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Radioligand labeling of *N*-methyl-D-aspartic acid (NMDA) receptors by [^{3}H](±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid in brain synaptic membranes treated with Triton X-100

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Some free acidic amino acids endogenous to the brain are thought to play a role as excitatory synaptic neurotransmitters in the mammalian CNS [1, 2]. Synaptic receptors for these amino acids are classified into at least three different subgroups according to sensitivity to typical exogenous compounds, such as *N*-methyl-D-aspartic acid (NMDA), quisqualic acid (QA) and kainic acid (KA) [3, 4]. The NMDA-sensitive receptors are supposed to be involved in neuronal plasticity [5], convulsive seizures [6] or neuronal cell death in cerebral ischemia [7], hypoglycemia [8] and Alzheimer's disease [9].

However, biochemical labeling of this subclass is rather difficult. Some aminophosphonic acids with NMDA antagonistic activity, such as DL-2-amino-5-phosphonovaleric acid (AP5) and DL-2-amino-7-phosphonoheptanoic acid (AP7), as well as NMDA itself, have been shown to be unsuitable as a radioligand to label these NMDA sites [10–12]. A recent study introduced a novel antagonist highly selective to NMDA receptors, (±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) [13]. Although this antagonist seems to label NMDA sites in brain synaptic membranes, the binding is detectable only when a centrifugation assay method is employed to separate the bound ligand from the free ligand [12]. A filtration assay method has been reported not to be useful for detecting [^{3}H]CPP binding in membranous preparations. Therefore, we have attempted in the present study to detect [^{3}H]CPP binding in brain synaptic membranes treated with Triton X-100 by employing a filtration assay method and we have demonstrated the usefulness of this method to determine NMDA-sensitive [^{3}H]L-glutamic acid (Glu) binding in these Triton membranes [14].

Materials and methods

Materials. [^{3}H]CPP (*propyl*-1,2-[^{3}H]CPP, 30.7 Ci/mmol) was purchased from New England Nuclear (Boston, MA). DL-α-Amino-3-hydroxy-5-methylisoxazol-4-propionic acid (AMPA), willardiine (WIL), D- and L-AP5, D- and L-AP7 and CPP were supplied by Tocris Neuramin (Buckhurst Hill, U.K.). (+)-5-Methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]-cyclohepten-5,10-imine maleate (MK-801) and phencyclidine (PCP) were donated by Dr L. L. Iversen (Merck Sharp & Dohme Research Laboratories, Harlow, U.K.) and Dr T Nabeshima (Meijo University, Nagoya, Japan) respectively. The other chemicals used were obtained from the Sigma Chemical Co. (St. Louis, MO).

Membrane preparation. Crude synaptic membrane fractions obtained from the brains of Wistar rats weighing 200–250 g [15] were washed four times by repeating the suspension in 50 mM Tris–acetate buffer (pH 7.4) [16] and subsequent centrifugation at 50,000 *g* for 20 min at 4° [17]. The final pellets were suspended, frozen, thawed, and treated with Triton X-100 [14].

[^{3}H]CPP binding. Membrane preparations (about 300 μg protein) were incubated with 10 nM [^{3}H]CPP in 0.5 mL of 50 mM Tris–acetate buffer (pH 7.4) at 2° for 10 min unless indicated otherwise. Incubation was terminated by the addition of 3 mL of ice-cold buffer and subsequent filtration

through a Whatman GF/B glass fiber filter under a constant vacuum of 15 mm Hg [18]. After rinsing the filter with 3 mL of cold buffer four times within 10 sec, the radioactivity trapped on the filter was measured by a liquid scintillation spectrometer using 5 mL of modified Triton–toluene scintillant at a counting efficiency of 40–42% [19]. Kinetic parameters such as K_d and B_{max} were calculated from the first order equation estimated by a computer with a non-linear regression analysis program [20]. Nonspecific binding was defined by 1 mM unlabeled Glu.

Binding assays were carried out at an interval of 15 sec in triplicate with a variation of less than 10%. Protein content was measured by the method of Lowry *et al.* [21]. Results are expressed as means ± SEM. and the statistical significance was determined by Student's *t*-test.

Results and discussion

Specific binding was inversely dependent on the incubation temperature and was reversible. The binding reached a plateau within 5 min after the initiation of incubation at 2°, whereas the time required to attain equilibrium at 30° was 1 min (data not shown).

Among various amino acids, L-Glu was the most potent inhibitor of the binding in a concentration-dependent manner at a concentration range from 10^{-8} to 10^{-4} M. L-Homocysteic acid induced more than five times less potent inhibition of the binding, with progressively lower potencies induced by D-aspartic acid (Asp), L-cysteinesulfinic acid, L-Asp, D-Glu, and L-cysteic acid. *In vitro* addition of *N*-methyl-L-aspartate as well as NMDA moderately inhibited the binding (Table 1). Competitive NMDA antagonists, such as CPP, α-aminoadipic acid and γ-D-glutamylglycine, were all effective in inhibiting the binding, whereas non-competitive antagonists including PCP and MK-801 did not affect the binding significantly at the concentrations used. QA, an agonist for the QA-sensitive receptors, also modestly inhibited the binding in a concentration-dependent fashion. However, the other QA agonists, AMPA and WIL, were ineffective as inhibitors of the binding. L-Glutamate diethylester elicited a relatively weak inhibition of the binding, whereas KA, an agonist for the KA-sensitive receptors, did not alter [^{3}H]CPP binding at the concentration range used. The binding was markedly abolished by two aminophosphonic acids with NMDA antagonistic activity, DL-2-amino-5-phosphonovaleric acid (AP5) and DL-2-amino-7-phosphonoheptanoic acid (AP7), but not by the other phosphonates, including DL-2-amino-3-phosphonopropionic and DL-2-amino-4-phosphonobutyric acids. However, DL-2-amino-6-phosphonohexanoic acid exerted a relatively strong diminution of the binding. The D-stereoisomers of both AP5 and AP7 exhibited more than 70 times as potent inhibition of the binding as their respective L-isomers. The binding was inhibited profoundly by some tryptophan metabolites endogenous to the brain, kynurenic, anthranilic and quinolinic acids, at a concentration of 10^{-4} M. Other metabolites such as kynurenine and xanthurenic acid had no such effect. Both GSH and GSSG rather potently inhibited the binding, whereas both *N*-acetyl-L-Asp-L-Glu and thyrotropin releasing hormone failed to affect the binding at the concentrations employed.

In addition, some nucleotides exhibited a relatively intensive inhibition of the binding (Fig. 1). Among some endogenous nucleotides examined, GTP was the most potent inhibitor of [^{3}H]CPP binding, followed by GDP and GMP. ATP also moderately inhibited the binding at a concentration of 10^{-4} M. Nonhydrolyzable GTP analogs, including guanosine 5′-O-(5-thiotriphosphate) and 5′-guanylylimidodiphosphate, were effective as inhibitors of the binding at a concentration range similar to that of GTP.

Scatchard analysis revealed that [^{3}H]CPP binding sites consisted of a single component in brain synaptic membranes treated or not treated with Triton X-100. Triton treatment resulted in a significant enhancement of the affinity for [^{3}H]CPP without altering the density of these binding sites (untreated membranes: $K_d = 238 \pm 31$ nM, $B_{max} = 1.45 \pm 0.12$ pmol/mg protein; Triton membranes: $K_d = 81 \pm 11$ nM, $B_{max} = 1.74 \pm 0.12$ pmol/mg protein) (Fig. 2A). GTP at 10^{-5} M lowered the affinity without significantly affecting the density of binding sites in Triton membranes (GTP: $K_d = 247 \pm 46$ nM, $B_{max} = 2.02 \pm 0.24$ pmol/mg protein) (Fig. 2B).

These data clearly indicate that a filtration assay method is indeed applicable to the detection of [^{3}H]CPP binding in brain synaptic membranes treated with Triton X-100. This fact obviously disagrees with the previous study in which the binding was detected exclusively by a centrifugation assay method, and not by a filtration assay method [12].

Table 1. Effects of various compounds on [^{3}H]CPP binding: K_i values and Hill coefficients

	K_i (μM)	Hill coefficient
L-Glu	0.057 ± 0.009	0.93 ± 0.02
L-HCA	0.296 ± 0.092	0.87 ± 0.08
D-Asp	1.94 ± 0.50	0.80 ± 0.07
L-CSA	2.29 ± 0.28	1.08 ± 0.13
L-Asp	2.89 ± 0.33	1.16 ± 0.17
D-Glu	11.8 ± 4.6	1.10 ± 0.18
L-CA	12.6 ± 1.42	1.06 ± 0.12
CPP	0.095 ± 0.042	0.99 ± 0.27
α-AAA	2.12 ± 0.59	0.93 ± 0.17
NMDA	5.15 ± 1.21	1.14 ± 0.11
γ-DGG	15.3 ± 3.4	0.99 ± 0.14
NMLA	20.0 ± 3.1	1.03 ± 0.04
QA	48.9 ± 15.1	0.86 ± 0.11
GDEE	83.0 ± 23.2	1.03 ± 0.04
DL-AP4	>100	
DL-AP5	0.124 ± 0.062	0.88 ± 0.07
DL-AP6	7.89 ± 2.31	0.88 ± 0.09
DL-AP7	0.462 ± 0.119	1.04 ± 0.14
D-AP5	0.083 ± 0.010	1.01 ± 0.05
L-AP5	6.38 ± 1.26	0.90 ± 0.12
D-AP7	0.270 ± 0.045	0.80 ± 0.01
L-AP7	5.54 ± 0.78	1.03 ± 0.25
ATA	25.6 ± 6.3	1.03 ± 0.29
KYNA	58.2 ± 15.8	1.01 ± 0.22
QNA	>100	
GSSG	1.13 ± 0.39	0.75 ± 0.07
GSH	3.79 ± 0.90	0.93 ± 0.15
NAAG	>100	
TRH	>100	

The routine binding assay was performed in the presence of five concentrations (in a range from 10^{-8} to 10^{-4} M) of each compound. Values (means ± SEM) were obtained from four independent determinations. K_i values were calculated according to the equation $K_i = IC_{50}/(1 + [L]/K_d)$. Compounds that did not inhibit the binding even at 10^{-4} M were: AMPA, DL-2-amino-3-phosphonopropionic acid, kynurenine, MK-801, PCP, WIL, and xanthurenic acid. Abbrevations: α-AAA, α-aminoadipic acid; AP4, 2-amino-4-phosphonobutyric acid; AP6, 2-amino-6-phosphonohexanoic acid; ATA, anthranilic acid; CA, cysteic acid; CSA, cysteinesulfinic acid; γ-DGG, γ-D-glutamylglycine; GDEE, glutamate diethylester; GSH, reduced glutathione; GSSG, oxidized glutathione; HCA, homocysteic acid; KYNA, kynurenic acid; NAAG, *N*-acetyl-L-aspartyl-L-glutamate; NMLA, *N*-methyl-L-aspartic acid; QNA, quinolinic acid; and TRH, thyrotropin releasing hormone.

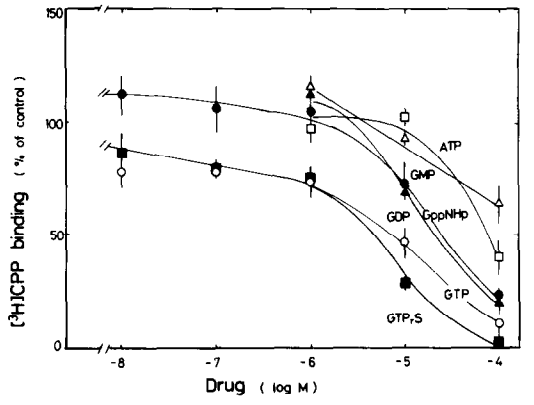

Fig. 1. Effects of several nucleotides on [^{3}H]CPP binding. Binding was determined in the presence of various concentrations of the nucleotides indicated. Each value is the mean ± SEM of four separate experiments. Control binding: 208 ± 20 fmol/mg protein. Abbreviations: GppNHp, 5′-guanylylimidodiphosphate; and GTPγS, 5′-O-(3-thiotriphosphate).

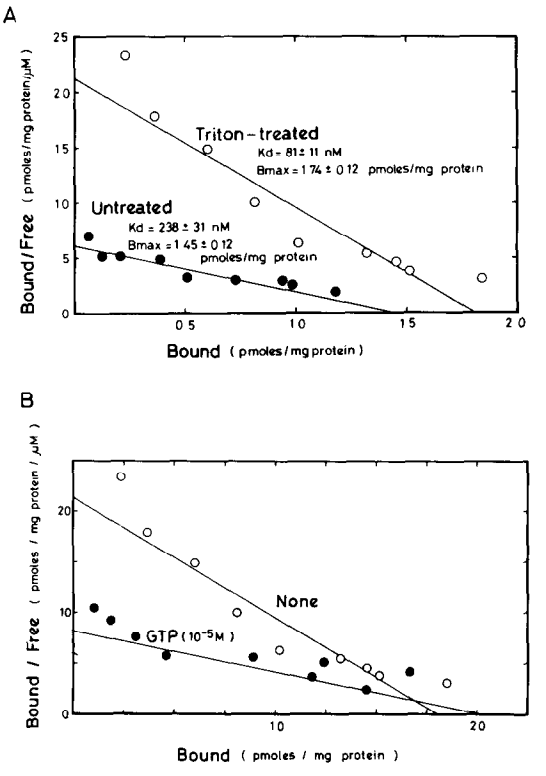

Fig. 2. Scatchard plots for [^{3}H]CPP binding. (A) Brain synaptic membranes treated or not treated with Triton X-100 were incubated with 10 nM [^{3}H]CPP in the presence of various concentrations of unlabeled CPP over a concentration range from 10 to 600 nM. (B) Triton membranes were incubated with [^{3}H]CPP as described above, in either the presence or absence of 10^{-5} M GTP. Values were calculated from ten separate determinations.

The latter discrepancy is easily explained by taking into consideration the difference in experimental conditions used in these two studies. The previous study had employed an incubation temperature of 23° [12], whereas the present study was carried out at 2°. Although all physiological responses are undoubtedly a temperature-dependent phenomenon, the incubation temperature seems to be maintained as low as possible to detect the steady-state level of the simple binding process of a ligand with the NMDA-sensitive receptors. Reduction of incubation temperature would often lower the affinity for Glu of numerous membrane-bound proteins, whereas the binding to receptor sites could be detected under these low temperature conditions due to their considerably higher affinity than the other proteins. A low incubation temperature is not optimal to determine the activity of receptor sites, but crucial to suppress the activities of membranous proteins other than receptors. Temperature could facilitate the dissociation of a ligand from the binding sites, in addition to stimulating the association rate.

The pharmacological characteristics of [^{3}H]CPP binding are consistent with those of NMDA-sensitive receptors evaluated electrophysiologically. Since AMPA is more specific to the QA-sensitive receptors than QA itself [22], the inhibition by QA seems to be derived from the non-specific action of QA on the NMDA-sensitive receptors. The exact mechanism as well as the functional significance of the relatively potent inhibition by GSH and GSSG remain to be elucidated. These endogenous peptides containing L-Glu abolish the Na^+-dependent and -independent bindings of [^{3}H]Glu in brain synaptic membranes not treated with Triton X-100 [23]. Brain synaptic membranes are shown to contain temperature-dependent and -independent binding activities of [^{3}H]GSH [24, 25]. These previous findings along with the present results raise the possibility that brain glutathione may play some important physiological role in the maintenance of central neuronal excitability by interacting with the NMDA-sensitive receptors.

Although selective inhibition by guanine nucleotides of a radioligand binding is strong supporting evidence for the involvement of a GTP-binding protein in the receptor–effector linkage system, the data provided here do not support this possibility. Guanine nucleotides are reported to inhibit [^{3}H]CPP binding [12], NMDA-sensitive [^{3}H]Glu binding [26], temperature-dependent and NMDA-insensitive [^{3}H]Glu binding [27], and DL-[^{3}H]AP4 binding [28], all of which are measured by employing the centrifugation assay method. Considering these previous findings together with the fact that the GTP effect is a physiologically important phenomenon seen specifically with agonists but not with antagonists [29, 30], it is reasonable to speculate that the GTP-binding protein is not involved in the coupling mechanism between the NMDA-sensitive receptors and effectors (cation channels?) in the brain. The possibility that CPP as well as AP4 may have some partially agonistic activity, however, cannot be excluded at present. From this viewpoint, it should be noted that GTP markedly inhibited the Glu-dependent [^{3}H]MK-801 binding activity by attenuating the stimulatory property of L-Glu [EC_{50} values (μM): control, 0.26 ± 0.04; 10^{-5} M GTP, 0.46 ± 0.06; 10^{-4} M GTP, 3.45 ± 0.89] (unpublished data). Therefore, electrophysiological and biochemical studies with respect to the modulatory effect of guanine nucleotides on receptor–effector interaction should be carried out before drawing any conclusions.

It thus appears that [^{3}H]CPP is a good biochemical tool to evaluate the pharmacological profile of the NMDA-sensitive subclass of the central excitatory amino acid recep-

tors, independently of the assay method employed. Removal of endogenous Glu by Triton treatment undoubtedly contributes to the usefulness of a filtration method. The use of a filtration assay method is evidently beneficial to the development of numerous neuroactive drugs acting at the latter subclass.

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