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3-[(±)-2-Carboxypiperazin-4-yl]propyl-1-Phosphonic Acid Recognizes Two *N*-Methyl-D-Aspartate Binding Sites in Rat Cerebral Cortex Membranes

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Abstract: Binding of 3-[(±)-2-carboxypiperazin-4-yl][^{3}H]-propyl-1-phosphonic acid ([^{3}H]CPP), a competitive inhibitor of *N*-methyl-D-aspartate (NMDA), has been studied in synaptic plasma membranes from rat cerebral cortex. Computer analysis of saturation and homologous displacement isotherms deriving from these plasma membranes indicated the existence of two binding sites: a specific, saturable, high-affinity binding site with a pK_D value of 7.53 ± 0.03 (29.5 n*M*) and a maximum binding value (B_{max}) of 2.25 ± 0.36 pmol/mg of protein, and a low-affinity site with a K_D of approximately 600 n*M* and a B_{max} of 7.0 pmol/mg of protein. It is argued that, in the light of current literature evidence, the low-affinity binding site may represent an agonist-dependent receptor, linked to physiological processes such as neurotransmitter release and channel regulation, whereas the high-affinity binding site may be linked to an antagonist-preferred receptor, for which no function has yet been reported. **Key Words:** Excitatory amino acids—*N*-Methyl-D-aspartate—3-[(±)-2-Carboxypiperazin-4-yl]-propyl-1-phosphonic acid binding. **van Amsterdam F. Th. M. et al.** 3-[(±)-2-Carboxypiperazin-4-yl]propyl-1-phosphonic acid recognizes two *N*-methyl-D-aspartate binding sites in rat cerebral cortex membranes. *J. Neurochem.* **59,** 1850–1855 (1992).

Extensive studies have indicated that excitatory amino acids (L-glutamate and L-aspartate) interact with three major subclasses of receptors, termed on the basis of selective agonists and antagonists *N*-methyl-D-asparate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), and kainate (Foster and Fagg, 1984; Cotman and Iversen, 1987; Collingridge and Lester, 1989; Watkins et al., 1990).

NMDA receptors are involved in the control of many processes related to neuronal excitability and enhancement of synaptic activity. The consequent potential importance of NMDA antagonists in the treatment of neurological diseases, such as epilepsy and ischemic brain damage, is becoming well recognized (Meldrum, 1985; Albers et al., 1989).

A continuous advance in the knowledge of the distribution and function of NMDA receptors in the brain has been facilitated by the introduction of selective radioactive ligands. Early available radioligands presented particular disadvantages, such as high nonspecific binding (NMDA), low selectivity for excitatory amino acid receptor subtypes (glutamate), poor correspondence of the binding profile with functional (electrophysiological) properties [DL-2-amino-7-phosphonoheptanoate (DL-AP7)], or a fast rate of dissociation from its binding site [D-2-amino-5-phosphonopentanoate (D-AP5)] (Olverman et al., 1986).

3-[(±)-2-Carboxypiperazin-4-yl][^{3}H]propyl-1-phosphonic acid ([^{3}H]CPP), a rigid analogue of AP7, overcomes most of these problems. It is a high-affinity, competitive antagonist at NMDA receptors. From electrophysiological, radioligand binding, and autoradiographic studies, CPP appears to act selectively at NMDA receptors, with a low affinity for kainate and AMPA binding sites (Harris et al., 1986; Lehmann et al., 1987). In addition, CPP does not show any activity in binding assays for 19 other puta-

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Abbreviations used: AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; AP5, 2-amino-5-phosphonopentanoate; AP7, 2-amino-7-phosphonoheptanoate; CPP, 3[(±)-2-carboxypiperazin-4-yl]propyl-1-phosphonic acid; GAMS, γ-glutamylaminomethylsulfonate; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate; NMDA, *N*-methyl-D-aspartate.

tive neurotransmitter receptors (Lehmann et al., 1987).

Originally, two studies have described the binding of [^{3}H]CPP to NMDA receptors. Olverman et al. (1986) reported an apparent equilibrium dissociation constant (K_D) of 280 n*M* and a maximum binding value (B_{max}) of 3.5 pmol/mg of protein in rat crude synaptic cerebrocortical membranes. Murphy et al. (1987) showed a K_D value of 201 n*M* and a B_{max} value of 6.4 pmol/mg of protein in Triton-treated crude synaptic membranes. Both used 1 m*M* glutamate to determine nonspecific binding, a concentration which is 1,000-fold higher than the apparent K_D for L-glutamate (reported to be between 0.15 and 1.1 μM).

In the present study, the binding of [^{3}H]CPP to crude synaptosomal membranes obtained from rat cerebral cortex, defined in the presence of variable concentrations of glutamate for determining nonspecific binding, resulted in the resolution of two CPP binding sites, which may reflect previously described agonist- and antagonist-preferred receptors of NMDA.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (200–250 g) were used. Animals were supplied by Charles River (Italy) and were kept under standard laboratory conditions.

Solutions and chemicals

[*propyl*-1,2-^{3}H]CPP (NET 962) was purchased from NEN–DuPont (U.S.A.) (specific activity in the batches used varied from 888 to 1,136 GBq/mmol). CPP was obtained from Tocris (U.K.). AMPA was purchased from RBI (Italy), and γ-D-glutamylaminomethylsulfonate (GAMS) from Cambridge Research Biochemicals (U.K.). NMDA was obtained from Aldrich Chimica S.r.l. (Italy). (+)-5-Methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate (MK-801) was synthesized in our laboratories. DL-AP5, glutamate HCl, L-aspartic acid, ibotenic acid, glycine HCl, quisqualic acid, and kainic acid were purchased from Sigma Chemical Co. (U.S.A.). Tris (hydroxymethyl)aminomethane was from Carlo Erba (Italy). Protosol was obtained from NEN-DuPont and Picofluor 40 from Packard. Other reagents were of the highest analytical grade available.

Preparation of crude synaptic membranes

Crude synaptic membranes were prepared according to Olverman et al. (1984), using rat cerebral cortex, an area showing a high density of [^{3}H]CPP binding sites (Jarvis et al., 1987). The preparation was performed at 4°C unless otherwise indicated. In brief, the tissue was homogenized in 15 volumes of ice-cold 0.32 *M* sucrose (pH 7) with a Potter homogenizer (12 strokes at 700 rpm). The homogenate was centrifuged at 1,000 *g* for 10 min. The supernatant was centrifuged at 17,000 *g* for 20 min, and the resultant pellet was lysed with 40 volumes of deionized water (Milli-Q system, Millipore) and homogenized with a Polytron (setting 5 for 35 s). Following incubation at 35°C for 30 min, the membranes were centrifuged at 48,000 *g* for 15 min and washed twice by resuspension in 40 volumes of water and centrifugation at 48,000 *g* for 15 min. The final pellet was frozen at −80°C. On the day of the binding experiment, the pellet was thawed and washed twice by resuspension in 10 volumes of water (Polytron setting 5 for 20 s) and centrifugation at 48,000 *g* for 15 min. The final pellet was resuspended in 35 volumes of 50 m*M* Tris-HCl buffer solution containing 2.5 m*M* $CaCl_2$ (final pH 7.6). $CaCl_2$ was added to the Tris buffer solution after preliminary experiments had indicated that Ca^{2+} increases the specific binding of [^{3}H]CPP by approximately 25%, without significant changes in the binding characteristics.

Binding assays

Incubations were carried out directly in scintillation Biovials (Beckman) in a final volume of 1 ml. [^{3}H]CPP was used in saturation experiments over a concentration range of 1–300 n*M* for the detection of the high-affinity site (using a specific activity of 88 GBq/mmol, obtained by a 10-fold isotopic dilution with unlabeled CPP), and over a range of 1–6,000 n*M* (sp. act. 8.8 GBq/mmol; 100-fold isotopic dilution) for the detection of the low-affinity site. Specific binding was defined initially as total binding of [^{3}H]CPP displaceable by 1 m*M* L-glutamate, whereas in the second series of experiments a concentration of 30 μM glutamate was used. Heterologous displacement binding curves were obtained with 20 n*M* [^{3}H]CPP (sp. act. 183 GBq/mmol), whereas homologous displacement curves were performed with 20 and 500 n*M* concentrations of radiolabel (sp. act. 12.2 GBq/mmol) in order to have the most favorable conditions for the detection of a high- and a low-affinity site, respectively.

The incubation was started by the addition of the membrane suspension (containing normally 350–450 μg of membrane protein) and lasted 25 min at 25°C, according to the method of Olverman et al. (1984). Samples were centrifuged at 30,000 *g* for 19 min at 4°C (Beckman centrifuge J2-21M/E equipped with a JA 14 rotor), after which the vials were washed immediately with 2 × 4-ml aliquots of ice-cold buffer solution. Pellets were digested overnight in 150 μl of Protosol. Subsequently, 3.5 ml of Picofluor 40 were added and the radioactivity was determined by liquid scintillation counting with a Packard Tri-Carb CA 1900. Protein contents were determined by the method of Lowry et al. (1951) as modified by Peterson et al. (1977), using bovine serum albumin as standard.

Data analysis

Data of saturation and homologous displacement experiments were analyzed using the nonlinear curve fitting program LIGAND (Munson and Rodbard, 1980). Total and specific binding of the saturation isotherms were analyzed and compared, using 30 and 1,000 μM glutamate to determine nonspecific binding. The presence of one or two binding sites was determined on the basis of the *F*-ratio test and was considered significant for two sites with $p < 0.05$.

Analysis of heterologous displacement curves to obtain the pharmacological profile of CPP binding was performed using ALLFIT (De Lean et al., 1978), a curve-fitting program based on a four-parameter logistic function describing sigmoidal curves, from which the concentration of compound inhibiting 50% of binding (IC_{50}) was obtained. The equilibrium dissociation constant (K_i) was determined from

these curves using the equation of Cheng and Prusoff (1973): $K_i = IC_{50}/(1 + L/K_D)$.

RESULTS

According to previous reports (Olverman et al., 1986; Murphy et al., 1987), a first series of saturation isotherms was performed using 1 m*M* L-glutamate to determine nonspecific binding. Computer analysis of this series of binding curves, obtained from experiments with radioligand concentrations in the range of 1–300 n*M*, indicated the existence of a saturable, high-affinity binding site with a pK_D of 7.53 ± 0.03 (29.5 n*M*) and a B_{max} of 2.25 ± 0.36 pmol/mg of protein (n = 5). Saturation experiments performed at radioligand concentrations up to 6,000 n*M* suggested a second, low-affinity binding site with a K_D varying widely from 100 to 700 n*M* and a B_{max} of about 7 pmol/mg of protein (Fig. 1). When the specific binding obtained from these experiments was forced to fit a model with one binding site, a pK_D of 6.85 ± 0.07 (141 n*M*) was found, which is very close to the previously mentioned literature values.

In homologous displacement binding studies performed with 20 n*M* [^{3}H]CPP (Fig. 2, open circles), the curve was described significantly better by a two-site than by a one-site fit ($p = 0.026$). The high-affinity site with a K_D of 32–36 n*M* unequivocally confirmed the results from the saturation isotherms, whereas the second, low-affinity site showed a K_D ranging from 1 to 6 μM. When using 500 n*M* [^{3}H]CPP to favor the detection of a potential low-affinity binding site, apparently a single, medium-affinity binding site was found, having a largely variable K_D of 60–210 n*M* and a level of nonspecific binding which on a percentage basis was three times higher than when using 20 n*M* [^{3}H]CPP (Fig. 2, filled circles). However, simultaneous fitting of the two experiments (20 and 500 n*M* concentrations of radioligand) showed that both curves were compatible with two binding sites, one with a K_D of 35 n*M* and a second, low-affinity site with a K_D of approximately 1 μM.

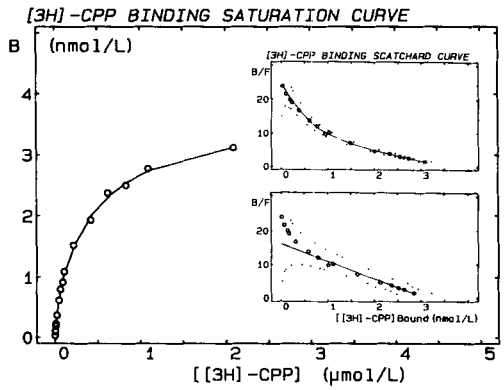

FIG. 1. Representative saturation isotherm of specific [^{3}H]CPP binding, using 1,000 μM glutamate to determine nonspecific binding. The curve represents a two-site model which was fit significantly better than a one-site model. **Insets:** Scatchard representations of the two-site fit (upper panel; K_D = 46 and 574 n*M*, B_{max} = 1.4 and 7.9 pmol/mg) and of the one-site fit (lower panel; K_D = 193 n*M*, B_{max} = 7.9 pmol/mg). The dotted lines indicate the 95% confidence interval. Fitted values are for the curve presented in this figure.

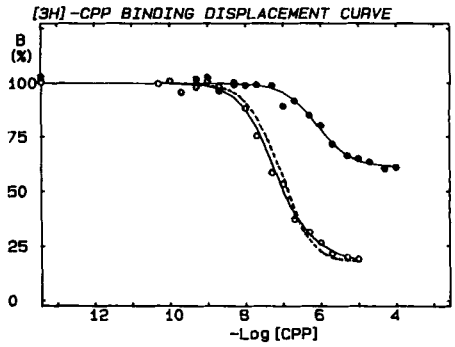

FIG. 2. Representative homologous displacement experiments using 20 n*M* (○) and 500 n*M* (●) [^{3}H]CPP. Simultaneous fitting of the two curves indicated the presence of a high-affinity site (K_D = 35 n*M*) and a low-affinity site (K_D = 1.1 μM). Dotted line represents single binding site model (K_D = 62 n*M*; two-site model was fit significantly better with $p = 0.014$; data show representative experiment).

The incongruency between the data obtained from these studies, indicating the presence of two binding sites with K_D values for CPP of around 30 and 1,000 μM, and those from the original literature studies, indicating only one site with intermediate affinity, prompted us to review the binding conditions utilized in the literature. Important consideration was given to the fact that in the presence of 100 μM L-glutamate already all specific CPP binding (with 20 n*M* [^{3}H]-CPP) was displaced. In fact, the IC_{50} value that we obtained for CPP displacement by glutamate was 0.28 μM (pIC_{50} = 6.55 ± 0.07, n = 4). Furthermore, it is well known that using a high concentration of displacer ligand (e.g., 1 m*M* glutamate) for the determination of aspecific binding may easily lead to erroneous interpretations of saturation binding and particularly to errors in Scatchard-type analyses (Bürgisser, 1984; Kermode, 1989).

Therefore, new saturation experiments were performed, using 30 μM instead of 1,000 μM glutamate to determine nonspecific binding. Under these conditions, in the concentration range of 1–300 n*M*, a high-affinity binding site with a pK_D value of 7.35 ± 0.05 (K_D = 45 n*M*) and a B_{max} of 3.2 ± 0.1 pmol/mg of protein were found, confirming the data for the high-affinity site obtained previously. In the range of 1–6,000 n*M*, however, 30 μM was not sufficient to fit the low-affinity binding site with significant improvement of the fit (Fig. 3).

In order to validate the pharmacological significance of the CPP-binding site, a closer characteriza-

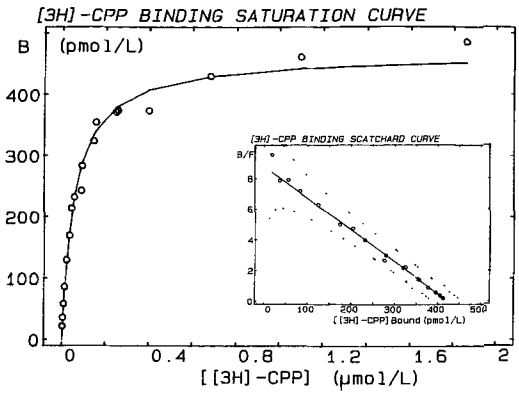

FIG. 3. Representative saturation isotherm of [^{3}H]CPP binding, using 30 μM glutamate to determine nonspecific binding. Conditions were identical to those of the experiments generating the data shown in Fig. 1, with the exception that the lower concentration of glutamate was used. **Inset:** Scatchard representation showing a one-site fit of the curve presented (K_D = 46 nM, B_{max} = 3.2 pmol/mg in this experiment). Dotted lines indicate 95% confidence interval.

tion of [^{3}H]CPP binding was performed by testing a series of amino acid agonists and antagonists selective for the three main subtypes of excitatory amino acid receptors in displacement experiments (Table 1). CPP was the most potent displacer of [^{3}H]CPP with an IC_{50} value of 0.094 μM, followed by L-glutamate, DL-AP5, and L-aspartic acid which were three, four, and 44 times less potent than CPP (IC_{50} values: 0.29, 0.40, and 4.15 μM, respectively). NMDA and ibotenic acid were approximately 100 times less potent than CPP (IC_{50} = 12.3 and 9.8 μM, respectively). Quisqualic acid was a moderate inhibitor of CPP binding (IC_{50} = 33.2 μM). Kainic acid and GAMS were very weak displacers (IC_{50} = 548 and 389 μM, respectively). Finally, AMPA, glycine, and MK-801 were inactive up to a concentration of 1 mM.

DISCUSSION

Computer analysis by nonlinear curve fitting of CPP saturation binding isotherms indicated the existence of a high-affinity binding site with a K_D of 29.5 nM and a B_{max} of 2.25 pmol/mg of protein. A second, low-affinity binding site was found by analysis of total CPP binding or by analysis of specific binding using 1 mM glutamate. When lower glutamate concentrations (30 μM) were used to determine nonspecific binding, the high-affinity site was unchanged, but the low-affinity binding site could not be detected anymore, indicating that two different CPP binding sites can be selectively displaced. Both binding sites could be confirmed in homologous displacement experiments.

The results apparently disagree with early data presented in the literature: Murphy et al. (1987) reported the presence of one class of binding sites (K_D = 217 nM) in Triton-treated membranes, even though they presented an apparently biphasic Scatchard curve. Reanalysis of the data by Murphy et al. (derived from Fig. 3A in the 1987 article) with the nonlinear curve fitting program LIGAND, however, provided a significant improvement of the fit (p = 0.045) when using a two-site model, and generated a high-affinity K_D value of 41 nM and a low-affinity K_D value of 301 nM, which is in good agreement with the data of the present study.

Displacement experiments of [^{3}H]CPP with a series of ligands indicated that the relative inhibitory potency was in good agreement with literature data, confirming the identity and the specificity of the NMDA receptor with respect to quisqualate or kainate receptors.

Recent literature studies show variable results. Cunningham and Michaelis (1990) reported a K_D of 540 and 590 nM in rat brain synaptic plasma membranes and in 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate-solubilized membranes, respectively, using the same procedure and fitted values for nonspecific binding. These data, however, were not detailed enough to detect a binding site in the low nanomolar range. Ogita and Yoneda (1990), using Triton-solubilized rat brain synaptic membranes in a filtration assay, reported a significantly lower equilibrium K_D of 81 nM, whereas the K_D derived from kinetic data (association and dissociation rate constants) appeared as low as 45.9 nM. Finally, Pelleymounter et al. (1990) reported that rat hippocampus membranes, prepared according to Olverman et al. (1984), showed a single binding site with a K_D for CPP of 39.5 nM and a B_{max} of 2.5 pmol/mg of protein, using low glutamate (10 μM) to determine nonspecific binding.

The emerging view, provided that data are analyzed in sufficient detail and using proper analytical tech-

TABLE 1. *Displacement of [^{3}H]CPP binding (10 nM) by excitatory amino acid agonists and antagonists*

Compound	IC_{50} (μM)	K_i (μM)
CPP	0.094 ± 0.031	0.070
L-Glutamate HCl	0.291 ± 0.047	0.217
DL-AP5	0.402 ± 0.052	0.300
L-Aspartic acid	4.15 ± 0.77	3.10
Ibotenic acid	9.81 ± 0.61	7.33
NMDA	12.32 ± 2.46	9.21
Quisqualic acid	33.23 ± 6.86	24.8
GAMS	389 ± 98	291
Kainic acid	548 ± 127	409
AMPA	>1,000	
Glycine	>1,000	
MK-801	>1,000	

Results are the means ± SEM of three to four experiments, each of which was performed in duplicate. IC_{50}, concentration at which 50% displacement was observed; K_i, equilibrium binding constant of the displacer ligand, calculated with the formula of Cheng and Prusoff (1973).

niques, supports the existence of high- and low-affinity CPP binding sites, as demonstrated in cortical membranes in this study.

Several relations can be made from literature with the low-affinity binding site. In fact, displacement binding studies of [^{3}H]glutamate have revealed K_i values for CPP that range from 820 n*M* (Fagg et al., 1990) to 1.6 μM (Grimwood et al., 1991). These values are in agreement with our low-affinity CPP binding site and seem to correlate with functional responses such as inhibition of NMDA-induced depolarizations in rat cortical slices ($pA_2 = 6.17$; Grimwood et al., 1991), inhibition of NMDA-evoked [^{3}H]-acetylcholine release in striatal brain slices ($pA_2 = 5.66$; Lehmann et al., 1987), or [^{3}H]dopamine release from fetal rat mesencephalon cell cultures ($IC_{50} \approx 5\ \mu M$; Mount et al., 1990).

On the significance of the high-affinity binding site of CPP, no clear concepts have been developed so far. Monaghan et al. (1988) proposed the existence of agonist- and antagonist-preferring binding sites on the basis of differential distribution and selective inhibition of glutamate and CPP binding in autoradiography studies. He also argued the possibility that still unknown factors may regulate the conversion from one site to the other. This concept was followed by Honoré et al. (1989), who reported different molecular weights (obtained from target size analyses) for the agonists glutamate, glycine, and *N*-[1-(2-thienyl)cyclohexyl]-3,4-piperidine (TCP), with respect to CPP, suggesting that antagonist binding requires an additional macromolecule to the agonist site. In fact, homologous displacement of these 209,000-Da large [^{3}H]CPP binding sites resulted in an IC_{50} value of 48 n*M*, which is very close to our high-affinity binding site.

In conclusion, our results show that [^{3}H]CPP binding performed in cerebral synaptic plasma membranes provides a specific and selective tool for the study of the NMDA receptor. Detailed analysis of binding data showed the existence of a high- and a low-affinity binding site in rat cortex membranes. The high-affinity binding site, which might coincide with an antagonist-preferring receptor, has an affinity of 29.5 n*M*, whereas the low-affinity site, possibly the agonist-preferring site related to the known physiological responses, has micromolar affinity. The function of the antagonist-preferring receptor and the possibility of interconversion remain to be elucidated.

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