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Polyamines potentiate responses of *N*-methyl-D-aspartate receptors expressed in *Xenopus* oocytes

(glutamate/excitatory neurotransmitter/spermine/synaptic transmission)

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ABSTRACT **Glutamate, the major excitatory neurotransmitter in the central nervous system, activates at least three types of channel-forming receptors defined by the selective agonists *N*-methyl-D-aspartate (NMDA), kainate, and quisqualate [or more selectively by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)]. Activation of the NMDA receptor requires glycine as well as NMDA or glutamate. Recent studies have provided evidence that certain polyamines potentiate the binding by NMDA receptors of glycine and the open channel blocker MK-801. To determine whether polyamines alter channel opening, we examined their effects on rat brain glutamate receptors expressed in *Xenopus* oocytes. Our results demonstrate that spermine potentiates the response of the NMDA receptor but has no effect on responses to kainate and quisqualate. Furthermore, spermine increases the maximum response to NMDA and glycine and acts, at least in part, by increasing the apparent affinity of the NMDA receptor/channel complex for glycine. The present findings and the fact that polyamines are a natural constituent of brain suggest that polyamines may play a role in the regulation of glutamatergic transmission.**

The *N*-methyl-D-aspartate (NMDA) receptor is a ligand-gated channel that mediates a slow response to glutamate in neurons of the central nervous system (1–3). An understanding of the pharmacology of agents that modulate activity of the NMDA receptor is important because of the putative role of the receptor in central nervous system processes such as long-term potentiation (4–6), learning (7), developmental structuring (8–11), excitotoxicity, ischemic cell death (12–15), epilepsy, kindling (16, 17), Huntington disease (12, 18), and schizophrenia (19).

Evidence from pharmacological and physiological studies indicates that the NMDA receptor has a number of regulatory domains. Glutamate and glycine have distinct binding sites on the NMDA receptor, both of which must be occupied for the channel to open (20–23). Both phencyclidine receptor ligands and Mg^{2+} block NMDA-induced currents in a voltage-dependent manner, suggesting that they bind within the lumen of the channel (24, 25). Zinc inhibits NMDA responses (26, 27), primarily by binding at a distinct site outside of the channel. It also appears to be a low-affinity channel blocker, much less effective than Mg^{2+} (28).

Recent evidence from receptor binding studies indicates that there is still another regulatory site on the NMDA receptor/channel complex. The polyamines *N,N'*-bis(3-aminopropyl)-1,4-butanediamine (spermine) and *N*-(3-aminopropyl)-1,4-butanediamine (spermidine) increased binding of the potent phencyclidine receptor ligand [^{3}H]MK-801 (29, 30) and of [^{3}H]glycine (31). These results and similar findings by others (32) suggested that certain polyamines might potentiate the NMDA response. Moreover, spermine increased the affinity of the receptor for glycine without affecting its interaction with NMDA (31, 32).

Polyamines have been shown to have a range of effects on responses of glutamate receptors in hippocampal neurons (33) and in *Xenopus* oocytes injected with messenger RNA (mRNA) from rat and chicken brain (34). Spermine potentiated NMDA-induced currents in hippocampal neurons while 1,10-diaminodecane decreased them; diethylenetriamine had no action on NMDA responses but antagonized actions of both spermine and 1,10-diaminodecane, which may therefore be considered positive and negative allosteric modulators (33). In *Xenopus* oocytes, currents induced by NMDA, kainate, and quisqualate were potentiated by spermine (34).

Polyamines are normal constituents of cells and play essential roles in cell proliferation and differentiation (35). Although they may have some influence on neurotransmission (36) and excitability of neurons during epileptiform kindling (37) and their synthesis may be regulated by neuronal activity (38), their role, if any, in modulation of NMDA receptors in the central nervous system is unknown.

The experiments described here were undertaken to examine the pharmacology of modulation of glutamate receptors by selected polyamines. We utilized receptors expressed in *Xenopus* oocytes injected with rat brain mRNA. This expression system is well suited to the study of receptor–ligand interactions, since it can translate channel-forming proteins from exogenous mRNA and provide necessary posttranslational modifications including phosphorylation, glycosylation, and assembly into the membrane. Exogenous excitatory amino acid receptors expressed in oocytes have been shown to have the pharmacological and biophysical characteristics of receptor proteins in neurons (39, 40).

MATERIALS AND METHODS

RNA was extracted from brains rostral to the brainstem of adult male Sprague–Dawley rats by a guanidinium isothiocyanate/cesium chloride density gradient centrifugation method as described (22, 41, 43, 44). Poly(A)$^+$ RNA was then purified by oligo(dT)-cellulose chromatography, dissolved in water, and stored at −70°C until use. Oocytes were removed from the ovarian lobes of anesthetized *Xenopus laevis*. Following incubation for 2 hr in Ca^{2+}-free ND-96 medium (82.5 mM NaCl/2 mM KCl/1 mM $MgCl_2$/5 mM Hepes buffer, pH 7.5) containing collagenase (2 mg/ml), penicillin (100 units/ml), and streptomycin (1 mg/ml), the follicular layer was removed manually. Stage V and VI oocytes (45) were then selected and injected with RNA (50 ng per oocyte). Oocytes were maintained for 5–9 days at 18°C in Leibovitz's L-15 medium supplemented with penicillin (100 units/ml) and streptomycin (1 mg/ml) and buffered with Hepes (5 mM, pH 7.6). Electrophysiological recordings took place in a chamber

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Abbreviations: NMDA, *N*-methyl-D-aspartate; I_{max}, maximum current.

with a volume of approximately 0.3 ml. This chamber makes rapid changes in the bath solution possible. While recording, oocytes were perfused with Mg^{2+}-free amphibian Ringer's solution (116 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/10 mM Hepes buffer, pH 7.2). NMDA, L-glutamate, and glycine were obtained from Sigma. 7-Chlorokynurenic acid was kindly supplied by R. Keith (ICI Americas, Wilmington, DE). Spermine and putrescine were obtained from Aldrich. Except where indicated, test drugs were applied 10–15 sec before the solution containing NMDA. If the glycine concentration was not varied during the experiment, all solutions contained the indicated concentration of glycine. Electrophysiological recordings were carried out using a two-electrode voltage clamp. The microelectrodes were filled with 1 M KCl (1–5 MΩ). Oocytes were clamped at a holding potential of −60 mV unless otherwise indicated.

Concentration–response data were fit to the equation $I = I_{max}[A/(A + K_d)]^n$, where I is the response amplitude, and A is the concentration of the active agent. The maximum current (I_{max}), the dissociation constant (K_d), and the Hill coefficient (n) were fit as free parameters using a nonlinear curve-fitting algorithm. All calculated values of I_{max}, K_d, and n are reported as mean ± SEM.

RESULTS

The typical response to NMDA in the presence of low glycine is an early peak current that declines to a steady state or plateau response as the receptor desensitizes (Fig. 1*A*, first record). Spermine reversibly potentiated both the peak and the plateau phases of the NMDA response (Fig. 1*A*), but it did not affect the short latency currents evoked by kainate or quisqualate or the long latency, inositol trisphosphate-mediated response induced by quisqualate (Fig. 1 *B* and *C*).

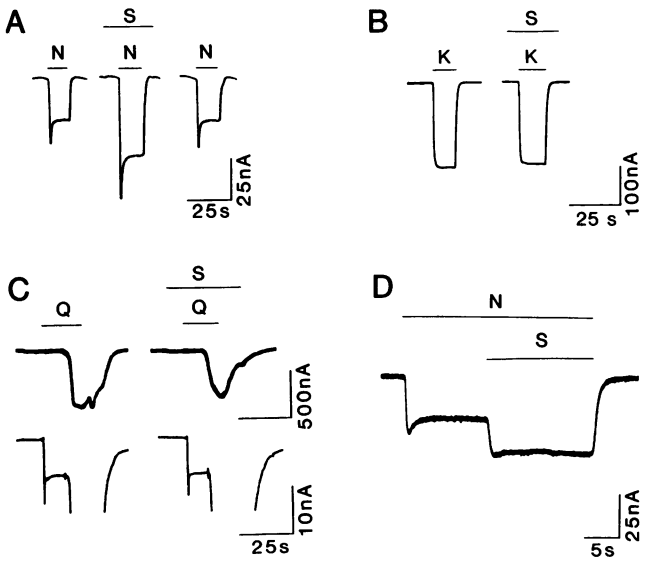

FIG. 1. Spermine selectively increases responses to NMDA in the presence of glycine. Oocytes were voltage clamped at −60 mV throughout the experiment. (*A*) Currents induced by application of 250 μM NMDA in the absence of spermine (N, first and third records) and with 250 μM spermine (S, second record) in the bath. The records were separated by 15–30 sec; 500 nM glycine was added to all solutions. (*B*) The response to 100 μM kainate (K, first record) was not augmented by 250 μM spermine (second record). (*C*) The response to quisqualate (Q) consists of both a large amplitude, long latency G-protein-linked response (upper left record) and a smaller, early ionotropic response (lower left record, higher gain). Neither of these responses was altered by 250 μM spermine (right records). (*D*) Application of 200 μM spermine in the presence of 250 μM NMDA and 1 μM glycine produces a rapid increase in the inward current. The oocyte was exposed to NMDA and glycine for 15 sec before spermine was applied.

Spermine (1–1000 μM) alone did not produce any current in the oocyte, nor did it alter the membrane resistance of the cell. The onset of the spermine effect was fast ($\tau \leq 0.5$ sec, Fig. 1*D*), which suggests that it was acting extracellularly and not through uptake, a second messenger, or some other slow signal transduction mechanism. The time constant of spermine wash-off was slower than the onset ($\tau \leq 1$ sec; data not shown). Spermidine (500 μM) also potentiated the NMDA response (data not shown).

Potentiation of the NMDA-induced current by spermine increased with spermine concentration over the range 1–250 μM (Fig. 2, ● and ▲); at higher concentrations the effect diminished (Fig. 2, ○ and △). Hill plots were fit to the rising phase; that is, for spermine concentrations up to 250 μM. The apparent K_d of the peak of the response was 39 ± 4 μM (Hill coefficient = 2.0 ± 0.1), while for the plateau response the K_d was 48 ± 6 μM (Hill coefficient = 1.9 ± 0.2, n = 9).

Spermine could increase the NMDA response by a number of mechanisms. It did not appear to alter the ion selectivity of the channel. The current–voltage relationship of the NMDA response was linear between −60 and −20 mV both in the presence and in the absence of spermine, and the extrapolated reversal potential was unchanged (Fig. 3). Spermine (250 μM) also did not alter the K_d or Hill coefficient for NMDA (with 10 μM glycine) but proportionally increased the response at each concentration of NMDA, including saturating levels (data not shown).

Spermine did affect the parameters of glycine action. Concentration–response curves in which glycine concentration was varied with 200 μM spermine or no spermine (in the presence of 250 μM NMDA) indicated that the K_d as well as the I_{max} were altered (Fig. 4). For three oocytes tested, the K_d for glycine of the peak response in the absence of spermine was 930 ± 102 nM while in the presence of 200 μM spermine it was 330 ± 18 nM. For the plateau response also, spermine decreased the K_d and increased the I_{max} by similar amounts. The Hill coefficients were unchanged. Because spermine changed the K_d of glycine, the degree of potentiation produced by spermine depended on the concentration of glycine. At saturating levels of both NMDA (250 μM) and glycine (10 μM) the peak response with 200 μM spermine was 178 ± 23% of the peak with no spermine in the bath. At low glycine concentration (0.1 μM, with 250 μM NMDA), 200 μM spermine resulted in a peak response that was 382 ± 78% of control. The preceding experiments provide strong evidence

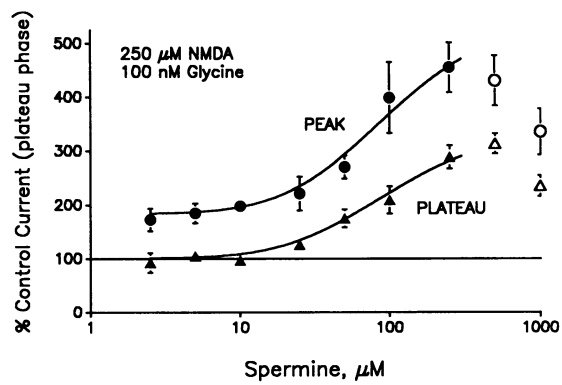

FIG. 2. Concentration–response curves for spermine. Spermine with 100 nM glycine was applied 10–15 sec prior to application of the same solution with 250 μM NMDA. The mean ± SEM of the peak current (● and ○) and the plateau current (▲ and △) are plotted for one oocyte as a percentage of the plateau current with no spermine in the bath. Two to five applications to a single oocyte were made at each concentration of spermine. The responses were depressed at the highest concentration (1 mM). Hill plots were fit to points on the rising phase of the response curves (● and ▲).

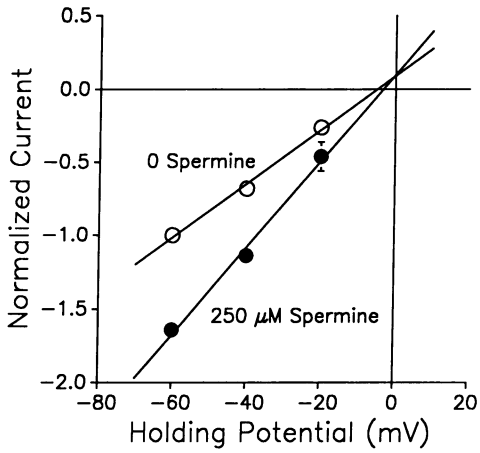

FIG. 3. Spermine did not alter the reversal potential of the NMDA response. The current–voltage relations for the peak of the NMDA response in the presence (●) and in the absence (○) of 250 μM spermine are shown. Data points are mean ± SEM for three oocytes; for each oocyte responses were normalized to its peak response at −60 mV in the absence of spermine. Lines represent the least-squares fit.

that one mechanism by which spermine influences the NMDA response is by increasing the affinity of the glycine site for glycine.

Binding studies have indicated that the polyamine putrescine does not potentiate [^{3}H]MK-801 binding (29, 30) or [^{3}H]glycine binding (32). In the oocyte, putrescine (1–500 μM) did not affect responses to NMDA with glycine, but at high concentrations (1 mM) it did reduce the potentiation produced by low concentrations of spermine (50 μM; Fig. 5). At higher spermine concentrations, putrescine had little or no effect, suggesting that putrescine is a competitive inhibitor of spermine. Thus, although putrescine does not directly alter NMDA responses, it may bind weakly to the polyamine site and compete with other polyamines.

DISCUSSION

This study demonstrates that spermine potentiates the responses of rat brain NMDA receptors expressed in oocytes.

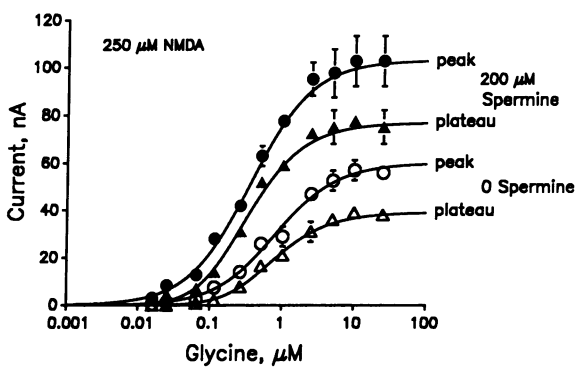

FIG. 4. Spermine increases the apparent affinity and the maximum of the concentration–response relation for glycine. Concentration–response curves for glycine are shown for a representative oocyte in the presence (●, peak; ▲, plateau) and absence (○, peak; △, plateau) of 200 μM spermine. Glycine and spermine were added to the bath 10–15 sec prior to 250 μM NMDA. Responses are plotted as mean ± SEM of inward current during NMDA application. Three to five applications were made at each concentration of glycine. The smooth curves were fit as described in *Materials and Methods*. Previous work has shown that there is some glycine contamination under our experimental conditions (23). Our data were consistent with there being 15–25 nM glycine in putatively "glycine-free" solutions and we assumed for the graph that the background glycine concentration was 15 nM.

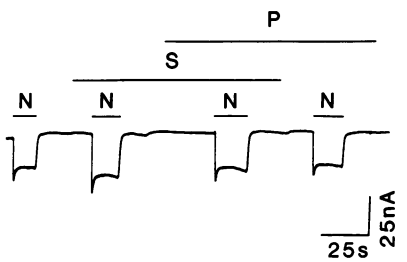

FIG. 5. High concentrations of putrescine inhibit spermine potentiation of the NMDA response. Current induced by 250 μM NMDA in the presence of 1 μM glycine is shown in the first response. At 50 μM, spermine increased the NMDA response by 45% (second response). Addition of 1000 μM putrescine reduced the response in the presence of spermine; the augmentation by spermine was reduced by 70% (third response). Putrescine (1000 μM) did not affect the NMDA response in the absence of spermine (fourth response).

The responses were potentiated over the entire range of NMDA and glycine effectiveness without change in apparent NMDA affinity but with some increase in apparent glycine affinity. Spermine potentiation is reduced somewhat at high concentrations, which may be due to autoinhibition at the binding site because of the polyvalent nature of the ligand. Others have suggested that there is a second, inhibitory polyamine site with somewhat lower affinity for spermine (47). The diminution of effect at high spermine concentration was also seen in studies of spermine potentiation of [^{3}H]MK-801 binding (29, 30) and NMDA-induced currents in cultured hippocampal neurons (47) but not in studies of [^{3}H]glycine binding (31, 32). In all binding studies the spermine concentration that elicited half-maximal increase in binding was 2- to 5-fold lower than that observed in our electrophysiological assay (29–32), and in cultured hippocampal neurons the spermine concentration producing half-maximal increase in NMDA-induced current was 10-fold lower (47).

Putrescine may be a weak competitive inhibitor of spermine action. Diethylenetriamine more effectively blocks the action of spermine of hippocampal neurons, possibly competitively (33, 47). Spermine does not act on non-NMDA-type glutamate receptors as has also been reported in hippocampal neurons (47). In contrast, others have reported that in oocytes spermine potentiates response to kainate and quisqualate as well as to NMDA (34). We have no explanation for the difference in their results for non-NMDA receptors.

Our results indicate that spermine acts, at least in part, by increasing the affinity of the NMDA receptor/complex for glycine. This finding is consistent with the result of others who have found a spermine-induced increase in [^{3}H]glycine binding in membrane preparations (31, 32). In a simple model of allosteric potentiation of NMDA responses by glycine, increase in affinity at the glycine site would not increase the response to saturating concentrations of NMDA and glycine, and spermine would have to have other effects on the NMDA receptor/complex. However, the action of glycine appears to be more complex (23, 48), and action of spermine limited to this site cannot be excluded at this point.

Although they are clearly capable of potentiating responses of NMDA receptors, the question remains whether spermine or spermidine have this effect in the central nervous system. The amount of spermine in mammalian brain tissue is about 100 nM/g (wet weight) and that of spermidine is several-fold higher (49, 50). However, levels in the cerebrospinal fluid are very low (42, 49) although it was not determined whether there was less spermine than 200 nM, which is sufficient to potentiate NMDA-induced currents in cultured hippocampal neurons (47). There is evidence for uptake of polyamines (35, 46) so that low levels in cerebrospinal fluid do not exclude

higher concentrations in some extracellular regions. Although there is no indication that polyamines are released into the extracellular space under physiological conditions, they still may play a role in modulation of NMDA receptors.

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