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INTERACTION OF RIGID POLYAMINE ANALOGUES WITH THE NMDA RECEPTOR COMPLEX FROM RAT BRAIN

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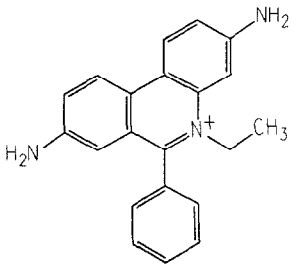
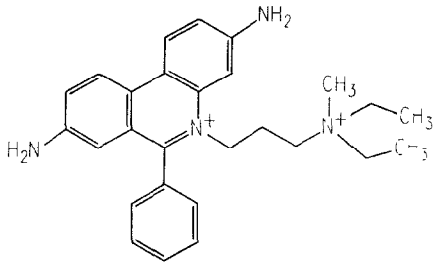
Abstract: We investigated four compounds that may be considered rigid analogues of triamine polyamines that bind to the NMDA receptor. Ethidium, propidium, safranine T and phenosafranine all inhibited [^{3}H] dizocilpine binding to the NMDA receptor (IC_{50} values 2-5μM). However, only propidium appeared to interact with the polyamine site.

Activity of the N-methyl-D-aspartate (NMDA) preferring subtype of glutamate receptor is controlled by a host of endogenous substances that includes glycine, Mg^{2+}, Zn^{2+}, H^+ and sulfhydryl redox reagents[1-4]. Any of these agents may alter the activity of the NMDA receptor *in vivo*, although in most cases their actual role remains unknown. The polyamines spermine and spermidine were recently added to the list of putative endogenous NMDA receptor modulators[5], based on their ability to allosterically increase [^{3}H] dizocilpine binding to the receptor complex. Subsequent studies have revealed that polyamines probably bind to at least two different sites on the receptor[6]. A high affinity site is responsible for increasing [^{3}H] dizocilpine binding, and the action of polyamines at this site can be competitively antagonized by dicationic compounds like arcaine (1,4-bisguanidinobutane)[7] and diaminodecane (DA10)[8]. The lower affinity site mediates polyamine inhibition of [^{3}H] dizocilpine binding, and is relatively insensitive to arcaine and DA10. The contribution of the polyamine site to the function of the NMDA receptor is poorly understood. However polyamine binding to the low affinity site may increase the affinity of glycine[9,10], thereby promoting activation of the receptor[11], while antagonists at the high affinity site may reverse the effects of drugs like dizocilpine and phencyclidine[6].

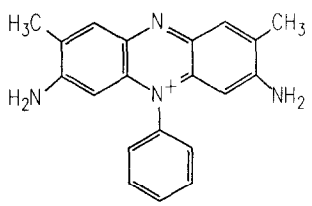
Structure activity studies with polyamines have largely focussed on linear di- tri- and tetra-amines[12]. These studies have shown that three amines with a minimum separation of 3 carbons each are required for full agonist activity at the high affinity polyamine site. Conversely, bisguanidines and diamines are effective antagonists[7,8]. To proceed beyond these simple compounds we chose four agents, ethidium, propidium, safranine T and phenosafranine that maintained a similar separation of amines but in structures made rigid and planar by the presence of aromatic rings.



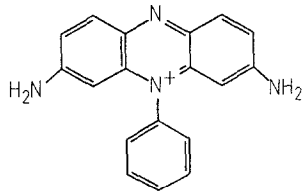
Ethidium



Propidium



Safranine T



Phenosafranine

Drug action was initially evaluated using [^{3}H] dizocilpine binding to well washed rat brain membranes as previously described[7]. All four compounds inhibited [^{3}H] dizocilpine binding in the concentration range 0.1-100μM (Figure 1). Interestingly, ethidium, safranine and phenosafranine inhibited [^{3}H] dizocilpine binding below the level of non-specific binding defined by 30μM dizocilpine.

Table 1. Effects of drugs on [^{3}H] dizocilpine binding. IC_{50} values were determined in the presence of 100μM glutamate and 30μM glycine (Control) with modifications as indicated. Data represent the mean (±SEM) of 4-8 determinations.

Drug	Control		+500μM Spermidine		DEPC Membranes	
	IC_{50}, μM	nH	IC_{50}, μM	nH	IC_{50}, μM	nH
Ethidium	2.74±.5	1.32±.04	7.34±1.3	0.98±.06	3.13±.4	1.29±.13
Propidium	4.71±.8	1.06±.05	>1000		6.80±1.4	1.20±.05
Safranine T	2.97±.2	1.20±.10	2.21±.2	1.46±.08	3.95±.6	1.01±.07
Phenosafranine	2.50±.2	1.34±.03	4.33±.6	1.52±.10	2.70±.5	1.18±.01

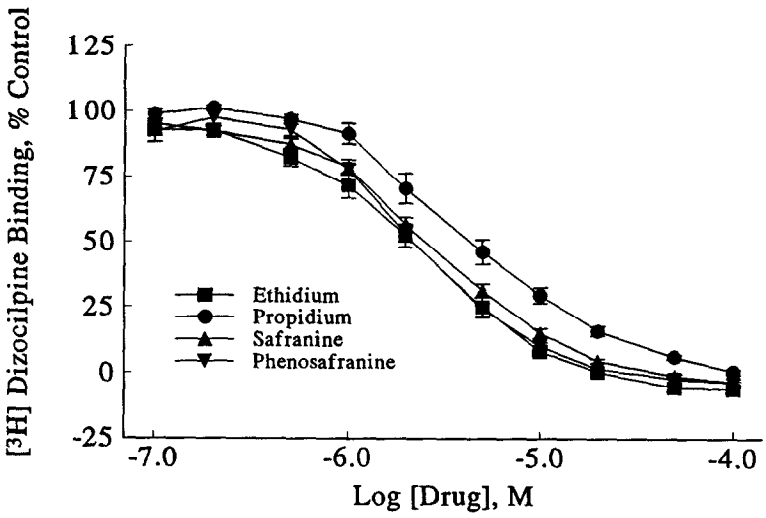


Figure 1. Inhibition of [^{3}H] dizocilpine binding by ethidium, propidium, safranine and phenosafranine. [^{3}H] Dizocilpine binding assays were performed in the presence of 100μM glutamate and 30μM glycine. Curves are the mean (± SEM) of 7-8 experiments.

To evaluate the possible site of action of these agents assays were performed in the presence or absence of 500μM spermidine. A 100-fold shift in the inhibitory potency in the presence of this concentration of spermidine would imply a competitive interaction of the drug with the high affinity polyamine site. As indicated in table 1, only the inhibition of [^{3}H] dizocilpine binding by propidium was significantly altered by the addition of spermidine. We have previously found that the histidine-modifying reagent diethylpyrocarbonate (DEPC) significantly decreases the ability of Zn^{2+} to inhibit [^{3}H] dizocilpine binding, and can be used to determine whether drugs bind to the Zn^{2+} site on the NMDA receptor[13]. Treatment of membranes with 1.2mM DEPC before addition of membranes to the binding assay had no effect on the potency of any of these compounds (Table 1).

To further investigate the possible mechanism of action of these agents, the ability of these drugs to alter the dissociation of [^{3}H] dizocilpine was examined. We have previously shown that drugs which prevent activation of the receptor slow the dissociation of [^{3}H] dizocilpine[14]. Each of the compounds tested slowed the dissociation of [^{3}H] dizocilpine (Table 2). As this effect was apparent at concentrations similar to those required to inhibit [^{3}H] dizocilpine binding, slowing the dissociation rate and presumably inactivating the

NMDA receptor, may be a primary mechanism by which the four drugs modulate the NMDA receptor.

Table 2. Effects of drugs on the dissociation of [^{3}H] dizocilpine binding. Rates were determined using 10 time points between 5 and 180min. Values are the mean (±SEM) of 3-8 experiments.

Drug	Dissociation Rate, min^{-1} x10^3	
	+3μM Drug	+30μM Drug
Control	3.76 ± 0.11	
Ethidium	3.17 ± 0.05**	1.60 ± 0.11**
Propidium	1.88 ± 0.10**	1.15 ± 0.08**
Safranine T	3.06 ± 0.18**	1.14 ± 0.13**
Phenosafranine	2.99 ± 0.18**	1.20 ± 0.04**

** Significantly different from control, $P<0.05$.

We also probed the functional properties of the four drugs of interest. NMDA together with glycine produce robust increases in $[Ca^{2+}]_i$ which can be monitored in individual neurons using the Ca^{2+}-sensitive fluorescent dye fura-2. Using previously described techniques[15] we tested the effects of a single concentration (10μM) of each agent on $[Ca^{2+}]_i$ increased produced by 30μM NMDA and 1μM glycine. This concentration is 2-4 fold greater that the IC_{50} observed in [^{3}H] dizocilpine binding assays. In the absence of antagonists these conditions produced mean $[Ca^{2+}]_i$ increases of 300-350nM. Pre-incubation of neurons with ethidium, safranine or phenosafranine for 2min prior to the addition of NMDA and glycine resulted in significant inhibition of the $[Ca^{2+}]_i$ increase (Table 3). Propidium did not inhibit the response at this concentration.

Table 3. Effects of drugs on increases in $[Ca^{2+}]_i$ produced by 30μM NMDA and 1μM glycine. Responses were obtained in the absence of drug and then following a 2min preincubation with 10μM of the indicated compound. Results are the mean (± SEM) 6-7 responses.

Drug	$[Ca^{2+}]_i$ Increase, % Control
Ethidium	84.7 ± 2.0**
Propidium	97.6 ± 5.3**
Safranine T	77.9 ± 5.6**
Phenosafranine	78.4 ± 3.3**

** Significantly different from control, $P<0.05$.

We have found two phenanthridiums and two analogues of safranine to be quite potent inhibitors of [^{3}H] dizocilpine binding, revealing an interaction with the NMDA receptor complex. These data show that propidium, but not ethidium, safranine or phenosafranine probably interacts with the high affinity polyamine site. This implies that the optimal configuration of the amines for binding to the polyamine site is different from a simple planar configuration represented by these molecules. The activity of propidium contrasted to that of ethidium suggests that the presence of a conformationally less restricted amine is important for binding to the polyamine site. It is possible that two of the planar amines, in conjunction with the third side chain nitrogen actually bind to the polyamine site, raising the possibility that triamines can also be effective antagonists of the polyamine site. However, this hypothesis cannot be critically assessed with presently available compounds. This study also shows that quaternary nitrogens can effectively bind to the amine recognition sites for polyamines. It will be interesting to determine if quaternary amine-substituted polyamines can act as agonists at the polyamine site. The failure of propidium to inhibit NMDA-induced $[Ca^{2+}]_i$ changes is also consistent with an action at the polyamine site, as concentrations of arcaine much greater than its IC_{50} value are required to inhibit functional NMDA responses[16].

Although they were not polyamine site antagonists, this study nevertheless provides the first demonstration that ethidium, safranine and phenosafranine bind to the NMDA receptor. Their mechanism of action cannot be precisely ascertained from the present data. However, there is a growing list of compounds that inhibit [^{3}H] dizocilpine binding in a non-competitive fashion, slow the dissociation of [^{3}H] dizocilpine and also inhibit functional responses to NMDA. Other members of this group include pentamidine[15] and 1,10-bis(guanidino)decane[17]. It is likely that propidium also has some action at this site, as it effectively slows the dissociation of [^{3}H] dizocilpine. This property is not usually associated with polyamine site antagonists[6]. Although the action of these compounds superficially resembles that of Zn^{2+}, DEPC which significantly reduces the potency of Zn^{2+} [13] had no effect on the inhibition of [^{3}H] dizocilpine binding by these drugs. As each of these agents has at least two free amines, it seems likely that the members of this group of compounds carry two or more positive charges at physiological pH, although pKa values were not established. With two or more charges it is possible that these drugs may interact with the NMDA receptor at one of the several metal ion recognition sites associated with the NMDA receptor complex. Although the carcinogenic potential of ethidium and the dye properties of the safranines may preclude their utility as therapeutic agents, it is possible that this novel class may yield some useful neuroprotective drugs.

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