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*ACCELERATED COMMUNICATION*

# Spermine Enhances Binding to the Glycine Site Associated with the *N*-Methyl-D-aspartate Receptor Complex

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**SUMMARY**

Spermine enhanced strychnine-insensitive [$^3$H]glycine binding 3-fold with an $EC_{50}$ of 27 ± 3.1 μM. Spermidine and putrescine were without effect, whereas the ethylenediamine analog of spermine had an intermediate effect. Eadie-Hofstee analysis revealed that spermine increased the affinity of glycine for its receptor without a significant change in receptor density. This effect persisted in the presence of glycine or *N*-methyl-D-aspartate receptor antagonists. Furthermore, spermine produced a leftward shift in the $IC_{50}$ of glycine agonists in displacing [$^3$H]glycine binding, without altering the $IC_{50}$ for glycine antagonists. These data indicate that spermine interacts with the glycine receptor through a novel binding site and, further, that spermine can be used to discriminate glycine agonist and antagonist binding.

Polyamines are ubiquitously distributed in body tissue. The concentrations of spermine and spermidine in the brain are reportedly in the high micromolar range (1). Recently, Ransom and Stec (2) reported that spermidine and spermine, but not putrescine or cadaverine, enhanced [$^3$H]MK 801 binding in a manner reversed by NMDA antagonists. We have confirmed this finding using [$^3$H]TCP binding (3). The [$^3$H]TCP and [$^3$H]MK 801 binding assays have been utilized by many laboratories including ours, as assays of the function of the NMDA receptor channel complex (4). In their study, Ransom and Stec (2) demonstrated a reciprocal interaction between glutamate, glycine, and spermidine. The $EC_{50}$ of each of these drugs in enhancing [$^3$H]MK 801 binding was decreased in the presence of either of the other two. Moreover, they showed an additive effect of spermidine when combined with maximal concentrations of glutamate and glycine on [$^3$H]MK 801 binding, suggesting a distinct site for the polyamines.

This study was conducted to further characterize the interaction between polyamines and the NMDA receptor-ionophore complex. Here we report that spermine, but not spermidine or putrescine, enhances [$^3$H]glycine binding by increasing the affinity of glycine for its receptor, without a significant change in receptor density. This effect was not blocked by glycine or NMDA antagonists. Further, spermine also increased the binding affinity for D-serine, but not 7-Cl-KYNA or cycloleucine, suggesting a unique ability of spermine to regulate agonist, but not antagonist, binding to the receptor.

## Experimental Procedures

**Materials.** [$^3$H]Glycine (49.0 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Spermine, spermidine, and putrescine were purchased from Sigma Chemical Co. (St. Louis, MO). *N,N'*-Bis-(3-aminopropyl)ethylenediamine and *N,N'*-bis-(3-aminopropyl)-1,3-propanediamine were purchased from Aldrich Chemical Co. (Milwaukee, WI). CPP was purchased from Research Biochemical Inc. (Natick, MA). 7-Cl-KYNA was kindly donated by Sterling-Winthrop Research Institute (Rensselaer, NY).

**Methods.** [$^3$H]Glycine binding assays were performed in a buffy coat rat cortical membrane preparation, as previously described (5). [$^3$H]Glycine was incubated at 20 nM with aliquots of approximately 200 μg protein/tube, in the presence or absence of the test drug dissolved in 50 mM Na-free HEPES containing 10 μM strychnine and buffered to pH 7.4 with KOH, for 30 min at 4°. The samples were then filtered over GF/C filters on a Brandel M-24 cell harvester, followed by washing with 6 ml of ice-cold buffer over a 6-sec period. In equilibrium saturation experiments, seven concentrations of [$^3$H]glycine, ranging from 20 to 1280 nM, were utilized. Nonspecific binding was determined in the presence of 100 μM glycine. Protein concentrations were determined by the method described by Bradford (6).

**Statistical analysis.** All experiments were replicated three to seven times. Saturation isotherms, as well as the $IC_{50}$ estimates, were analyzed by an iterative nonlinear regression program (7) as adapted for the IBM PC (8). $EC_{50}$ values were estimated by fitting the data to a

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**ABBREVIATIONS:** [$^3$H]MK 801, (+)-[$^3$H]-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine; [$^3$H]TCP, [$^3$H]1-[1-(2-thienyl)cyclohexyl]piperidine; NMDA, *N*-methyl-D-aspartate; 7-Cl-KYNA, 7-chlorokynurenic acid; CPP,3-((+)-2-carboxypiperazin-4-yl)prop-1-phosphonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

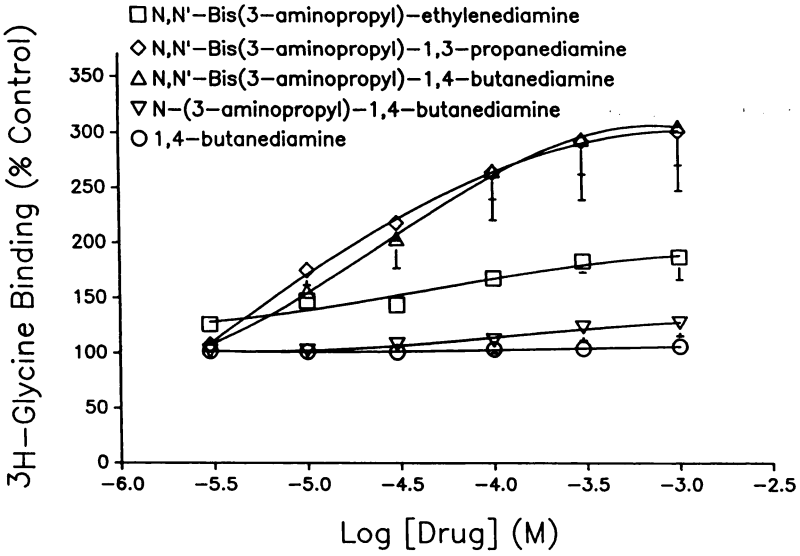


**Fig. 1.** Concentration-response curves of various polyamines on [$^3$H]glycine binding to rat cortical buffy coat membranes. Each *point* is the mean ± standard error of three or four independent determinations, each performed in triplicate.

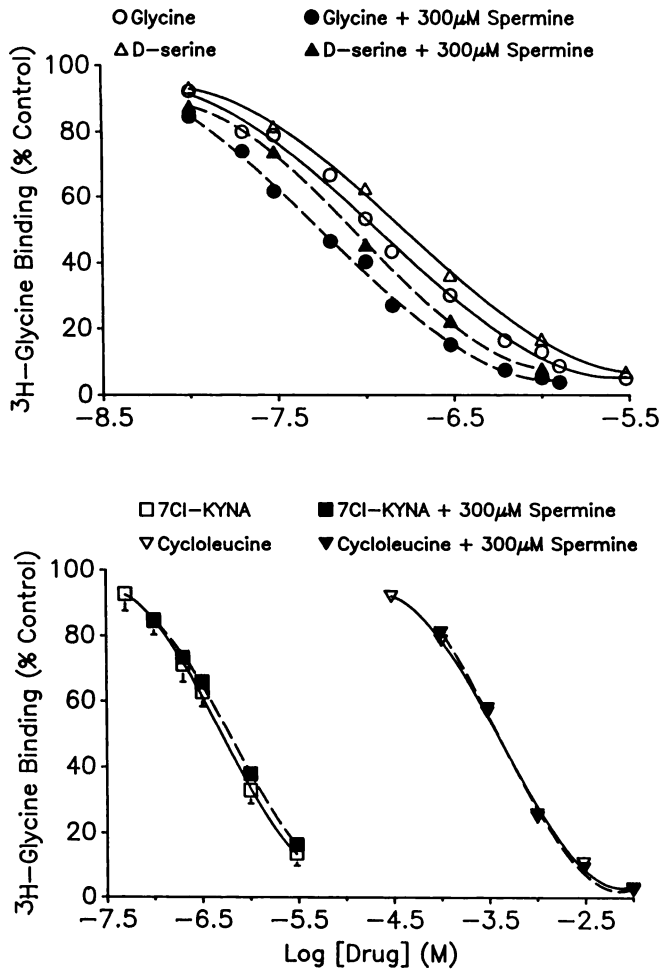


**Fig. 2.** *Upper*, displacement of [$^3$H]glycine binding by glycine and D-serine. The range of concentration used was 10 to 3000 nM. Each *point* is the mean ± standard error of three or four independent experiments, performed in triplicate. *Lower*, displacement of [$^3$H]glycine binding by 7-Cl-KYNA and cycloleucine. The range of concentration used was 50 to 3000 nM and 0.03 to 10 mM, respectively. Each *point* is the mean ± standard error of three independent experiments, performed in triplicate.

sigmoidal logistic equation with the assistance of an iterative curve-fitting program (ALLFIT), according to the method of DeLean and colleagues (9). Results were analyzed by Student's $t$ test, where $p < 0.05$ was considered significant.

## Results

The effects of various polyamines on [$^3$H]glycine binding are shown in Fig. 1. *N,N'*-Bis-(3-aminopropyl)-1,4-butanediamine, better known as spermine (N-3C-N-4C-N-3C-N), enhanced [$^3$H]glycine binding 3-fold, in a concentration-dependent manner, with an $EC_{50}$ of 27 ± 3.1 μM. *N,N'*-bis-(3-aminopropyl)-1,3-propanediamine (N-3C-N-3C-N-3C-N) mimicked the effects of spermine with an $EC_{50}$ of 18 ± 0.7 μM. On the other hand, *N,N'*-bis-(3-aminopropyl)ethylenediamine (N-3C-N-2C-N-3C-N) was less efficacious, producing less than a 2-fold increase at 1 mM. The dose-response curve was very shallow, making a precise estimate of its $EC_{50}$ value impossible. *N*-(3-Aminopropyl)-1,4-butanediamine, most commonly known as spermidine (N-3C-N-4C-N), was without effect, as was 1,4-diaminobutane, (putrescine, N-4C-N).

Because allosteric interactions between the NMDA and glycine receptors have been previously reported (10, 11), it is possible that blocking the NMDA receptor would alter the effect of spermine on [$^3$H]glycine binding. In the presence of 100 μM CPP, a maximally effective concentration (11), spermine still enhanced [$^3$H]glycine binding with an $EC_{50}$ of 35 ± 7.9 μM, a value not significantly different from control ($t$ = 0.98, degrees of freedom = 4). Similarly 0.3 μM 7-Cl-KYNA, a concentration that selectively inhibits [$^3$H]glycine binding by about 50%, had no significant effect on the enhancement of [$^3$H]glycine binding by spermine ($EC_{50}$ = 23 ± 1.5 μM). The effect of spermine was still seen in the presence of 3 μM 7-Cl-KYNA, a concentration that inhibits about 80% of [$^3$H]glycine binding (data not shown).

The persistence of the effect of spermine on [$^3$H]glycine binding in the presence of 7-Cl-KYNA suggested the possibility that spermine selectively alters the way glycine agonists interact with the receptor, without necessarily altering the way antagonists recognize the receptor. To test this hypothesis, we displaced [$^3$H]glycine binding by various glycine agonists and antagonists, in the absence or presence of 300 μM spermine. As

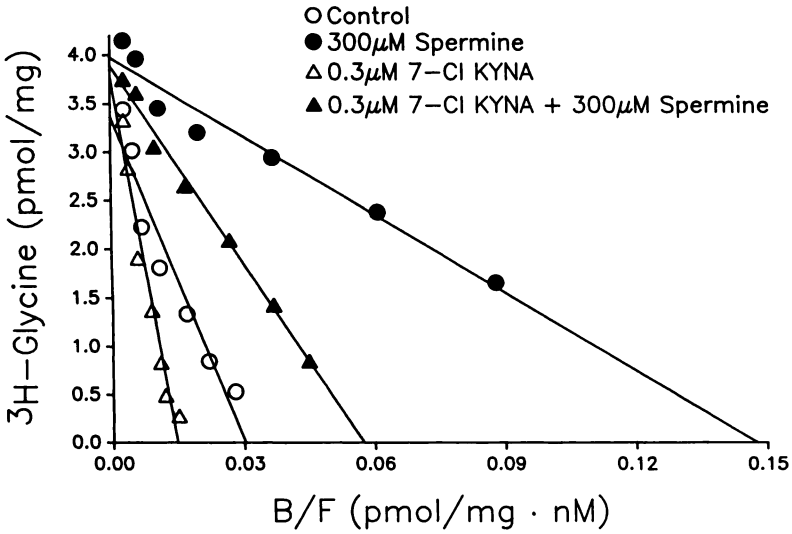


**Fig. 3.** Representative Eadie-Hofstee plots of [$^3$H]glycine binding in 50 mM Na-free HEPES, pH 7.4, at 4° for 30 min, utilizing seven concentrations of [$^3$H]glycine ranging from 20 to 1280 nM, each performed in triplicate. Each treatment was repeated 3 to 7 times. The average effect of spermine (three experiments) was a 2.95-fold decrease in the $K_D$ and, in the presence of 0.3 μM 7-Cl KYNA, a 2.96-fold decrease (three experiments).

shown in Fig. 2, the $IC_{50}$ values for glycine and D-serine displacement of [$^3$H]glycine binding were 109 ± 10.1 and 154 ± 13.7 nM, respectively, similar to those previously reported (12). In the presence of 300 μM spermine, the $IC_{50}$ values for glycine and D-serine were 45 ± 4.0 and 74 ± 5.7 nM, respectively, both of which were significantly different from those in the absence of spermine (Student's $t$ test). On the other hand, Fig. 2 shows that the displacement curves of the competitive glycine antagonists 7-Cl-KYNA (13) and cycloleucine (14) were superimposable in the presence or absence of spermine ($IC_{50}$ for 7-Cl-KYNA is 0.46 ± 0.10 and 0.53 ± 0.04 μM in the absence and presence of 300 μM spermine, respectively; the $IC_{50}$ for cycloleucine was 323 ± 20.8 and 298 ± 21.5 μM in the absence and presence of 300 μM spermine, respectively).

In order to verify that the enhancement of [$^3$H]glycine binding by spermine was due to an increased affinity, as suggested above, we constructed saturation isotherms of [$^3$H]glycine binding in the presence or absence of spermine. Eadie-Hofstee analysis of the data (Fig. 3) revealed that 300 μM spermine produced an approximate 3-fold increase in affinity without a significant change in $B_{max}$ (control, $K_D$ = 145 ± 9.0 nM, $B_{max}$ = 3.14 ± 0.35 pmol/mg of protein; 300 μM spermine, $K_D$ = 49.0 ± 12.0 nM, $B_{max}$ = 3.98 ± 0.24 pmol/mg of protein). The magnitude of the spermine effect was not changed in the presence of 7-Cl-KYNA (control, $K_D$ = 254 ± 23.8 nM, $B_{max}$ = 3.71 ± 0.27 pmol/mg of protein; 300 μM spermine, $K_D$ = 85.8 ± 6.9 nM, $B_{max}$ = 4.84 ± 0.23 pmol/mg of protein). These data completely account for the 3-fold increase in binding observed using 20 nM [$^3$H]glycine (Fig. 1) and the approximate 3-fold shift in the displacement curve by glycine and D-serine (Fig. 2).

## Discussion

From the few polyamine analogs tested in this study, it is difficult to draw definite conclusions about the structural requirements for the activation of this novel polyamine site. However, our results point out that at least four amino groups are required for activation, because spermidine (N-3C-N-4C-N) and putrescine (N-4C-N) were without effect (Fig. 1). Secondly, it seems that the distance between the two central amino groups is also important for activation, because both the butane-containing compound (spermine, N-3C-N-4C-N-3C-N) and the propane derivative (N-3C-N-3C-N-3C-N) were considerably more efficacious than the ethylene derivative (N-3C-N-2C-N-3C-N) (Fig. 1). Perhaps aliphatic or amino substitutions on the spermine backbone would result in compounds with greater or lesser potency.

This enhancement of [$^3$H]glycine binding could explain the mechanism by which spermine enhanced the effect of glycine on [$^3$H]MK 801 (2) or [$^3$H]TCP binding (3). However, similar effects were reported for spermidine (2, 3), a polyamine that was without effect on [$^3$H]glycine binding in this study. In addition, we recently observed that *N,N′*-bis(3-aminopropyl)ethylenediamine, a weak partial agonist in this study, was as effective as spermine and spermidine in enhancing [$^3$H]TCP binding ($EC_{50}$ value of 2.3 μM; data not shown). This suggests either that spermine and spermidine have different mechanisms for enhancing [$^3$H]TCP or [$^3$H]MK 801 binding or that this effect of spermine on [$^3$H]glycine binding is distinct from that on [$^3$H]MK 801 and [$^3$H]TCP binding. The observations that the polyamines still enhanced [$^3$H]MK 801 binding in the presence of saturating concentrations of glycine (2) and that CPP blocked the stimulatory effect of spermine and spermidine on [$^3$H]MK 801 binding (2) but had no effect on spermine-induced [$^3$H]glycine binding can be construed to argue in favor of the hypothesis that the effect of spermine on [$^3$H]MK 801 binding is unrelated to its effect on [$^3$H]glycine binding.

If this is the case, then one might question the potential relevance of the effect of spermine on the glycine receptor. One interesting possibility is suggested by the data shown in Figs. 2 and 3. Eadie-Hofstee analysis (Fig. 3) showed that the $K_D$ for glycine was decreased by about 3-fold in the presence of 300 μM spermine, whereas the $B_{max}$ was not significantly altered. The magnitude of this response was not changed in the presence of the competitive glycine antagonist 7-Cl-KYNA, suggesting that the actions of spermine may be mediated through a site associated with agonist but not antagonist binding. This hypothesis is supported by the data in fig. 2, which show that spermine increased the apparent affinity of glycine and D-serine for the glycine receptor but had no effect on the binding affinity of the glycine antagonists 7-Cl-KYNA and cycloleucine. This suggests that the glycine receptor may exist in agonist and antagonist-preferring states and that spermine selectively increases the affinity with which agonists bind to the agonist-preferring state. In order to account for 7-Cl-KYNA

displacement of [$^3$H]glycine binding in the presence and absence of spermine, the agonist and antagonist binding domains must overlap but must be independently regulated by spermine. Thus, if there are endogenous glycine antagonists, the physiological relevance of this action of spermine becomes obvious.

Magnesium, a divalent cation, was previously demonstrated to enhance [$^3$H]glycine binding (15). Because polyamines are also polyvalent cations, it is possible that the effect of $Mg^{2+}$ and the polyamines are the same. However, in our hands, $Mg^{2+}$ had no effect on [$^3$H]glycine binding (up to 10 mM $MgCl_2$; data not shown). Furthermore, the absence of effect of spermidine (a trivalent cation) and putrescine (a divalent cation), as well as the differences between the similarly charged polyamines spermine and *N,N'*-bis-(1,3-aminopropyl)ethylenediamine, suggests that the effect observed here on [$^3$H]glycine binding is not simply a matter of charge.

In summary, we have demonstrated that spermine interacts with a novel site with specific structural requirements to increase the affinity of [$^3$H]glycine for its receptor in rat cortex. The effects of spermine are unique in that its effects are restricted to glycine agonists, suggesting that agonist and antagonist binding domains of the strychnine-insensitive glycine receptor are independently regulated by spermine. The complete physiological relevance of this effect is not yet understood but, inasmuch as NMDA has been reported to activate ornithine decarboxylase (16), the rate-limiting enzyme in polyamine synthesis, it is important to resolve the interactions between the polyamines and the various effector sites on the NMDA receptor/ion channel complex.

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