Multiple Receptor Liquid Chromatographic Stationary Phases: The Co-Immobilization of Nicotinic Receptors, γ-Amino-Butyric Acid Receptors, and *N*-Methyl D-Aspartate Receptors

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INTRODUCTION

Drugs active in the central nervous system (CNS) exert their pharmacologic activities by affecting a number of CNS receptors. These receptors include a variety of neurotransmitter receptors classified as ligand-gated ion channels (LGIC), which are composed of three groups of receptors: the nicotinic, excitatory amino acid, and ATP purinergic receptors (1). Nicotinic (NCT), γ-aminobutyrate ($GABA_A$), glycine, and 5-HT receptors are all members of the nicotinic receptor family, whereas glutamate, *N*-methyl D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazoleproplonic acide (AMPA), and kainate receptors are members of the excitatory amino acid receptor family.

Although there are great differences between these receptors, there are also significant overlaps. As such, a drug specifically designed for one receptor subtype may also elicit a response at another. For example, risperidone binds to both the dopamine (D_2) and the 5-HT receptors (2). At the present time, it is difficult to determine the effect of a drug on the individual members of a multiple receptor system. However, the development of receptor-based liquid chromatographic stationary phases has opened up the possibility for the development of on-line multiple-receptor screens.

Previous studies have reported the immobilization of the α4/β2 and α3/β4 NCT receptors to create NCT receptor-based stationary phases (NCT-SPs; References 3,4). The NCT-SPs were used in liquid chromatographic studies employing known NCT receptor ligands. The resulting NCT-SPs could not only be used to assess ligand binding affinities, they could also be used to determine differences between the two receptor subtypes (4).

The ability to use rat forebrain tissue to prepare a functioning NCT-SP raises the possibility of developing a liquid stationary phase containing more than one functioning receptor, a multiple-receptor stationary phase (MR-SP). The object of this study was the development of a MR-SP containing different members of the LGIC superfamily. Solubilized rat forebrain tissue was immobilized on an immobilized artificial membrane liquid chromatographic stationary phase using previously described techniques (4), and binding affinities were obtained using frontal chromatography techniques. On-line competitive binding experiments were performed using marker and displacer ligands for the NCT, $GABA_A$, and NMDA receptors.

MATERIALS AND METHODS

Materials

[^{3}H]-EB, [^{3}H]-FTZ, and [^{3}H]-MK-801 were purchased from Amersham Life Science Products (Boston, MA). NMDA, (–)-NCT, benzamidine, salts, cholate, leupeptin, phenyl methyl sulfonyl fluoride (PMSF), EDTA, and Trizma were purchased from Sigma Chemical Co. (St. Louis, MO). Immobilized Artificial Membrane PC Stationary Phase (IAM) was obtained from Regis Chemical Co. (Morton Grove, IL). Rat brains were purchased from Pel-Freez Biologicals (Rogers, AR).

Preparation of MR-SPs

Solubilization of Rat Brain Tissue

Four rat brains were homogenized in 30 mL of Tris-HCl buffer [50 mM, pH 7.4] (Buffer A) containing 5 mM EDTA, 3 mM benzamidine, and 0.2 mM PMSF for 3 × 20 s using a Polytron homogenizer (Brinkman Intruments, Westbury, NY) at setting 6, intermittently placing the tissue in an ice bath. The mixture was centrifuged for 10 min at 4 °C at 32,000 × g, and the supernatant was then discarded. The pellet was suspended in 10 mL of Buffer A containing 100 mM NaCl, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 5 mM KCl, 2% sodium cholate, and 10 μg/ml leupeptin. The resulting mixture was stirred for 12h at 4 °C and centrifuged at 32,000 × g.

Immobilization of Solubilized Receptors

The supernatant (receptor–cholate suspension) was mixed with 200 mg of dried IAM-PC packing material and stirred gently for 1 h at 25°C, transferred into dialysis tubing, and dialyzed for 48 h at 4°C against 3 × 600 mL of Buffer A containing 5 mM EDTA, 100 mM NaCl, 0.1 mM $CaCl_2$, and 0.1 mM PMSF.

The resulting mixture was centrifuged for 3 min at 4°C at 32,000 × g, and the supernatant was then discarded. The pellet (MR-SP) was washed with Buffer A and centrifuged. This

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process was repeated until the supernatant was clear. The MR-SP was then collected.

Determination of Binding Affinities Using Frontal Chromatography

Chromatographic Procedures

The MR-SP (200 mg) was packed into a HR 5/2 glass column (Amersham Pharmacia Biotech, Uppsala, Sweden) to yield a 150 mm × 5 mm (ID) chromatographic bed. The column was then connected to a P1000 isocratic HPLC pump (Thermo Separations, San Jose, CA). The mobile phase consisted Buffer A delivered at 0.4 mL/min at room temperature. Detection of the [^{3}H]-marker ligands was accomplished using an on-line scintillation detector (525 TR, Packard Instruments, Meriden, CT).

In the chromatographic studies, a 50-mL sample Superloop (Amersham Pharmacia Biotech) was used to apply a series of [^{3}H]-marker ligand concentrations through the MR-SP column to obtain elution profiles showing a front and plateau regions. The chromatographic data was summed up in 1-min intervals and smoothed using the Microsoft Excel program with a 10-point moving average.

Data Analysis

The dissociation constants, K_d, for the marker and displacer ligands were calculated using a previously described approach (3, 4). The experimental approach is based upon the effect of escalating concentrations of a competitive binding ligand on the retention volume of a marker ligand that is specific for the target receptor. For example, if the NCT receptor is the target, epibatidine (EB) can be used as the marker ligand (4, 5). Then the association constants of EB, K_{EB}, and the test drug, K_{drug}, as well as the number of the active binding sites of the immobilized NCT receptor, P, can be calculated using Equations 1 and 2.

$$(V_{max} - V)^{-1} = (1 + [EB]\,K_{EB})(V_{min}\,[P]\,K_{EB})^{-1} + (1 + [EB]\,K_{EB})^2\,(V_{min}\,[P]\,K_{EB}\,K_{drug})^{-1}\,[drug]^{-1} \quad (1)$$

$$(V - V_{min})^{-1} = (V_{min}\,[P]\,K_{EB})^{-1} + (V_{min}\,[P])^{-1}\,[EB] \quad (2)$$

where V is retention volume of EB; V_{max}, the retention volume of EB at low concentration (60 pM); V_{min}, the retention volume of EB when the specific interaction is completely suppressed (this value can be determined by running [^{3}H]-EB in a series of concentration of drugs and plotting $1/(V_{max} - V)$ vs. 1/(drug) extrapolating to infinite (drug). From the above plot and a plot of $1/(V - V_{min})$ vs. (EB), dissociation constant values, K_d, for [^{3}H]-EB and the drugs can be obtained, c.f. References 3 and 4).

Ligands Used in This Study

The following ligands were used in this study. NCT receptor: 60 pM [^{3}H]-EB was used as the marker, EB (60-450 pM) and (–)-NCT (0.1-1000 nM) were used as displacers. $GABA_A$ receptor: 25 pM [^{3}H]-FTZ was used as the marker, FTZ and diazepam (0.05–500 nM) were used as displacers. NMDA receptor: 2 nM [^{3}H]-MK-801 was used as the marker, NMDA (1–2 mM) and MK-801 (0.5–200 nM) were used as a displacer. The chromatographic buffer for the NMDA receptor studies contained 1μM L-glutamate and 1 μM glycine because the presence of both amino acids was required to insure proper functioning of the NMDA receptor (5).

RESULTS

Presence and Activity of Immobilized NCT, $GABA_A$, and NMDA Receptors

A representative chromatogram depicting the reduction in the breakthrough volume of 60 pM [^{3}H]-EB produced by the addition of 1 μM (–)-NCT to the mobile phase is presented in Figure 1. In control experiments, no specific binding of [^{3}H]-EB was detected on IAM particles. The results of the chromatographic studies are presented in Table I. The K_d values obtained for the ligands from the frontal chromatographic studies were consistent with the values for the corresponding non-immobilized receptor obtained from the literature [NCT (6), NMDA (9, 10)], from previous binding studies in this laboratory [NCT (3)] and in the case of the $GABA_A$ receptor, from studies performed in connection with this study using previously described methods (3).

Interactions between Co-Immobilized $GABA_A$ and NCT Receptors

As described above, the K_d of EB for the immobilized NCT receptors and FTZ for the immobilized $GABA_A$ receptors were determined to be 0.044 nM and 1.3 nM, respectively (Table I). During these experiments, the [^{3}H]-EB or [^{3}H]-FTZ concentrations were held constant at 60 pM or 25 pM, respectively. At the completion of these studies, the MR-SP was washed, and 60 pM [^{3}H]-EB or 25 pM[^{3}H]-FTZ was reinjected onto the column. No significant change was seen in the shape or elution volume of the [^{3}H]-EB or [^{3}H]-FTZ. Then, 60 pM [^{3}H]-EB or 25 pM [^{3}H]-FTZ was again injected onto the column, however, at this time, the $GABA_A$ receptor ligand FTZ, or the NCT receptor ligand (–)-NCT, respectively, had been added to the mobile phase at a 1 μM concentration. Under these experimental conditions, no decrease in the elution volume of [^{3}H]-EB (Fig. 2) or [^{3}H]-FTZ (Fig. 3) was observed, indicating that the $GABA_A$ receptor ligand did not affect the binding of [^{3}H]-EB at the NCT receptor and vice versa.

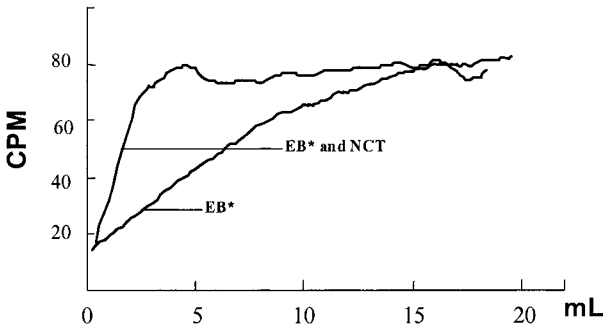

Fig. 1. Example of the elution profiles of [^{3}H]-EB on the MR-SP (0.5 × 1.7 cm). 60 pM [^{3}H]-EB with the presence of 1 μM NCT in the mobile phase.

Table I. The Affinities of Ligands for Individual Receptors Expressed as Dissociation Constant Determined by Frontal Chromatography on the MR-SP (K_d Frontal) and Classical Binding Assays to Solubilized Receptors (K_d Standard)

	K_d Frontal (nM)	K_d Standard (nM)
Nicotinic receptor		
Nicotine	1.0	0.8 (3, 6)
Epibatidine	0.044	0.05 (3, 6)
$GABA_A$ receptor		
Flunitrazepam	1.3	1.7
Diazepam	1.0	1.3
NMDA receptor		
NMDA	1.2/0.6	1.5/0.4 (9, 10)

Note: The K_d standard values were obtained from the literature [NCT (3, 6), NMDA (9, 10)] or from studies carried out in association with this study ($GABA_A$) using previously described methods (3).

DISCUSSION

In previous studies, NCT receptors were isolated from rat brain tissues and immobilized on the IAM liquid chromatographic support (3, 4). The resulting NCT receptor-stationary phase contained active NCT receptors that closely resembled the activity of the α4/β2 NCT receptor subtype isolated from rat brain tissues (3, 4).

In this study, the previously described protocol was repeated with the same results relative to the immobilized NCT receptor. The stability and reproducibility of these columns was examined: The NCT and $GABA_A$ receptors were stable and reproducible for over a thousand experiments; however, the NMDA receptor was less stable (one month, c.a. 250 experiments). The source of this difference is under investigation.

Although the co-immobilization of multiple receptors from rat brain tissue onto a stationary phase is novel, it should not be surprising that the co-immobilized receptors identified were the NCT, $GABA_A$, and NMDA receptors. These receptors, albeit pharmacologically independent, are part of the same receptor superfamily (7), and the same solubilization and isolation processes can be used for each of the receptors (8).

In displacement chromatography, the displacer ligand is placed in the mobile phase and the chromatographic phase is brought into contact with this phase. Once the system has reached equilibrium, the target ligand is then introduced into the system. Therefore, in the competitive displacement experiments undertaken in this study, the MR-SP had been equilibrated with the specific $GABA_A$ receptor ligand before introduction of specific NCT receptor ligand or, conversely, equilibrated with the specific NCT receptor ligand before introduction of the specific $GABA_A$ receptor ligand. The 1 μM concentrations were used to assure that the displacer ligand saturated the MR-SP. The fact that these experiments produced no observable changes in the elution volumes of the marker ligands reflects the specificity of the marker ligands used as well as the independence of the immobilized receptors. Under the experimental conditions, an overlap in either of these factors would have been reflected in a change in the elution volume.

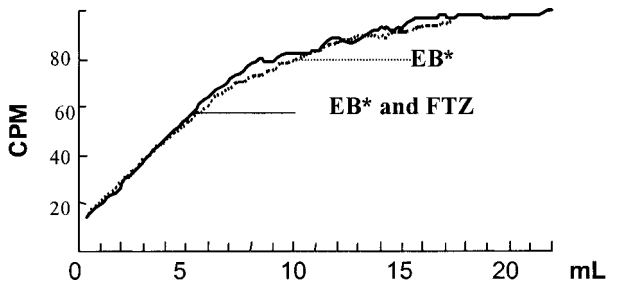

Fig. 2. Frontal chromatography on the co-immobilized MR-SP where the elution profiles of a 60 pM solution of the NCT receptor ligand [^{3}H]- EB* alone and with the $GABA_A$ ligand FTZ added to the mobile phase at a 1 μM concentration.

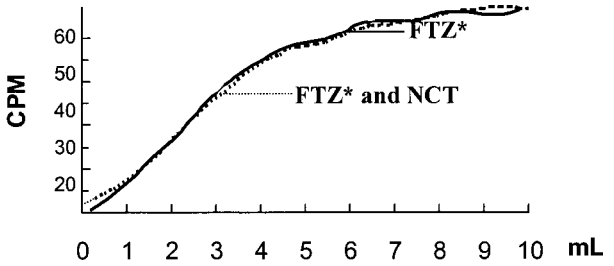

Fig. 3. Frontal chromatography on the co-immobilized MR-SP where the elution profiles of a 25 pM solution of the $GABA_A$ ligand [^{3}H]-FTZ* alone and with the NCT receptor ligand (–)-NCT added to the mobile phase at a 1 μM concentration.

CONCLUSIONS

The results of this study demonstrate that LGIC receptors can be solubilized and then immobilized on an IAM stationary phase to create a MR-SP. In this study, the MR-SP was shown to contain LGIC from rat brain, including the NMDA, $GABA_A$, and NCT receptors. The results also demonstrate that the co-immobilized $GABA_A$ and NCT receptors act independently of each other suggests that MR-SP columns might be useful in the investigation of overlapping affinities; for example, a new NCT antagonist might be readily screened for $GABA_A$ binding properties.

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