Two Affinity States of N-Methyl-d-Aspartate Recognition Sites: Modulation by Cations

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ABSTRACT

Previous studies have indicated that inorganic and organic cations can markedly affect parameters of the function of the N-methyl-d-aspartate receptor ionophore complex. As these effects may involve modulation of agonist binding, the purpose of our study was to investigate the stimulatory effect of mono- and divalent cations on binding properties of glutamate/N-methyl-d-aspartate recognition sites on the N-methyl-d-aspartate receptor complex, using [3H]CGP 39653 as the specific ligand for these sites. In well-washed membranes from rat brain, [3H]CGP 39653 binding sites were present at two affinity states when assayed at 10 mM HEPES-KOH buffer. About 75% of these sites were in a low-affinity state (Kd = 210 ± 30 nM) although 25% were in a high-affinity state (Kd = 6.4 ± 0.4 nM).

Addition of mono- or divalent cations to the incubation medium stimulated [3H]CGP 39653 binding, measured at a radioligand concentration of 4 nM. Maximal increases in binding were to ~230 and 400% of control, in the presence of mono- and divalent cations, respectively. Values of EC50 for stimulation were 5 to 7 mM for monovalent cations and 0.2 to 0.4 mM for divalent cations. At these concentrations, cations increased the Bmax for the high-affinity population of [3H]CGP 39653 sites and decreased the Bmax for low-affinity ones. These findings suggest that, like spermidine, inorganic cations stimulate binding by converting [3H]CGP 39653 binding sites from the low- to high-affinity state.

The NMDA receptor-ionophore complex plays a fundamental role in important physiological and neuropathological processes in brain (Wroblewski and Danysh, 1989; Daw et al., 1993). The receptor consists of at least two families of protein subunits that form an ion channel (Moriyoshi et al., 1991; Ikeda et al., 1992; Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992; Ishii et al., 1993; Watanabe et al., 1993), and variation in the combination of these subunits probably causes heterogeneity of NMDA receptors among brain regions. In most cases, the complex contains an NMDA-recognition domain, a strychnine-insensitive glycine recognition domain and a polyamine-recognition domain. Occupation of each domain with appropriate agonist leads to positive modulation in opening of the channel (Wroblewski and Danysh, 1989; Yoneda and Ogita, 1991; Williams et al., 1991).

The ion channel, which is permeable to Ca2+, Na+ and K+, contains binding sites for Mg2+ and Zn2+ (Anis et al., 1983; Mayer et al., 1984; Nowak et al., 1984; Peters et al., 1987) as well as sites where drugs, such as dizocilpine (MK 801) (Reynolds et al., 1987; Yoneda and Ogita, 1991) and TCP, bind to induce channel blockade. Because binding sites for dizocilpine and TCP are located within the channel, [3H]-labeled species of these compounds have been widely used in radioligand studies to characterize functioning (opening, closing) of the channel (Foster and Wong, 1987; Wong et al., 1987; Wroblewski and Danysh, 1989; Yoneda and Ogita, 1991). For example, it was shown that NMDA or glutamate, glycine and polyamines all increase binding of [3H]dizocilpine and [3H]TCP, while antagonists at the same sites
decrease binding of these radioligands (Wroblewski and Danyz, 1989; Yoneda and Ogita, 1991).

Cloning and sequencing of the NMDA receptor subunits, and identification of the sequences that confer selectively for cations or for channel blockade by Mg$^{2+}$ and noncompetitive NMDA receptor antagonists have advanced our understanding of the functions of the NMDA receptor complex (Yamakura et al., 1993; Hume et al., 1991). Nevertheless, the mechanisms by which modulators of different kinds, including divalent cations, can affect opening of the NMDA receptor channel are still under investigation.

One approach to this question is study of the biphasic effect of divalent cations (e.g., Ca$^{2+}$, Mg$^{2+}$) on binding of channel ligands. Low concentrations of divalent cations increase the binding of $[^3H]$dizocilpine, whereas high concentrations reduce binding, in preparations of well-washed membranes without added glutamate (Reynolds and Miller, 1988; Wong et al., 1988; Reynolds, 1990; Enomoto et al., 1992). Using unwashed membranes (containing endogenous glutamate, glycine and other modulators) or in the presence of saturating concentration of glutamate, only the inhibitory effect of high concentrations of divalent cations has been observed (Reynolds and Miller, 1988; Enomoto et al., 1992). Similarly, low concentrations of the polyamine (spermidine) enhance $[^3H]$dizocilpine binding, whereas higher concentrations reduce binding to control levels; the stimulatory effect is 6-fold greater in the absence of added glutamate than in the presence of 90 $\mu$M glutamate (London and Mukhin, 1995). These observations are consistent with the view that divalent cations and polyamines have different effects on the NMDA receptor channel, depending on their concentrations, and that they exert a positive effect on the activity of the NMDA channel at least in part by modulation of NMDA (glutamate) recognition sites. In this regard, recent studies have indicated that cations, such as Mg$^{2+}$ and polyamines, stimulate the binding of $[^3H]$CGP 39653, a competitive antagonist that is highly selective for NMDA recognition sites (Reynolds, 1994), and that this effect is due to an increase in the affinity for the radioligand (Reynolds, 1994).

Although previous studies identified a single component of $[^3H]$CGP 39653 binding (Sills et al., 1991; Reynolds, 1994), equilibrium binding studies performed in low molarity buffer with a wider concentration range of this radioligand provided data that were consistent with a two-site model for binding to membranes of rat forebrain (London and Mukhin, 1995). The results of these latter studies also suggested that spermidine converted $[^3H]$CGP 39653 binding sites from a low- to a high-affinity state (London and Mukhin, 1995). It therefore seemed possible that, as with spermidine, inorganic cations might effect more than an increase in the affinity of the NMDA recognition site. The purpose of our work was to further clarify the mechanisms by which mono- and divalent cations might affect NMDA receptor function. Specifically, we sought to determine if, as with polyamines, inorganic cations can increase binding to the NMDA receptor by effecting conversion from a low- to a high-affinity state.

Materials and Methods

Materials. Male Fischer rats were obtained from Charles River Breeding Laboratories (Wilmington, MA). Rats were shipped at 86 days of age, and were housed in a temperature- and light-controlled vivarium for at least 2 wk before being used for this study.

Chemicals were obtained from the following sources: CPP, D-AP5, NMDA, (+)-quisqualic acid, kainic acid (trans-(-)-ACPD) were obtained from Research Biochemicals Inc. (Natick, MA). L-Cystine was purchased from Calbiochem (San Diego, CA). Spermidine was obtained from Aldrich Chemical Company (St. Louis, MO). $[^3H]$CGP 39653 (32.0 Ci/mmol), and $[^3H]$CGS 19755 (50.0 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Membrane preparation. Rats were decapitated. The frontal cortex and hippocampus were dissected and homogenized together in 10 volumes of ice-cold 0.32 M sucrose using a motor-driven Teflon-glass homogenizer. The homogenate was centrifuged at 800 g for 10 min; the pellet was resuspended in 10 volumes of ice-cold 0.32 M sucrose using a motor-driven Teflon-glass homogenizer, and centrifuged at 18,000 $\times$ g for 90 min. The pellet was stored at -20°C overnight. On the next day, the pellet was resuspended in 30 volumes of ice-cold deionized water using a Polytron, and was centrifuged at 30,000 $\times$ g for 20 min. This wash step was repeated five additional times, and the final pellet was stored in portions at -70°C for at least 16 hr, but no more than 4 wk before use. On the day of assay, the pellets were resuspended in 10 volumes of deionized water (20°C), homogenized using a motor-driven Teflon-glass homogenizer, and centrifuged at 18,000 $\times$ g for 15 min. This wash step was repeated two more times, and the final pellet was resuspended in ice-cold assay buffer (10 mM HEPES-KOH, pH 8.0).

$[^3H]$CGP 39653 and $[^3H]$CGS 19755 binding assays. For saturation studies and for assays to test the effects of cations, membrane preparations (20–30 $\mu$g of protein) were incubated in polystyrene tubes containing 10 mM HEPES-KOH buffer, pH 8.0, with or without addition of the salts of different cations in an incubation volume of 0.3 ml. Because 12 mM KOH was used to adjust pH of the HEPES buffer, all assays were performed at a background level of 12 mM K$^+$. Saturation studies were performed by adding increasing concentrations of radioligand (0.3–400 nM $[^3H]$CGP 39653, 0.8–840 nM $[^3H]$CGS 19755). In competition studies, 3 nM $[^3H]$CGP 39653 was incubated with 60 to 90 $\mu$g membrane protein in a total volume of 0.6 ml. Nonspecific binding was determined in the presence of 100 $\mu$M NMDA. After a 1-hr incubation at 4°C, binding was terminated by rapid filtration onto Whatman GF/B filters (soaked for at least 2 hr in 1 M KCl containing 100 mM sodium salt of glutamate, pH 7.8–8.0) using a Brandell 48-channel cell harvester (Biochemical Research Laboratory, Gaithersburg, MD). For this purpose, 4 ml of ice-cold 50 mM Tris HCl pH 7.4 buffer were added to membrane suspension and samples were immediately filtered with four separate 4-ml rinses of the same buffer. All filtration procedures were conducted at 4°C in a cold room, and were completed in ≤7 sec. Pretreatment of GF/B filters with KCl and glutamate resulted in a substantial (30–40%) reduction of nonspecific binding of the radioligand to the filters. Radioactivity was measured after 24 hr using LSC 989 scintillation cocktail (New England Nuclear, Boston, MA) and a Beckman LS-3801 liquid scintillation counter at a counting efficiency of 47%.

Protein assay. Protein measurements were performed using a concentrated dye reagent (Bio-Rad, Richmond, CA) (Bradford, 1976), and bovine serum albumin as the standard.

Data analysis. The LIGAND program (Munson and Rodbard, 1980), as modified for the IBM PC (McPherson, 1985), were used to determine parameters of ligand binding in membrane preparations. The concentration of salts required to produce half-maximal enhancement (EC50) of $[^3H]$CGP 39653 binding above the control level was determined using linear regression analysis of ln (–) logit plots.

To model the biphasic effect of salts on $[^3H]$CGP 39653, we used the following equation, which describes the ratio of specific binding of $[^3H]$CGP 39653 in the presence of added salts (B2) to that observed...
under the control condition (B0) (i.e., without addition of salts, but in the presence of 12 mM K+ as a function of added salt:

\[ \frac{B_s}{B_0} = \left( 1 + \frac{K_{dc}}{K_0} \right) \cdot \left( \frac{K_{dc} \cdot K_t + K_{dc} \cdot C}{K_t \cdot K_{dc} + K_{dc} \cdot C} \right) \cdot \left( \frac{1}{1 + (IC_{50})^n} \right) \]  

(1)

in which \( K_0 \) is the concentration of K+ in the control condition, C is the concentration of added cations, I is the concentration of inhibitor (chloride) introduced by addition of salts, \( K_{dc} \) is the dissociation constant for K+, \( K_t \) is the dissociation constant for added cations, IC0 is the concentration of inhibitor that reduces specific binding by 50%, and n is the Hill coefficient. Equation 1 describes the interactions of one or two activators (e.g., mono- and divalent cations) of different affinities with radioligand binding in the presence of varying concentrations of a competitive inhibitor (anions, e.g., Cl-).

This model assumed a complex interaction in which: 1) stimulation due to cations is a positive allosteric modulation, 2) cations compete for binding to allosteric modulatory sites, 3) inhibition of [3H]CGP 39653 binding due to anions is competitive and 4) only high-affinity binding sites are considered.

Equation 1 was derived from the Langmuir absorption isotherms for interaction of the NMDA receptor with radioligand, allosteric modulators (cations), and a competitive inhibitor (anion, e.g., Cl-). Considering the aforementioned assumptions, binding in the absence of added salts can be characterized as follows:

\[ \frac{B_s}{B_0} = \frac{B_{\text{max}0} \cdot F}{K_0 + F} \]  

(2)

in which \( B_{\text{max}0} \) is the density of high-affinity binding sites in the absence of added salts, and \( K_0 \) and F represent the dissociation constant and concentration of the radioligand, respectively. When salts are added, specific binding can be expressed as follows:

\[ \frac{B_s}{B_0} = \frac{B_{\text{max}h} \cdot F \cdot K_t}{K_{t} + F \cdot K_t + K_{dc} \cdot (1 + (IC_{50})^n)} \]  

(3)

where \( B_{\text{max}h} \) is the density of high-affinity binding sites in the presence of added salts, and \( K_t \) is the dissociation constant of the inhibitor.

Based on the relationship between \( K_t \) and IC0, \( K_t = (IC_{50})^n \cdot K_0/(K_0 + F) \), Cheng and Prusoff, 1973, substitution into equation 3 yields the following expression:

\[ \frac{B_s}{B_0} = \frac{B_{\text{max}h} \cdot F}{(K_d + F) \cdot (1 + (IC_{50})^n)} \]  

(4)

From equations 2 and 4, the ratio of specific binding in the presence of added salts to that observed in the absence of salts is as follows:

\[ \frac{B_s}{B_0} = \frac{B_{\text{max}h}}{B_{\text{max}0}} \cdot \frac{1}{1 + (IC_{50})^n} \]  

(5)

The density of high-affinity sites is related by a constant, p, to the sum of the quantities of modulatory sites bound by K+ (introduced in buffer) and added cations, \( M_{K^+} \) and \( M_c \) respectively:

\[ B_{\text{max}h} = p \cdot (M_{K^+} + M_c) \]  

(6)

Although the concentration of K+ introduced into the incubation medium by the buffer is constant (12 mM), \( M_{K^+} \) is variable because added cations compete with K+ for binding to allosteric modulatory sites. In the absence of added cations,

\[ B_{\text{max}0} = p \cdot M_0 \]  

(7)

in which \( M_0 \) represents the quantity of allosteric sites occupied by K+ in the control condition (no added salts). By substitution from equations 6 and 7 into equation 5 and simplification, the following ratio is obtained:

\[ \frac{B_s}{B_0} = \frac{(M_{K^+} + M_c)}{M_0} \cdot \frac{1}{1 + (IC_{50})^n} \]  

(8)

If cations compete for binding to the same modulatory sites, the modulatory sites bound could be determined using equations based on the ligand binding to a receptor in the presence of a competitive inhibitor (similar to equation 3), as follows:

\[ M_K = \frac{M \cdot K_{dc} \cdot K_{0}}{K_{dc} + K_{0}} \]  

(9)

\[ M_c = \frac{M \cdot K_{dc} \cdot C}{K_{dc} + K_{0}} \]  

(10)

in which \( K_{dc} \) and \( K_{0} \), respectively, are the dissociation constants for added cations and K+, and \( K_0 \) is the concentration of potassium introduced in the buffer (12 mM), and C is the concentration of added cations. When cations are not added (C = 0), the modulatory sites occupied by K+ can be determined as follows:

\[ M_0 = \frac{M \cdot K_{0}}{K_{dc} + K_{0}} \]  

(11)

Therefore, by substituting the definitions of \( M_{K^+} \), \( M_c \), and \( M_0 \) from equations 9 to 11 into equation 8 and simplifying, we obtained equation 1. Finally, substituting 12 mM for \( IC_{50} \), the concentration of added cations (S) for the concentration of added cations, and \( S \cdot v \) (where \( v \) is the valence of the cation) for concentration of Cl- (I) in equation 1, we obtained equation 12, describing the ratio of binding of the radioligand in the presence of added salts relative to binding observed in the control condition, as a function of the concentration of added salts:

\[ \frac{B_s}{B_0} = \left( 1 + \frac{K_{dc}}{12mM} \right) \cdot \left( \frac{12mM \cdot K_{dc} + K_{dc} \cdot C}{K_{dc} + 12mM \cdot K_{dc} + K_{dc} \cdot C} \right) \cdot \left( \frac{1}{1 + (S \cdot v \cdot IC_{50})^n} \right) \]  

(12)

To determine how well this function describes the data that were obtained, we applied it to the values of \( B/B_0 \), shown in figure 1 for added Ca2+ and K+, and estimated of IC50, \( K_{dc} \), and the Hill coefficient, using nonlinear regression analysis.

**Results**

**Effects of mono- and divalent cations on [3H]CGP 39653 binding.** Addition of the chloride salts of various mono- and divalent cations to the incubation medium had biphasic effects on the binding of [3H]CGP 39653 (4 nM) (fig. 1). Similar biphasic effects of these cations on specific binding to NMDA recognition sites were found in two additional experiments using [3H]CGS 19755 as the radioligand (data not shown). All of the cations caused concentration-dependent stimulation of binding, with a return to control at high concentrations. Divalent cations were more potent and effective in every case than monovalent cations (table 1; fig. 1). Maximal levels of binding were about 230 and 400% of control in every case than monovalent cations (table 1; fig. 1).
of divalent cations produced a much greater stimulation than salts of monovalent cations when the concentrations of chloride were equal (fig. 1). For example, in the presence of 16 mM Cl\textsuperscript{2-} as the magnesium salt (8 mM MgCl\textsubscript{2}), [3H]CGP 39653 binding was stimulated to a maximum of 400% of control, whereas the level of binding in the presence of 16 mM Cl\textsuperscript{2}, as the sodium salt (16 mM NaCl), was 180% of control.

In addition, increasing the concentration of sucrose (up to 0.5 M) had no stimulatory effect on [3H]CGP 39653 or on [3H]CGS 19755 binding (data not shown).

To assess the possibility that mono- and divalent cations share a common mechanism for stimulation of [3H]CGP 39653 binding, we examined the effect of coincubation with mono- and divalent cations, at different concentrations, on [3H]CGP 39653 binding (4 nM) (fig. 2). Although NaCl alone simulated the binding of [3H]CGP 39653, when combined with various concentrations of MgCl\textsubscript{2}, NaCl did not produce any stimulation of binding beyond that which was obtained with MgCl\textsubscript{2} alone (figs. 2A and C). In fact, at concentrations of 32 mM or more, added NaCl decreased the stimulatory effects of MgCl\textsubscript{2} (fig. 2A), and added KCl decreased the stimulatory effects of CaCl\textsubscript{2} on [3H]CGP 39653 binding (fig. 2B), at concentrations of MgCl\textsubscript{2} ≥ 0.5 mM, NaCl was unable to stimulate [3H]CGP 39653 binding (fig. 2C). Furthermore, at the highest concentrations of added salts, binding was reduced to basal levels or below (figs 2A to C). Similar interactions between MgCl\textsubscript{2} and NaCl on stimulation of [3H]CGS 19755 binding were also observed (data not shown). The lack of additivity between the effects of mono- and divalent cations was consistent with the view that inorganic cations act on NMDA recognition sites through the same stimulatory mechanism.

**Fig. 1.** Effect of the addition of mono- and divalent cations on [3H]CGP 39653 binding. The data are from a single experiment that was repeated at least twice for each salt with similar results (see table 1). Each point is the mean of four replicates with S.E.M. < 5%. The concentration of [3H]CGP 39653 was 4 nM. In the absence of added cations (control condition, 12 mM K’), specific binding was 0.55 ± 0.03 pmol/mg protein.

**TABLE 1**

<table>
<thead>
<tr>
<th>Cation</th>
<th>EC\textsubscript{50} mM</th>
<th>Maximal Stimulation (% of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li\textsuperscript{+}</td>
<td>5.3 ± 0.7</td>
<td>236 ± 24</td>
</tr>
<tr>
<td>Na\textsuperscript{+}</td>
<td>6.4 ± 1.0</td>
<td>227 ± 23</td>
</tr>
<tr>
<td>K\textsuperscript{+}</td>
<td>6.2 ± 1.1</td>
<td>226 ± 17</td>
</tr>
<tr>
<td>Cs\textsuperscript{+}</td>
<td>5.5 ± 1.0</td>
<td>233 ± 34</td>
</tr>
<tr>
<td>Mg\textsuperscript{2+}</td>
<td>0.23 ± 0.02</td>
<td>388 ± 40</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+}</td>
<td>0.28 ± 0.04</td>
<td>427 ± 23</td>
</tr>
<tr>
<td>Sr\textsuperscript{2+}</td>
<td>0.32 ± 0.05</td>
<td>397 ± 51</td>
</tr>
<tr>
<td>Ba\textsuperscript{2+}</td>
<td>0.37 ± 0.04</td>
<td>390 ± 43</td>
</tr>
</tbody>
</table>

The data represent the means ± S.E.M. of three independent experiments performed in quadruplicate for each condition. Each EC\textsubscript{50} estimate was based on data from at least five concentrations of salts.

Effects of cations on parameters of [3H]CGP 39653 binding to NMDA recognition sites. To study the possible mechanism of the stimulatory effects of cations, we used a Scatchard analysis of [3H]CGP 39653 binding data obtained in the absence and presence of cations. In preparations of well-washed membranes in 10 mM HEPES-KOH buffer without added cations, there appeared to be at least two populations of binding sites for [3H]CGP 39653. A Scatchard plot from pooled data obtained in 4 independent experiments is shown in figure 3. Results of these four and eight additional experiments are presented in table 2. Approximately 25% of specific binding sites (see table 2) were in a high-affinity conformation (K\textsubscript{d1} = 6.4 ± 0.4 nM; B\textsubscript{max1} = 1.0 ± 0.1 pmol/mg protein), although the remainder were in a low-affinity conformation (K\textsubscript{d2} = 210 ± 30 nM; B\textsubscript{max2} = 3.2 ± 0.2 pmol/mg protein).
pmol/mg protein). Under the same conditions, [3H]CGP 19755 binding also revealed the presence of two binding sites, with similar values of Bmax1 and Bmax2, but affinities of both populations were lower (around 20 and 400 nM for high and low-affinity populations, respectively, n = 2 experiments, data not shown).

Scatchard analysis provided a different picture (fig. 4) when binding of [3H]CGP 39653 was measured in the presence of high concentrations of cations. In the presence of 10 mM MgCl2, a concentration close to that which produced maximal stimulation of [3H]CGP 39653 binding (see fig. 1), data on [3H]CGP 39653 binding failed to fit significantly better to a two-site than to a one-site model (by F-test). Under these conditions, Kd (5.6 ± 0.9 nM) for binding was close to that of the high-affinity component in the absence of added cations, and Bmax (4.3 ± 0.3 pmol/mg protein) was almost equal to total Bmax (Bmax1 + Bmax2) measured in incubations without added cations (see fig. 4A). These results suggested that divalent cations converted the low-affinity [3H]CGP 39653 binding sites into high-affinity sites.

Monovalent cations apparently converted low-affinity sites to high-affinity sites in the same manner, but the magnitudes of the changes in percentages of high and low-affinity sites, in the stimulatory range of concentration of the cations, were less remarkable (fig. 4C). Thus, in the presence of 50 mM NaCl (the concentration that produced maximal stimulation of [3H]CGP binding, fig. 1), [3H]CGP 39653 binding was characterized better by a two-site model than by a one-site model (P < .05 by F-test). The population of high-affinity sites in this case represented about 70% of all specific binding sites and had about 2.5 times the density observed in the control condition. Extremely high concentrations of mono- and divalent cations caused inhibition of [3H]CGP 39653 binding. Under these conditions [see results obtained with 80 mM MgCl2 (fig. 4B) and 200 mM NaCl (fig. 4D)], Scatchard analysis revealed only one population of binding sites with lower affinity than that seen in assays with optimal concentrations of the MgCl2 (fig. 4A). Table 2 presents results of the estimation of [3H]CGP 39653 binding parameters under these different conditions.

As assays of [3H]CGP 39653 under all of the aforementioned conditions yielded almost the same value of total Bmax (about 4 pmol/mg protein summing Bmax1 and Bmax2 when a two-site model fit the data better than a one-site model, see table 2), it appears that mono- and divalent cations increase [3H]CGP 39653 binding by conversion of binding sites from the low- to the high-affinity state. Inhibition of [3H]CGP 39653 binding by salts at high concentrations apparently resulted from decreasing affinity of the binding sites.

Pharmacological sensitivity of [3H]CGP 39653 binding sites converted to a high-affinity state. To determine whether the low-affinity component of [3H]CGP 39653 binding assayed in the absence of cations was indeed NMDA recognition sites rather than other sites labeled with [3H]CGP 39653, we tested the competition of several agonists and antagonists for NMDA recognition sites as well as ligands for other types of glutamate receptors or for a glutamate transporter against 3 nM [3H]CGP 39653. These assays were performed in the absence and presence of 10 mM MgCl2.

At 10 mM MgCl2, all [3H]CGP 39653 binding sites were in the high-affinity state (fig. 4A; table 2) and their density was equal to the summed densities of low- and high-affinity binding sites (75% and 25% of total Bmax, respectively), observed in the absence of cations (see fig. 3; table 2). Thus, in the presence of 10 mM MgCl2 most (about 75%) of [3H]CGP 39653 binding reflected interactions with binding sites that were converted by MgCl2 from the low-affinity state (in absence of added cations).

Competition assays with a number of ligands for different types of glutamate binding sites in the brain tissue demonstrated that those [3H]CGP 39653 binding sites converted to the high-affinity state by MgCl2 have a pharmacological profile typical of NMDA recognition sites (table 3). Ligands for NMDA recognition sites (glutamate, CPP, AP5, NMDA) had much higher potencies as inhibitors of [3H]CGP 39653 binding than ligands for non-NMDA glutamate binding sites (quisqualate, kainate, cystine, trans-ACPD). When the potencies of inhibitors of [3H]CGP 39653 binding in the presence of 10 mM MgCl2 were compared to the potencies of the same inhibitors without added cations, a strong correlation (r = 0.985) was obtained (fig. 5). Therefore, the close correlation in the rank order of potencies of the competing drugs tested indicates that the high-affinity sites assayed under the basal, unstimulated condition have the same pharmacological specificity (vis-à-vis binding sites on glutamate receptors) and that the sites that are converted by cations are indeed NMDA recognition sites.

Lack of additivity between stimulatory effects of inorganic cations and spermidine on [3H]CGP 39653 binding. Consistent with previous results indicating that polyamines increase [3H]CGP 39653 binding by conversion of low- to high-affinity sites (London and Mukhin, 1995), Scatchard analysis of [3H]CGP 39653 binding in the presence of 0.5 mM spermidine yielded data that fit a one-site model, with Kd = 3.8 ± 0.1 nM and Bmax = 3.5 ± 0.1 pmol/mg.

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**Fig. 3.** Scatchard plot of [3H]CGP 39653 binding (concentration range: 0.3–400 nM) in the absence of cations. Data were pooled from four independent experiments performed in quadruplicate with S.E.M. < 4% for quadruplicates. Similar results were obtained in eight additional experiments on membrane samples from different preparations (see table 2).
protein (fig. 6). As only one population of high-affinity sites were observed in the presence of spermidine and the density of sites was similar to that obtained by summing the densities of high- and low-affinity sites assayed in the absence of added inorganic cations or polyamines, the data supported the hypothesis that spermidine shared the same mechanism of stimulation of [3H]CGP 39653 binding as observed with inorganic cations.

To test this hypothesis, we determined the effect of increasing concentrations of spermidine on [3H]CGP 39653 binding in the absence of added inorganic cations, as well as in the presence of either 5 mM MgCl2 or 50 mM NaCl, which are maximally activating salt concentrations. Although progressively increasing concentration of spermidine alone increased radioligand binding, with a maximal effect at 0.64 mM, spermidine did not enhance the level of binding beyond the maximal level obtained with either MgCl2 or NaCl (fig. 7A). Similarly, although progressively increasing concentrations of MgCl2 alone enhanced binding, with a maximal effect at a concentration of about 5 to 10 mM, adding this inorganic salt had no further stimulatory effect at a maximally stimulating concentration of spermidine (0.5 mM) (fig. 7B).

TABLE 2
Effects of cations on parameters of [3H]CGP 39653 binding

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control (n = 12)</th>
<th>10 mM MgCl2 (n = 5)</th>
<th>80 mM MgCl2 (n = 5)</th>
<th>50 mM NaCl (n = 4)</th>
<th>200 mM NaCl (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kd1 nM</td>
<td>6.4 ± 0.4</td>
<td>5.6 ± 0.9</td>
<td>47 ± 6</td>
<td>6.2 ± 0.5</td>
<td>30 ± 4</td>
</tr>
<tr>
<td>Kd2 nM</td>
<td>210 ± 30</td>
<td>165 ± 40</td>
<td>20.0 ± 0.2</td>
<td>3.6 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Bmax1 pmol/mg</td>
<td>1.0 ± 0.1</td>
<td>4.3 ± 0.3</td>
<td>4.2 ± 0.3</td>
<td>2.2 ± 0.4</td>
<td>4.2 ± 0.1</td>
</tr>
<tr>
<td>Bmax2 pmol/mg</td>
<td>3.2 ± 0.2</td>
<td>4.3 ± 0.3</td>
<td>4.2 ± 0.3</td>
<td>3.6 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Bmax total pmol/mg</td>
<td>4.3 ± 0.2</td>
<td>4.3 ± 0.3</td>
<td>4.2 ± 0.3</td>
<td>3.6 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

Well-washed membranes were incubated at 9 to 11 concentrations of [3H]CGP 39653 (0.3–400 nM) in the presence of salt concentrations as indicated. In control conditions, incubation was done in 10 mM HEPES · KOH. Results represent the means ± S.E.M. of 4 to 12 experiments performed in triplicate or quadruplicate.

**Discussion**

Our results demonstrate that mono- and divalent inorganic cations convert NMDA recognition sites from a low- to a high-affinity state, as does spermidine. This conclusion is supported by the fact that the density of [3H]CGP 39653 binding sites, assayed in the presence of added divalent cations (one-site model), is equal to the summed densities of low- and high-affinity sites assayed without added cations (two-site model). Furthermore, [3H]CGP 39653 binding sites converted by MgCl2 from the low- to the high-affinity state show a pharmacological profile (rank order of potencies of competing drugs) that is typical of NMDA recognition sites.

Conversion of NMDA recognition sites from the low- to the high-affinity state would increase the sensitivity of NMDA receptors to relevant ligands, and thereby would enhance the sensitivity of the channel to activation. Indeed, such effects of divalent cations have been described previously. In particular, low concentrations of CaCl2 or MgCl2 stimulate [3H]dizocilpine binding (an index of channel activation) at low, but not at high concentrations of glutamate (Reynolds and Miller, 1988; Enomoto et al., 1992). The purported increase in sensitivity was not, however, seen as a change in the IC50.
data for NMDA and glutamate shown in table 3, because at the radioligand concentration used (3 nM), $85\%$ of the specific binding reflected labeling of high-affinity sites only, whether MgCl$_2$ was added or not. With regard to monovalent cations, increasing the molarity of Tris HCl increases the potencies of competitive agonist and antagonists of NMDA receptors in modulating $[3H]dizocilpine binding (Hood et al., 1992).

The demonstration of two populations of NMDA recognition sites by binding assay of rat forebrain is not unique. Two populations of sites were observed previously by centrifugation assays using $[3H]CPP$ (van Amsterdam et al., 1992) and $[3H]CSG 19755$ (Murphy et al., 1988) as radioligands. The use of $[3H]CGP 39653$, which has higher affinity than that of previously available radioligands (Sills et al., 1991), allowed rapid filtration assay to detect two affinity states of NMDA recognition sites and to demonstrate the possibility of cation-dependent conversion of these sites from a low- to a high-affinity state.

The conclusion from a previous report, which demonstrated that MgCl$_2$ and polyamines enhanced $[3H]CGP 39653$ binding, was that the stimulation of binding reflected an increase in the affinity for the radioligand (Reynolds, 1994). Support presented for this view was derived primarily from

### TABLE 3

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Control IC$_{50}$ (μM) $[K_i$ (μM)]</th>
<th>$n_H$</th>
<th>10 mM MgCl$<em>2$ IC$</em>{50}$ (μM) $[K_i$ (μM)]</th>
<th>$n_H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate</td>
<td>0.032 ± 0.004 [0.022 ± 0.003]</td>
<td>0.84</td>
<td>0.043 ± 0.003 [0.028 ± 0.002]</td>
<td>0.89</td>
</tr>
<tr>
<td>CPP</td>
<td>0.055 ± 0.005 [0.037 ± 0.003]</td>
<td>0.83</td>
<td>0.048 ± 0.005 [0.031 ± 0.003]</td>
<td>1.13</td>
</tr>
<tr>
<td>AP5</td>
<td>0.22 ± 0.03 [0.15 ± 0.02]</td>
<td>1.0</td>
<td>0.17 ± 0.06 [0.11 ± 0.04]</td>
<td>1.05</td>
</tr>
<tr>
<td>NMDA</td>
<td>1.19 ± 0.06 [0.81 ± 0.04]</td>
<td>1.0</td>
<td>1.05 ± 0.13 [1.36 ± 0.20]</td>
<td></td>
</tr>
<tr>
<td>Quisqualate</td>
<td>5.5 ± 0.3 [3.7 ± 0.2]</td>
<td>1.0</td>
<td>10.9 ± 0.4 [7.1 ± 0.3]</td>
<td>1.03</td>
</tr>
<tr>
<td>Kainate</td>
<td>197 ± 2 [134 ± 2]</td>
<td>1.05</td>
<td>233 ± 45 [152 ± 29]</td>
<td>1.07</td>
</tr>
<tr>
<td>Cystine</td>
<td>1150 ± 70 [783 ± 48]</td>
<td>0.96</td>
<td>1600 ± 40 [1040 ± 26]</td>
<td>0.95</td>
</tr>
<tr>
<td>Trans-ACPD</td>
<td>1980 ± 180 [1350 ± 120]</td>
<td>1.04</td>
<td>2010 ± 570 [1310 ± 370]</td>
<td>1.23</td>
</tr>
</tbody>
</table>

Values represent the mean IC$_{50}$ $[K_i$ $\mu M$ ± SEM values and pseudo-Hill coefficients $(n_H)$ obtained in three independent experiments performed in quadruplicate. At 3 nM $[3H]CGP 39653$, the proportion of binding to high affinity sites exceeded 85%, estimated using $K_d$ and $B_{max}$ values for the high affinity and low affinity sites; therefore, $K_i$ values were obtained from the IC$_{50}$ values, using values of $K_d$ for high affinity sites in the presence and absence of MgCl$_2$ (table 2).
determinations of $K_d$ in the absence and presence of added MgCl$_2$ and spermine. However, in that study, [$^3$H]CGP 39653 binding was assayed using radioligand concentrations (0.5–20 nM) that did not allow detection of the low-affinity binding component, which has $K_d$ about 200 nM, well above the concentration range tested.

The biphasic effect of salts on [$^3$H]CGP 39653 binding reflects two mechanisms. Stimulatory effects of salts on binding appear to reflect conversion of NMDA recognition sites from low- to high-affinity states. At the same time, decrements in binding at high salt concentrations reflect a reduction in affinity of the sites, all of which are in the same high-affinity conformation.

The experiments performed allowed direct comparison of the effects of mono- vs. divalent cations at equal concentrations of anion (chloride). Chloride salts of divalent cations produced a much greater stimulation than salts of monovalent cations when the concentration of chloride was equal under both conditions. Furthermore, as seen in figure 1, salts of divalent cations produced greater stimulation than salts of monovalent cations at every concentration assayed, despite the fact that the salts of the divalent cations had twice the concentration of chloride as salts of monovalent cations. Therefore, the stimulatory effects of increasing salt concentrations apparently were due to cations.

Whereas the stimulation of [$^3$H]CGP 39653 binding by salts of mono- and divalent ions is an effect of cations, the mechanism by which binding is reduced by concentrations of salts higher than those that produce maximal stimulation has not been elucidated. It appears that inhibition at high salt concentrations reflects a reduction in affinity of the sites while they still remain in a high-affinity conformation (fig. 4). Our preliminary observations suggest that anions play an important role in this reduction in affinity (Mukhin et al., 1994).

To illustrate the difference between the effects of mono- and divalent cations regarding maximal stimulation and the role of anions in the biphasic nature of this effect, we applied a mathematical model for the interactions of two activators (mono- and divalent cations) of different affinities with radioligand binding in the presence of varying concentrations of a competitive inhibitor (anions, e.g., Cl$^-$. The present evidence that the primary mechanism of positive modulation is conversion from a low- to a high-affinity state (figs. 3 and 4) supports the first assumption of the model (equations 1 and 12, see "Materials and Methods"), i.e., that stimulation due to cations is a positive allosteric modulation. Our assumption that mono- and divalent cations compete for binding to modulatory sites is supported by the lack of additivity in their stimulatory effects on radioligand binding (fig. 2). Furthermore, the assumption that inhibition due to anions is competitive was validated by the effect of increasing Cl$^-$ to reduce $K_d$ (fig. 4). Finally, in view of the determined values of $K_d$ and $B_{max}$ for low- and high-affinity sites, and the fact that all assays on the effects of added salts on [$^3$H]CGP 39653 binding were performed at a radioligand concentration of 4 nM, the Law of Mass Action dictated that more than 75% of the labeling always represented interactions with high-affinity sites. Therefore, the exclusion of binding to low-affinity sites in this model was acceptable. In conclusion, it appears that equation 12 is applicable to description of the ratio of radioligand binding in the presence of added salts relative to binding observed in the control condition, as a function of the concentration of added salts.

$$B_S = \left( 1 + \frac{K_{dK}^{\cdot} \cdot C}{12mM} \right) \cdot \left( \frac{12mM \cdot K_{dK}^{\cdot} \cdot C}{K_{dK}^{\cdot} \cdot K_{dC}^{\cdot} + 12mM \cdot K_{dC}^{\cdot} + K_{dK}^{\cdot} \cdot C} \right) \cdot \left( 1 + (S \cdot \nu IC_{50})^z \right)$$

On the right side of the equation, the left-most component denotes the magnitude of stimulation of [$^3$H]CGP 39653 binding; the middle component reflects the potentials of added cations as stimulatory modulators; and the right-most component indicates the inhibitory potency of Cl$^-$. To determine how well this function describes the data that were obtained, we applied it to the values of B/B$_0$ shown in figure 1 for added Ca$^{2+}$ and K$^+$, and estimated IC$_{50}$, $K_{dC}^{\cdot}$, $K_{dK}^{\cdot}$, and the Hill coefficient, using nonlinear regression analysis. The graph shown in figure 8 indicates that the function reflects the processes measured. The values obtained were as follows: $K_{dC} = 0.3$ mM, $K_{dK}^{\cdot} = 46$ mM, IC$_{50} = 90$ mM for Cl$^-$, and the Hill coefficient for the interaction of Cl$^-$ = 2.3. The value of $K_{dC}$ corresponded well with the ED$_{50}$ determined experimentally for Ca$^{2+}$ (table 1) although $K_{dK}^{\cdot}$ generated by the model was about 7-fold more than the ED$_{50}$ determined experimentally. The discrepancy primarily reflects the fact that the equation determines the value of $K_d$ independent of added cations and anions whereas the ED$_{50}$ calculations were determined under conditions (i.e., addition of Cl$^-$) that masked maximal stimulation. In addition, the ED$_{50}$ determination did not take into consideration any stim-

Fig. 7. Absence of additivity between the stimulatory effects of spermidine and mono- and divalent cations. A. Effects of increasing concentration of spermidine (SPD) in the absence of other added cations (control, CNTRL) and in the presence of 5 mM MgCl$_2$ or 50 mM NaCl on [$^3$H]CGP 39653 binding (4 nM). B. Effect of increasing concentration of MgCl$_2$ in the absence of other added cations (control) and in the presence of 0.5 mM spermidine on [$^3$H]CGP 39653 binding (4 nM). The data (A and B) represent results of a single experiment performed with four replicates per condition (S.E.M. < 5% for replicates), and repeated once with the similar results.
Cations and NMDA Receptor Binding

Fig. 8. Modeling of the effect of increasing salt concentration on [3H]CGP 39653 binding. Experimentally obtained values of B/B0 shown in figure 1 for effects of added CaCl2 (open circles) and KCl (closed circles) were fit to the curve generated by equation 12, where:

\[
\frac{B_b}{B_c} = \left( \frac{K_{ac}}{12 \text{mM}} \right) \cdot \left( \frac{1}{1 + \left( S \cdot IC_{50} \right)^n} \right)
\]

and by which \(K_{ac} = 0.3 \text{mM for Ca}^{2+}\), \(IC_{50} = 90 \text{mM for Cl}^{-}\) and the Hill coefficient for the interaction of Cl\(^{-}\) (\(n = 2.3\)) were estimated using nonlinear regression analysis.

ulation due to the 12 mM K\(^{+}\) introduced in the buffer. Thus, our experimental determination of ED\(_{50}\) was influenced by an underestimation of the degree of stimulation due to K\(^{+}\). The IC\(_{50}\) value for Cl\(^{-}\) is close to the value (130 mM) that we obtained experimentally in preliminary assays (Mukhin et al., 1994 and unpublished data), and the Hill coefficient of 2.3 suggests positive cooperativity.

Our study did not demonstrate substantial selectivity in effects of mono- and divalent cations and spermidine (figs. 2 and 7). These findings suggest that all of the cations studied affect binding by a common mechanism to produce conversion of low- and high-affinity sites. Nonetheless, our results do not exclude the possibility that cations can also increase affinity of the high affinity binding sites as well. Notably, the affinity measured in the presence of 0.5 mM spermidine was higher than generally assayed for the high-affinity site in the absence of added cations or even in the presence of maximally stimulating concentrations of inorganic cations (fig. 6; table 2).

The present findings provide insight into the complexity of ligand interactions with NMDA recognition sites, and are relevant to the interpretation and design of in vitro binding studies of this receptor. Furthermore, they are important for the interpretation of assays on the effect of agonists and antagonists on the binding of [3H]dizocilpine and other ligands for the phencyclidine receptor. These assays generally have been performed in a low molarity buffer without any additional salts (Ransom and Stec, 1988; Reynolds and Miller, 1988; Javitt and Zukin, 1989; Enomoto et al., 1992). Under such conditions, most of the NMDA recognition sites are in the low-affinity state. However, the concentrations of Na\(^{+}\), Mg\(^{2+}\) and Ca\(^{2+}\) measured in extracellular fluid are in the range of those that produce a shift from low- to high-affinity in vitro. Although the implication of this observation is that the sites would be in the high-affinity conformation in vivo, the affinity state of the receptor obviously could be influenced by the actions of a variety of cations, anions, and other potential modulatory substances.

References


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