % cholic acid and 0.02 % asolectin (buffer A). The solubilized preparation (90 ml) was applied to the affinity column, and it was washed with 50 ml of buffer A at the flow rate of 16.6 ml/h. The receptor protein bound to the affinity gel was then eluted by 1 M KCl.

Assay of $GABA_B$ Receptor Binding Each receptor preparation was incubated with 12.5 nM [^{3}H]GABA at 4 C for 60 min, and bound ligand was separated from its free form by polyethyleneglycol precipitation method (8). The specifically bound [^{3}H]GABA to $GABA_B$ receptor was defined as the total binding minus the binding obtained in the presence of 1 mM baclofen.

Protein Assay Protein concentrations were determined by the method of Bradford (9), using bovine serum albumin as a standard.

RESULTS

Affinity Column Chromatography of Cerebral $GABA_B$ Receptor

Baclofen, which possesses a high affinity for $GABA_B$ receptor, was immobilized on epoxy-activated Sepharose 6B via an amino group. The presumed structure of the baclofen-coupled Sepharose is shown in Fig. 1. The prepared gel was stable for at least 6 months at 4 C. A chromatographic profile of the solubilized $GABA_B$ receptor on baclofen-Sepharose is shown in Fig. 2. The treatment of synaptic membrane from the bovine cerebral cortex with 1.2 % CHAPS

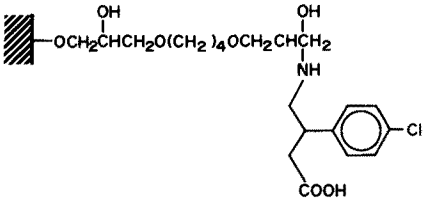

Fig.1. Presumed structure of the baclofen-epoxy-activated Sepharose 6B.

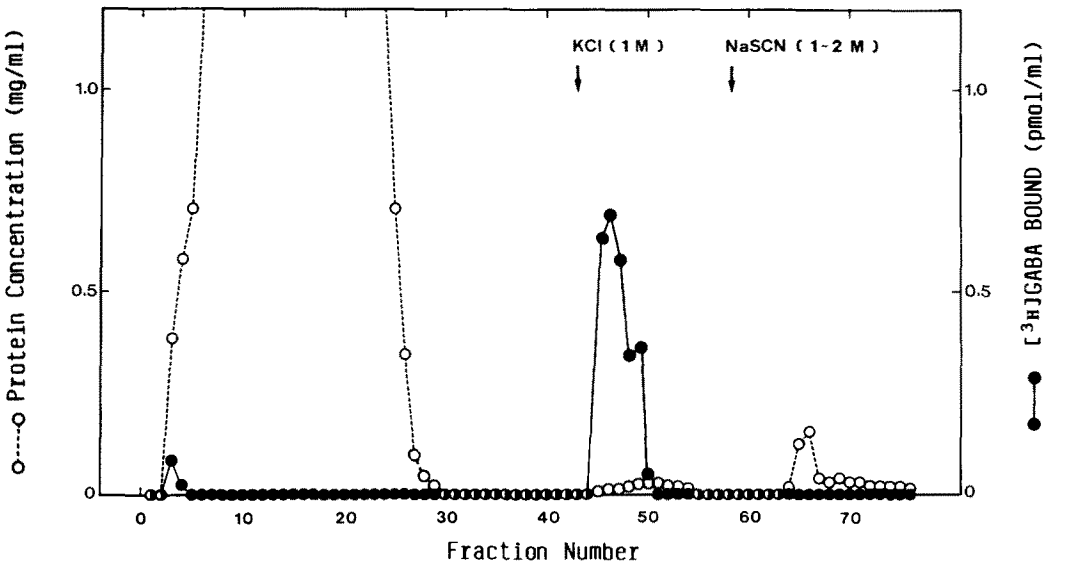

Fig.2. Affinity column chromatography of CHAPS/asolectin-solubilized preparation of $GABA_B$ receptor from bovine cerebral cortex on baclofen-coupled Sepharose 6B. The solubilized $GABA_B$ receptor preparation was applied to a 1 x 40 cm column at 4°C. [^{3}H]GABA binding was determined as described under "MATERIALS AND METHODS". The arrows indicate the initiation of each elution buffer application.

resulted in the solubilization of 30-40 % of $GABA_B$ receptor in the membrane, but no enrichment of the binding activity in the solubilized fraction was found. On the other hand, more than 80 % of $GABA_B$ receptor binding activity was adsorbed to the affinity gel of baclofen-Sepharose. Washing with equilibrating buffer caused the removal of most of the applied protein as indicated by absorbance at 280 nm. The subsequent application of 1 M KCl to the affinity column resulted in the elution of approximately 2800-fold purified $GABA_B$ receptor with a recovery of 75 %. A typical purification scheme is summarized in Table I. Although some proteins were also eluted by the post-application of a linear gradient of NaSCN, no significant [^{3}H]GABA binding was not detected in these fractions.

Properties of Purified $GABA_B$ Receptor by Affinity Column Chromatography

[^{3}H]GABA binding to the purified $GABA_B$ receptor was saturable with approximately 100 nM of the ligand. As shown in Fig. 3, The Scatchard analysis

Table 1. Summary on affinity chromatographic separation of CHAPS-solubilized $GABA_B$ receptor from bovine cerebral cortex

step	Total protein (mg)	Total activity (p mol)	Specific activity (p mol/mg)	Recovery (%)	Purification (- fold)
Solubilized	345.6	3.51	0.01	100	1
Baclofen-Sepharose 6B	0.09	2.64	28.7	75	2828

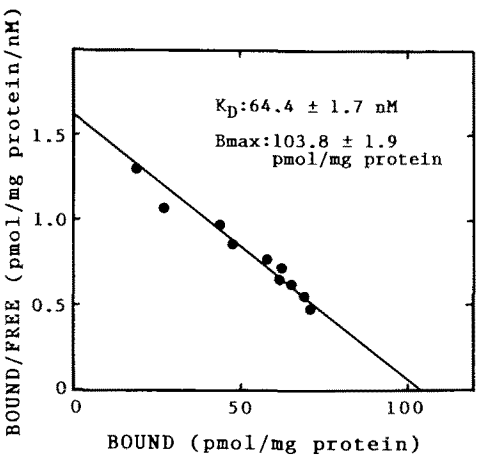

Fig.3. Saturation isotherm and Scatchard plot for specific binding of [^{3}H]GABA to $GABA_B$ receptor. [^{3}H]GABA binding was determined as described under "MATERIALS AND METHODS". The K_D and Bmax values were 64.4 nM and 103.8 p mol/mg of protein, respectively. Each value is the mean of three separate determinations.

of [^{3}H]GABA binding to $GABA_B$ receptor showed a linear and single line, suggesting the presence of a single class of binding sites in the preparation. The K_D and Bmax values were estimated to be approximately 64.4 nM and 103.8 p mol/mg of protein, respectively. The purified receptor was stable and no loss in its binding activity was found during storage at 4 C for at least 20 days. The binding was specifically displaced by GABA and baclofen in a concentration-dependent manner as shown in Fig. 4. 2-Hydroxy saclofen, which is known to be a $GABA_B$ antagonist, also displaced the binding, although its efficacy was found to be lower than those of GABA and baclofen.

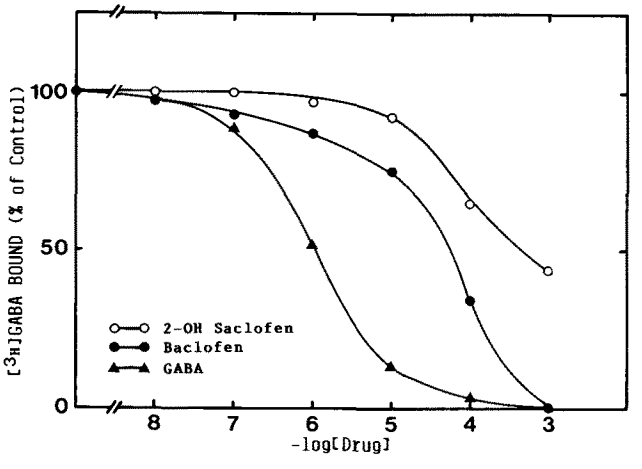

Fig.4. Displacement by various GABAergic drugs of [^{3}H]GABA binding to purified $GABA_B$ receptor. [^{3}H]GABA binding was determined as described under "MATERIALS AND METHODS". Total binding activity in these preparations (100 % Control) was approximately 14.03 p mol/mg of protein.

DISCUSSION

Among various affinity gels coupled with compounds possessing the selective affinity for $GABA_B$ receptor examined, the baclofen-epoxy-activated Sepharose 6B demonstrated successful achievement with regard to adsorption of the soluble $GABA_B$ receptor. Approximately 2800-fold purification was obtained by the use of a single affinity chromatography step, with an overall recovery of 75 % as compared with the solubilized fraction applied. The binding capacity of the affinity matrix was relatively high and more than 80 % of the applied soluble $GABA_B$ receptor could be adsorbed on the affinity column. In contrast, no significant adsorption of [^{3}H]GABA binding activity occurred when the solubilized receptor was passed over epoxy-activated Sepharose 6B in which the activated groups had been blocked with ethanolamine. Furthermore, 75 % of the adsorbed $GABA_B$ receptor could be eluted by 1 M KCl. These results suggest that the applied soluble $GABA_B$ receptor does not adsorb non-specifically to the matrix of Sepharose 6B but recognizes baclofen, which is used as an immobilized ligand, within affinity gel. In this study, it has been found that $GABA_B$ receptors adsorbed to the affinity gel can be also eluted by GABA or baclofen. These elution procedures, however, were found to be unsuitable, since the complete removal of GABA or baclofen used for the elution was not possible even after the extensive dialysis and/or ion exchange column chromatography.

The partially purified receptor obtained in this study fulfilled the following pharmacological criteria for $GABA_B$ receptor. (a) The binding of [^{3}H]GABA was specifically displaced by not only GABA, baclofen but also 2-hydroxy saclofen which was reported to be a specific antagonist for $GABA_B$ receptor (10). Although it was shown that the efficacy of 2-hydroxy saclofen for displacing $GABA_B$ receptor binding was significantly lower than those of GABA and baclofen, one possible reason for such a low potency of 2-hydroxy saclofen may be that this antagonist has very low affinity for $GABA_B$ receptor. (b) The equilibrium dissociation constant (K_D) was in a range similar to that of the membrane-bound receptor which was reported previously (11). The purified $GABA_B$ receptor preparation obtained in this study showed several protein bands on sodium dodecyl sulfate polyacrylamide gel electrophoresis. Therefore, further purification steps are obviously necessary to obtain its homogeneity. The usefulness of the affinity column chromatographic procedures reported herein, however, should be emphasized as an important initial step in such future studies.

Acknowledgment

This work was supported, in part, by research grants from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1. Wojcik,W.J., and Neff,N.H. (1984) Mol.Pharmacol. 25, 24-28.
2. Crawford,M.L.A., and Young,J.M. (1988) J.Neurochem. 51, 1441-1447.
3. Ohmori,Y., and Kuriyama,K. (1989) Neurochem.Int. 15, 359-363.
4. Holz,G.G., Rane,S.G., and Dunlap,K. (1986) Nature 319, 670-672.
5. Haga,K., and Haga,T. (1985) J.Biol.Chem. 260, 7927-7935.
6. Lomasney,J.W., Leeb-Lundberg,L.M.F., Cotecchia,S., Regan,J.W., DeBernardis,J.F., Caron,M.G., and Lefkowitz,R.J. (1986) J.Biol.Chem. 261, 7710-7716.
7. Zukin,S.R., Young,A.B., and Snyder,S.H. (1974) Proc.natn.Acad.Sci.U.S.A. 71, 4802-4807.
8. Ohmori,Y., Hirouchi,M., Taguchi,J., and Kuriyama,K. (1990) J.Neurochem. 54, 80-85.
9. Bradford,M.M. (1976) Anal.Biochem. 72, 248-254.
10. Kerr,D.I.B., Ong,J., Johnston,G.A.R., Abbenante,J., and Prager,R.H. (1988) Neurosci.Lett. 92, 92-96.
11. Bowery,N.G., Hill,D.R., and Hudson,A.L. (1983) Br.J.Pharmacol. 78, 191-206.