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*ACCELERATED COMMUNICATION*

# 1,5-(Diethylamino)piperidine, a Novel Spermidine Analogue that More Specifically Activates the *N*-Methyl-D-aspartate Receptor-Associated Polyamine Site

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**SUMMARY**

We have investigated the action of 1,5-(diethylamino)piperidine (DEAP), a novel spermidine analogue that activates the polyamine site associated with the *N*-methyl-D-aspartate receptor. DEAP increased [$^3$H]dizocilpine ([$^3$H]MK801) binding to rat brain membranes with a potency similar to that of spermine and spermidine, but with a somewhat greater efficacy. Unlike other polyamines, however, DEAP did not exhibit low affinity inhibition of [$^3$H]dizocilpine binding, suggesting that it binds more selectively to the polyamine site. DEAP increased the equilibrium affinity of [$^3$H]dizocilpine. The increase in affinity was due to slowed dissociation, as well as a small increase in the association rate of [$^3$H]dizocilpine. All of these effects of DEAP could be reversed by arcaine. These data illustrate the utility of DEAP as a novel polyamine agonist at the *N*-methyl-D-aspartate receptor complex. However, these data fail to support the hypothesis that polyamines activate the *N*-methyl-D-aspartate receptor by a mechanism similar to that of glutamate and glycine.

The NMDA complex is subject to modulation by a wide variety of putatively endogenous agents that may profoundly alter its activation by the neurotransmitters glutamate or aspartate *in vivo* (see Ref. 1 for review). The study of the mechanism of action of these modulators *in vitro* has been facilitated by the use of ligands, such as [$^3$H]dizocilpine ([$^3$H]MK801) and [$^3$H]TCP, that bind within the NMDA-operated ion channel (2–4). The channel location for the binding site of these ligands results in the rates of association and dissociation being increased by agents that activate the receptor, thereby opening the receptor-associated channel, and *vice versa* (5–7). Thus, glutamate, NMDA, and glycine increase rates of binding and unbinding, whereas AP5, 7-chlorokynurenate, and $Zn^{2+}$ decrease these rates.

It was recently demonstrated that the polyamines spermine and spermidine can profoundly increase [$^3$H]dizocilpine binding to the NMDA receptor complex (8). Because polyamines are found in the brain at concentrations that would produce this effect, these observations suggested that polyamines might normally contribute to the activation of the NMDA receptor complex. Moreover, the finding that divalent cations, including $Mg^{2+}$ and $Ca^{2+}$, also act as agonists at the polyamine site (9, 10) also implied that the normal activation of this site may be of physiological importance. However, the contribution of the polyamine site to the activation of the NMDA receptor complex ultimately depends on the precise mechanism by which polyamines modulate [$^3$H]dizocilpine and [$^3$H]TCP binding. The increase in binding produced by these agents could, in principle, arise either from an increase in the rate of binding, if binding is not measured at equilibrium, or from an allosteric alteration of the [$^3$H]dizocilpine site that increases the affinity for the ligand. The former process would be analogous to the effects of NMDA and glycine and would imply that polyamines contribute to the activation of the receptor and channel opening. The contribution of the latter effect of NMDA receptor activation in the absence of channel-blocking drugs is much harder to predict.

The mechanism of action of polyamines has proved to be difficult to determine. This is due, in part, to the biphasic concentration-response curves that spermine and spermidine produce (10–13). The high affinity increase in binding produced by activation of the polyamine site is reversed by competitive antagonists like arcaine (14) and diaminodecane (12). Superimposed on this is a lower affinity inhibition of binding that is insensitive to arcaine (14) and is apparently mediated by polyamines binding to the $Mg^{2+}$ site (9). It has previously been shown that occupation of the $Mg^{2+}$ site profoundly alters the kinetics of [$^3$H]dizocilpine binding (7, 12). Thus, studies that have examined the effects of polyamines on the kinetics of [$^3$H]

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**ABBREVIATIONS:** NMDA, *N*-methyl-D-aspartate; AP5, aminophosphonovaleric acid; DEAP, 1,5-(diethylamino)piperidine; TCP, thienylphencyclidine.

dizocilpine or [$^3$H]TCP binding have generally been confounded by the overlapping effects of spermine or spermidine on both the polyamine site and the $Mg^{2+}$ site. Indeed, at least one study has shown that the increase in the dissociation of [$^3$H]dizocilpine produced by polyamines is insensitive to arcaine, implying that these polyamine effects are not mediated by the high affinity polyamine site (9). Thus, the mechanism of action of polyamines remains controversial.

In this study, we describe the actions of DEAP, a novel spermidine analogue (Fig. 1) that mimics the high affinity effects of spermidine on the polyamine site but lacks the low affinity effects of spermidine on the $Mg^{2+}$ site of the NMDA receptor complex. The specificity of this novel probe allowed a careful analysis of the mechanism by which polyamines increase [$^3$H]dizocilpine binding. We report that the actions of DEAP may be entirely due to allosteric modulation of the [$^3$H]dizocilpine binding site and that we are unable to demonstrate a polyamine-induced increase in channel opening.

## Experimental Procedures

**Materials.** [$^3$H]Dizocilpine (22.5 Ci/mmol) was obtained from DuPont/New England Nuclear (Boston, MA). DEAP (MDL 26630, trihydrochloride salt) was supplied by Marion Merrell Dow Research Institute (Cincinnati, OH). Unlabeled dizocilpine was provided by Merck, Sharp, and Dohme (West Point, PA). Other materials were obtained from commercial sources.

**Methods.** [$^3$H]Dizocilpine binding assays were performed using well washed rat brain membranes prepared as previously described (9). The assay conditions for nonequilibrium binding and saturation curves (9) have been described previously. The assays were incubated for 2 and 4 hr, respectively. Association curves were performed using about 100 $\mu$g of tissue, 100 $\mu$M glutamate, 30 $\mu$M glycine, drugs as appropriate, and about 3 nM [$^3$H]dizocilpine, unless otherwise indicated. Twenty-two points between 1 and 240 min were measured, together with estimates of nonspecific binding at 1 and 60 min. No difference in nonspecific binding was noted at these time points, and the 1-min point was used in calculations. For dissociation curves, tissue was incubated with radioligand for about 2 hr before 120-fold dilution into buffer containing 100 $\mu$M glutamate, 30 $\mu$M glycine, and drugs as appropriate. Assays were further incubated for 5–180 min before filtration.

Binding data were analyzed using the EBDA, LIGAND, and KINETIC routines (Elsevier Biosoft, New York, NY). Association curves were always better described by two rate constants ($p < 0.05$, $F$ test). Most association experiments used a single concentration of radioligand. Values for the association rate constant, $k_{+1}$, were obtained using measured $k_{obs}$ rates and dissociation rates measured in separate experiments.

## Results

We first examined the effects of DEAP on nonequilibrium [$^3$H]dizocilpine binding (Fig. 2). Spermine, spermidine, and DEAP all increased [$^3$H]dizocilpine binding that had been maximally stimulated by glutamate and glycine. Whereas DEAP had a potency similar to that of the other polyamines,

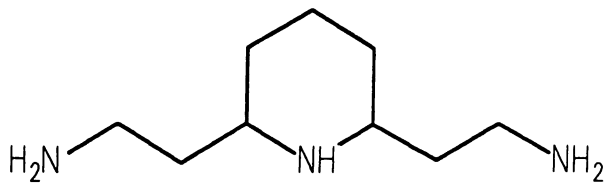


**Fig. 1.** Structure of DEAP.

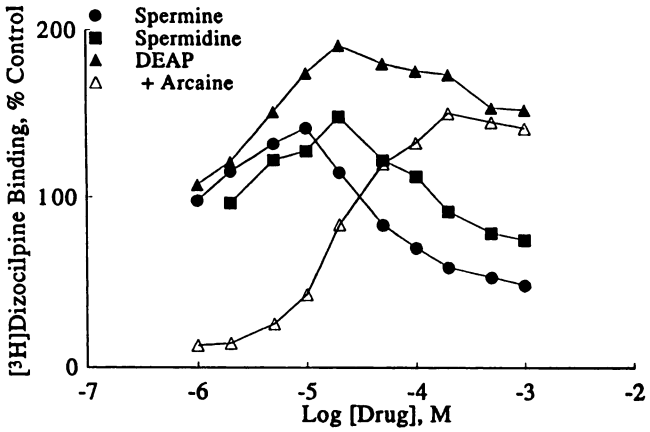


**Fig. 2.** Comparison of the effects of polyamines on [$^3$H]dizocilpine binding. *Curves* were generated in the presence of 100 $\mu$M glutamate and 30 $\mu$M glycine and, as indicated, 50 $\mu$M arcaine. Data are taken from a single representative experiment performed in duplicate that was repeated two additional times with similar results.

it displayed an apparently greater efficacy. These conditions also maximized the low affinity inhibitory actions of spermidine and spermine that are believed to be associated with the $Mg^{2+}$ site. Fig. 2 clearly demonstrates that DEAP is much less effective as a low affinity inhibitor of [$^3$H]dizocilpine, implying that it essentially has minimal effects on the $Mg^{2+}$ site. Fig. 2 also demonstrates the ability of DEAP to reverse the inhibition of [$^3$H]dizocilpine binding produced by 50 $\mu$M arcaine, a competitive polyamine antagonist. In the following experiments, we used 20 $\mu$M DEAP to produce maximal effects and used 50 $\mu$M arcaine with 20 $\mu$M DEAP to reduce the effects of the novel polyamine to approximately control levels.

We next examined the actions of DEAP on the kinetics of [$^3$H]dizocilpine binding. With the NMDA and glycine sites saturated, [$^3$H]dizocilpine exhibited a two-phase association, characterized by observed rate constants of 0.86 and 0.026 $min^{-1}$, with 23% of the binding residing in the fast phase. Addition of DEAP under these conditions increased the fraction associating in the fast phase to 51% (Fig. 3A). The fast association rate was not significantly altered by DEAP, whereas the slow rate was increased slightly, an effect that was significant if the rate was compared with the rate in the presence of DEAP and arcaine (Table 1). As previously observed, [$^3$H]dizocilpine dissociates from its binding site slowly (7, 12, 15, 16). The addition of DEAP decreased the dissociation rate to about 25% of control. This could be reversed by the addition of arcaine (Fig. 3B; Table 1). DEAP also increased the affinity of [$^3$H]dizocilpine binding, without altering the number of binding sites, in an arcaine-reversible fashion (Table 1).

## Discussion

In this study we report the effects of DEAP, a novel analogue of spermidine that activates the NMDA receptor-associated polyamine site. This drug has an affinity similar to that of spermine and spermidine and shows a slightly greater efficacy. Most notable, however, is the relative lack of the low affinity inhibitory effects of spermine and spermidine. Because these latter effects have been attributed to polyamines binding to the $Mg^{2+}$ site on the receptor complex, the absence of these effects suggest that DEAP is more selective for the polyamine site on the NMDA receptor complex. The data in Fig. 2 also suggest that DEAP has a greater efficacy than does spermine or sper-

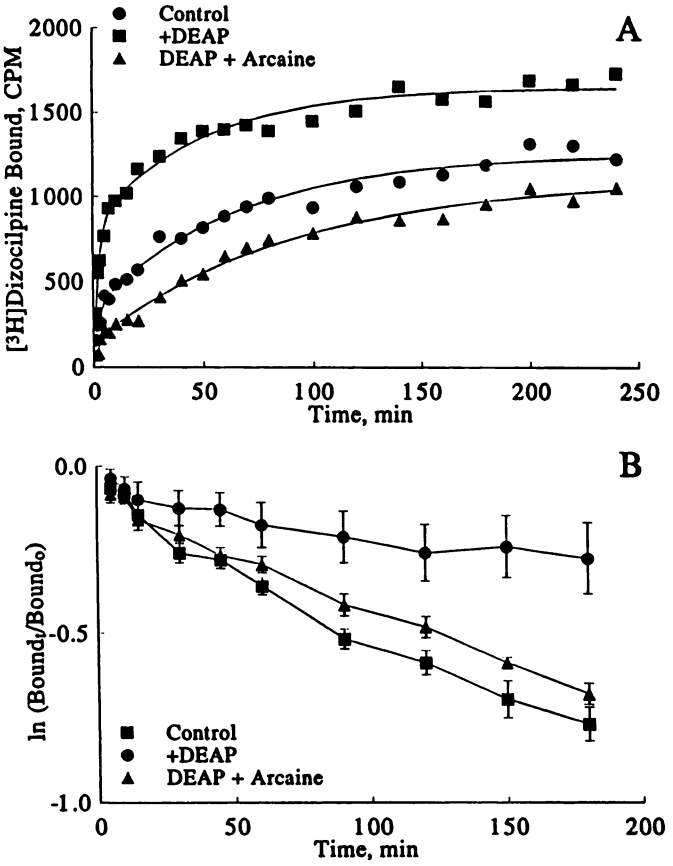


**Fig. 3.** Effects of DEAP on the association and dissociation of [$^3$H]dizocilpine. A, Association curves were generated in the presence of 100 $\mu$M glutamate and 30 $\mu$M glycine alone (control (●) or in the presence of 20 $\mu$M DEAP (■) or with 20 $\mu$M DEAP and 50 $\mu$M arcaine (▲). *Symbols*, data points; *lines*, fitted curves. These data points were generated in duplicate and are typical of four to seven additional experiments. B, Dissociation curves were generated in the presence of 100 $\mu$M glutamate and 30 $\mu$M glycine alone (control) (■) or in the presence of 20 $\mu$M DEAP (●) or with 20 $\mu$M DEAP and 50 $\mu$M arcaine (▲). Data represent the mean ± standard error of six experiments performed in duplicate. Kinetic constants derived from these data are given in Table 1.

midine. However, the apparent difference may be due to the absence of the inhibitory component that is superimposed on the stimulatory effects of spermine and spermidine.

We have taken advantage of the specificity of action of DEAP to probe further the mechanism by which polyamines modulate the NMDA receptor. Previous studies of NMDA receptor modulation suggest that NMDA and glycine site agonists increase binding of ligands like [$^3$H]dizocilpine primarily by increasing access of the ligand to its binding site in the ion channel (5–7). This property increases the rate of ligand binding but does not increase the equilibrium affinity. In contrast, modulators like $Mg^{2+}$ interact with [$^3$H]dizocilpine binding by way of a direct allosteric coupling between the respective binding sites (7). Several previous studies have shown that polyamines increase equilibrium [$^3$H]dizocilpine affinity in an arcaine-sensitive fashion (9, 17). DEAP also increased equilibrium [$^3$H]dizocilpine binding (Table 1), which shows that at least some of the actions of polyamines can be attributed to allosteric coupling between the two sites, rather than a change in access of [$^3$H]dizocilpine for its binding site.

Several prior studies have also shown that spermine or spermidine can increase [$^3$H]dizocilpine association and dissociation rates (9, 12). However, because $Mg^{2+}$ can also increase these rates, it is important to exclude the possibility that these effects are due to the low affinity, nonspecific effects of polyamines. Indeed, we have previously found that the effects of both spermidine and spermine on the dissociation of [$^3$H]dizocilpine are not sensitive to arcaine, consistent with an action at the $Mg^{2+}$ site (9). In the present study, we found that DEAP showed the dissociation of [$^3$H]dizocilpine. This might be anticipated from the observed increase in equilibrium affinity of [$^3$H]dizocilpine but is not consistent with a simple increase in activation of the receptor, such as that produced by NMDA or glycine. DEAP modestly increased the association rate of [$^3$H]dizocilpine binding, which is also consistent with the observed increase in affinity. This effect was arcaine sensitive, indicating that this action of DEAP is due to an interaction at the polyamine site. Because the profile of effects of DEAP on the rates of [$^3$H]dizocilpine binding are distinct from those of glutamate and glycine, our observations do not provide any clear support, as such, for the hypothesis that polyamines increase the opening of the NMDA receptor-associated ion channel.

A clear effect of DEAP on the association of [$^3$H]dizocilpine was the increase in the fraction of [$^3$H]dizocilpine binding in the fast phase of the association curve. The functional correlates of the fast and slow components of [$^3$H]dizocilpine are currently not well understood. Moreover, equilibrium binding studies do not reveal multiple phases of the [$^3$H]dizocilpine saturation curve. It is possible that there is a second component of the dissociation curve that was not resolved by our experiments. However, to generate monophasic saturation curves it is necessary to postulate a rapid dissociation rate that can

TABLE 1

**Effects of DEAP on kinetic parameters of [$^3$H]dizocilpine binding**

Association rates were derived from measured $k_{obs}$ values, the dissociation constants shown, and the ligand concentration for individual experiments. The mean ligand concentration used in this series of experiments was 3.1 nM. Kinetic $k_d$ values were derived from association and dissociation rates, whereas actual $k_d$ values were determined in saturation experiments, as described in Experimental Procedures. All experiments contained 100 $\mu$M glutamate and 30 $\mu$M glycine and included 20 $\mu$M DEAP and 50 $\mu$M arcaine as indicated.

| Condition | Phase | $k_{+1}$ | Fast phase | $k_{-1}$ | Kinetic $k_d$ | Measured $k_d$ | $B_{max}$ |
|---|---|---|---|---|---|---|---|
| | | $M^{-1}\ min^{-1} \times 10^{-8}$ | % | $min^{-1} \times 10^3$ | nM | nM | pmol/mg of protein |
| Control | Fast | 2.78 ± 0.39 | 23 ± 2 | 4.19 ± 0.22 | 0.015 | 0.89 ± 0.03 | 0.67 ± 0.09 |
| | Slow | 0.072 ± 0.014 | | | 0.586 | | |
| DEAP | Fast | 1.79 ± 0.22 | 51 ± 3[a] | 1.28 ± 0.41[a] | 0.007 | 0.21 ± 0.03[a] | 0.88 ± 0.15 |
| | Slow | 0.111 ± 0.010 | | | 0.115 | | |
| DEAP + arcaine | Fast | 2.56 ± 0.72 | 15 ± 2[b] | 3.34 ± 0.02[b] | 0.013 | 1.23 ± 0.07[a,b] | 0.83 ± 0.14 |
| | Slow | 0.036 ± 0.005[b] | | | 0.917 | | |

[a] Significantly different from control, $p < 0.05$, analysis of variance with Bonferroni correction.
[b] Significantly different from DEAP condition, $p < 0.05$, analysis of variance with Bonferroni correction.

account for about 50% of the total binding. It is clear from our studies that this situation does not exist. It has been suggested that the fast component of binding represents binding to the activated receptor, whereas the slow component reflects ligand approaching the binding site via hydrophobic route (16, 18). However, in this study the equilibrium affinity most closely corresponds to the kinetic constant obtained from the slow component of association. It is noteworthy that the fast association rate, $2.78 \times 10^8$ $M^{-1}$, compares closely with the dizocilpine binding rate to open channels reported by Huettner and Bean ($1.8 \times 10^9$ $M^{-1}$ $min^{-1}$) (19) and by Jahr ($1.4 \times 10^8$ $M^{-1}$ $min^{-1}$) (20), whereas the slow rate is >200-fold slower. It is possible that this initial fast phase of [$^3$H]dizocilpine binding represents binding to an open channel, whereas the slow phase (accounting for the majority of equilibrium binding) reflects a desensitized form of the receptor. The latter state is capable of transiently cycling through the open state from the desensitized state, which would account for the continued slow binding of [$^3$H]dizocilpine and, also, the ability of drugs that decrease channel opening to inhibit this phase of binding. In this scheme, a primary action of DEAP would be to increase the fraction of [$^3$H]dizocilpine binding to open channels. The increased availability of open channels is consistent with a recent report that showed that low concentrations of spermine increase channel opening frequency (21). However, if dizocilpine stabilizes the NMDA receptor in the desensitized state, as has been reported for the interaction of analogous channel-blocking drugs with the nicotinic acetylcholine receptor (22), the action of polyamines may simply prevent this "desensitizing" effect of dizocilpine. In the latter case, it remains difficult to predict the effects of polyamines on NMDA receptors not occupied by channel-blocking drugs. It has also been reported that spermine alters the onset of NMDA receptor desensitization (23), which may be consistent with this hypothesis.

Several laboratories have reported effects of polyamines on NMDA responses measured electrophysiologically (12, 21, 23–26). These experiments appear to be based on the hypothesis that polyamines activate the receptor and should, therefore, increase NMDA responses. However, there are several reasons why the functional studies may be difficult to interpret. Firstly, polyamines may produce several effects on the receptor, some of which may not be related to activation of the polyamine site. In addition, it has been shown that divalent cations, including $Mg^{2+}$ and $Ca^{2+}$, can activate the polyamine site, and the functional studies have not taken this into consideration. Finally, the functional studies have generally not demonstrated that the reported effects are pharmacologically consistent with the polyamine actions described in the binding assay. For example, Maciver *et al.* (26) found that spermidine could not reverse the effects of arcaine on electrophysiological responses. Because so many of the drugs used to define this site lack reasonable specificity, demonstrating appropriate pharmacological selectivity must remain an important consideration.

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