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A Phencyclidine Recognition Site Is Associated with *N*-Methyl-D-aspartate Inhibition of Carbachol-Stimulated Phosphoinositide Hydrolysis in Rat Cortical Slices

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SUMMARY

The effects of *N*-methyl-D-aspartate (NMDA) on muscarinic receptor-stimulated phosphoinositide (PI) hydrolysis in rat cortical slices were studied. NMDA inhibits carbachol-stimulated PI hydrolysis with an IC_{50} of 9.8 ± 1.4 μM and a maximal inhibition of 70% at 100 μM. The inhibitory effect of NMDA is not due to increased metabolism of accumulated inositol phosphates. NMDA inhibition of carbachol-stimulated PI hydrolysis was significantly reduced in the absence of extracellular calcium. Although the inhibitory effect of NMDA is observed in the presence of 1.18 mM Mg^{2+}, the concentration-response curve is slightly shifted to the left (5-fold) in the absence of extracellular Mg^{2+}. Antagonists of NMDA-evoked excitations were effective inhibitors of the NMDA modulation of PI hydrolysis, including the competitive antagonist 2-amino-5-phosphonopentanoic acid and the noncompetitive antagonist MK-801. The rank order of potencies of the antagonists were MK-801 > phencyclidine = (−)-cyclazocine > ketamine = etoxadrol > *N*-allylnormetazocine > 2-amino-5-phosphonopentanoic acid. (+)-MK-801 and (−)-cyclazocine were more potent inhibitors, by 4–5-fold, of the NMDA response than their respective isomers, whereas *N*-allylnormetazocine isomers were approximately equipotent antagonists. The activity of dexoxadrol against NMDA inhibition of carbachol-stimulated PI hydrolysis could not be determined because of its antimuscarinic effects. The rank order of potencies of antagonists, the stereoselectivity of the isomers of MK-801, cyclazocine, and *N*-allylnormetazocine, and Mg^{2+} sensitivity of the NMDA inhibitory response suggest that a phencyclidine binding similar to the one located in the cation channel gated by NMDA receptors is associated with the NMDA receptor that modulates muscarinic-stimulated PI hydrolysis.

The precise definition of glutamate receptor subtypes has been hampered by the lack of selective antagonists for the proposed subtypes and instead has relied on agonist responses for preferred compounds, kainate, quisqualate, or NMDA. Antagonists in general are more suitable for the pharmacological categorization of receptors. Recently, rapid progress has been made in the development of compounds that are selective for the NMDA receptor, because of their potential as anticonvulsants and protective agents in cerebral ischemia (1, 2). Thus, it is now possible to establish a potency series for antagonists at NMDA receptors, facilitating the comparison of the various NMDA-mediated responses.

The coupling of the NMDA subtype of glutamate receptor to cellular biochemical effectors has also been the focus of intense investigation. Activation of NMDA receptors opens cation channels that are permeable to calcium and sodium (3, 4). These channels are thought to mediate fast excitatory responses in the central nervous system. NMDA receptor agonists have also been reported to stimulate cyclic GMP production (2, 5) and have a small stimulatory effect on PI hydrolysis (6, 7). In addition, NMDA inhibits muscarinic receptor-stimulated PI hydrolysis in hippocampus and striatum (8, 9). The cerebral cortex has high levels of NMDA binding sites (10, 11), but the interaction of NMDA with muscarinic-stimulated PI metabolism in this brain region has not been studied in detail (12). The purpose of this study was to examine the interaction between NMDA and muscarinic receptor-mediated PI hydrolysis in rat cortical slices, with regard to defining a potency series of antagonists for receptor classification, and to further explore the mechanisms that underlie this interaction.

Experimental Procedures

Materials. Experiments were performed on male Sprague-Dawley rats (200–300 g) obtained from Harlan Sprague-Dawley (Indianapolis, IN). myo-[2-^{3}H]Inositol (specific activity, 19.6 Ci/mmol) was obtained

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartic acid; PI, phosphoinositide; MK-801, 5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclophepten-5,10-imine hydrogen maleate; AP-5, 2-amino-5-phosphonopentanoic acid.

from Amersham (Arlington Heights, IL). Isomers of MK-801 and ketamine were obtained from Research Biochemicals, Inc. (Natick, MA). Phencyclidine, isomers of cyclazocine, isomers of *N*-allylnormetazocine, etoxadrol, and dexoxadrol were obtained from the Research Technology Branch of the National Institute on Drug Abuse (Rockville, MD). Other drugs were from Sigma Chemical Co. (St. Louis, MO).

PI hydrolysis assay. The method of Berridge *et al.* (13), as described by Gonzales and Crews (14), was used for determining PI hydrolysis. Briefly, cerebral cortical slices were cross-chopped by a McIlwain tissue chopper (350-μm thickness) and washed in Krebs-Ringer bicarbonate buffer (118 mM NaCl, 4.7 mM KCl, 0.75 mM $CaCl_2$, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_4$, 24.8 mM $NaHCO_3$, and 10 mM glucose, bubbled with 5% CO_2 in O_2 to give pH 7.5). [^{3}H]Inositol was added to the tissue in a concentration of 0.2–0.3 μM, and the slices were incubated in a shaking water bath for 1 hr at 37°. At the end of the incubation, slices were washed with fresh buffer and distributed into tubes that contained buffer in which 10 mM LiCl had been substituted isotonically for NaCl, to give a final concentration of 8 mM LiCl in a total volume of 0.25 ml. Antagonists, when present, were added to the lithium-containing buffer before addition of the slices. After a 10-min incubation in the lithium buffer, agonists were added and the tubes were gassed, capped, and incubated for 60 min. Reactions were terminated by addition of 1.0 ml of chloroform/methanol (1:2, v/v), and additional chloroform and water were added (0.35 ml). Tubes were shaken and centrifuged, and an aliquot of the upper phase was taken for batch analysis of total ^{3}H-inositol phosphates. A portion of the lower phase was also taken to dryness for determination of radioactivity in lipids by liquid scintillation counting. The aqueous aliquot was diluted with water and poured into a column containing 0.5 ml of Dowex-1 (X8, formate form). The column was washed with water, and the ^{3}H-inositol phosphates were eluted into a scintillation vial with 6.0 ml of 1.0 M ammonium formate in 0.1 M formic acid. Radioactivity in the eluate was determined, after addition of 8 ml of Beckman Ready Gel, in a liquid scintillation counter. Data are expressed as the ratio of dpm recovered from the column to total dpm incorporated into lipids (dpm from column plus dpm in lipids), with blank values subtracted.

Statistical analysis. IC_{50} values were determined using the method of Chou (15). Data were transformed to percentages of maximal response using the maximal response obtained in each experiment, including cases where increasing concentrations produced less than maximal responses. Significant differences between control and experimental treatment groups were determined by analysis of variance followed by Newman-Keuls test for comparisons between individual means. Differences were considered significant if $p < 0.05$.

Results

The effects of NMDA alone and in combination with carbachol, a muscarinic agonist, on PI hydrolysis in cortical slices were determined initially. NMDA alone caused no significant stimulation of PI hydrolysis above basal levels at concentrations from 0.1 to 3 mM (Fig. 1). However, when added simultaneously with carbachol, NMDA caused a dramatic concentration-dependent inhibition of the carbachol-stimulated PI hydrolysis response. The threshold for this effect was 3 μM and the maximal inhibition of 70% was attained at 100 μM NMDA. The IC_{50} value was determined to be 9.8 ± 1.4 (mean ± SE; five experiments). These values agree with those reported by Schmidt *et al.* (8), obtained using striatal neurons in primary culture, and Baudry *et al.* (9), who used hippocampal slices.

We also examined the time course of this inhibitory effect of NMDA on the carbachol-induced PI response to determine whether the effect was dependent on the time of addition of NMDA, relative to carbachol. When added simultaneously with carbachol, the inhibitory effect of NMDA on the level of ^{3}H-inositol phosphates were observed at 15 and 30 min, similar to that seen at 60 min (Fig. 2). Addition of NMDA to slices that had been stimulated with carbachol for 15 min caused a slowing of the accumulation of ^{3}H-inositol phosphates compared with control. Similarly, if NMDA is added after 30 min of incubation with carbachol, the amount of ^{3}H-inositol phosphates is slightly lower in the NMDA-treated slices, compared with control. In no instance did NMDA cause a reduction in the level of inositol phosphates below that which had been attained up to that point.

The effect of the divalent cations calcium and magnesium, in the medium, on the NMDA inhibition of the carbachol response was investigated because these ions have been shown to be involved in the neurophysiological responses to NMDA (3, 16). Deletion of calcium from the medium significantly reduced the basal and carbachol-stimulated accumulation of inositol phosphates to 50% of control (Fig. 3). NMDA failed to significantly inhibit the carbachol response in the absence of added calcium up to concentrations of 300 μM.

Initial experiments investigating the effects of magnesium ions on NMDA inhibition of carbachol-stimulated PI hydrolysis suggested that Mg^{2+} did not have an influence on the NMDA effect. Removal of Mg^{2+} from the medium did not change the inhibition curve for NMDA or the maximal inhibitory effect, using the experimental protocol of adding carbachol and NMDA to the slices simultaneously (data not shown). However, a modification of the procedure, in which the slices are preincubated with NMDA for 10 min, allowed an effect of Mg^{2+} to be observed (Fig. 4). The slices were first washed in a Mg^{2+}-free buffer and treated with various concentrations of NMDA in buffer to which 10 mM, 1.2 mM, or no extra Mg^{2+} had been added. All slices were then washed and stimulated with buffer, 1 mM carbachol, or carbachol plus NMDA for 30 min. This procedure still allowed the inhibitory effect of NMDA to be expressed, even though NMDA was not present during the carbachol stimulation period. However, NMDA was not as potent using this protocol, with significant effects observed only at 30 and 100 μM (Fig. 4) instead of 10 μM as in Fig. 1. Deletion of Mg^{2+} from the buffer during this preincubation with NMDA caused a 5-fold shift to the left of the NMDA concentration-effect curve, with significant inhibition observed at 10 μM. The basal level of inositol phosphate formation was significantly increased in slices that had been preincubated in 10 mM Mg^{2+}. However, the NMDA-induced inhibitory response was not significant even at a concentration of 30 μM. Increasing the NMDA concentration to 100 μM did cause the inhibition of net carbachol-stimulated response by 90% in one experiment (data not shown).

To fully characterize the subtype of glutamate receptor that mediates the negative modulation of muscarinic-stimulated PI hydrolysis, a series of compounds previously shown to have antagonist properties for glutamate-evoked responses were tested for their ability to reverse the NMDA effect. In general, the results of these studies agree with literature values for the rank order of potencies of NMDA antagonists (Fig. 5 and 6; Table 1). As shown in Fig. 5, AP-5, D-α-aminoadipic acid, ketamine, phencyclidine, and (+)-MK-801 antagonized the inhibitory effect of 100 μM NMDA on carbachol-stimulated PI hydrolysis in a concentration-related manner. (+)-MK-801 was the most potent compound tested, with an IC_{50} of 830 nM. D-α-aminoadipic acid was the least potent antagonist tested for which an IC_{50} estimate was obtained (IC_{50} = 260 μM) and

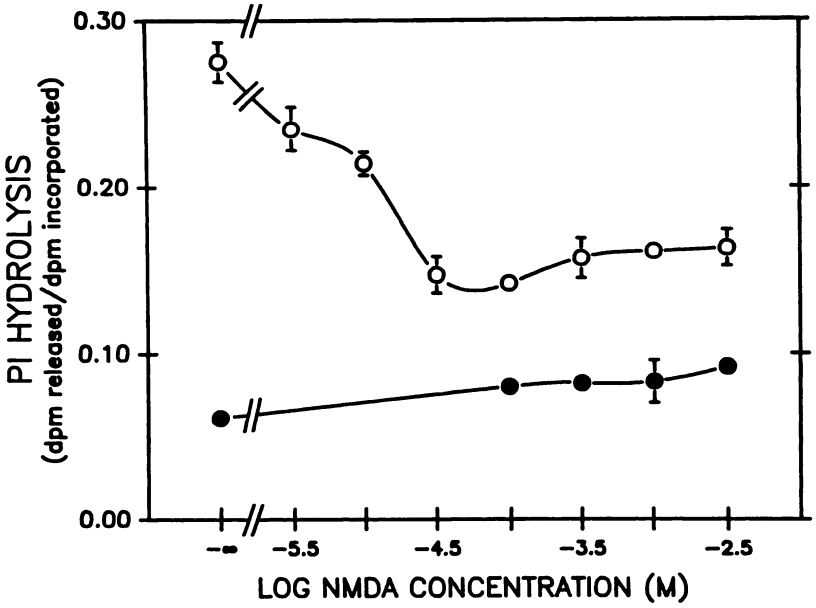

Fig. 1. Concentration-effect curve for NMDA alone and in combination with 1 mM carbachol in rat cortical slices. Prelabeled cortical slices were incubated with the indicated concentrations of NMDA (●) or NMDA plus 1 mM carbachol (○) for 60 min, and the accumulation of ^{3}H-inositol phosphates was determined as described in Materials and Methods. Each *point* represents the mean ± standard error of two to seven experiments, each performed in triplicate. Significant inhibition of carbachol-stimulated PI hydrolysis was observed at all NMDA concentrations tested ($p < 0.05$ by analysis of variance and Newman-Keuls test).

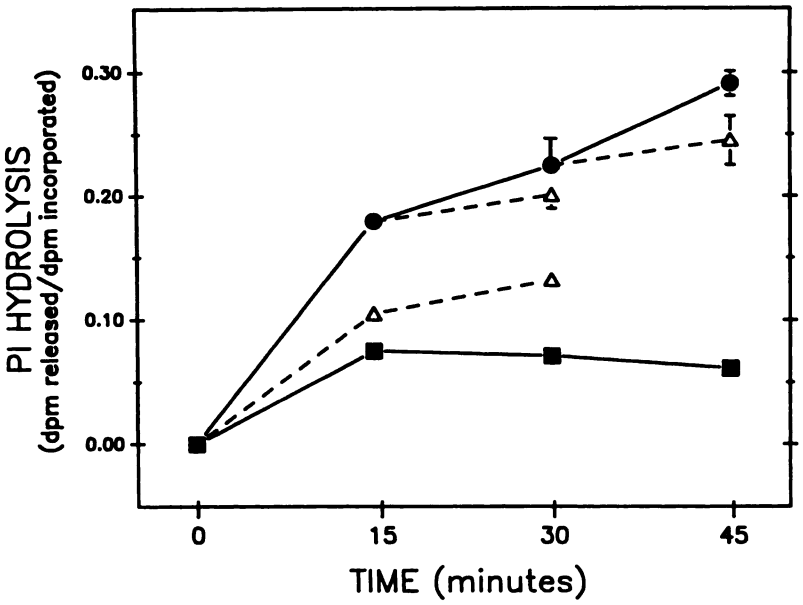

Fig. 2. Effects of varying time of addition of NMDA on carbachol-stimulated PI hydrolysis in cortical slices. Prelabeled cortical slices were incubated with buffer, carbachol, or carbachol plus NMDA. Reactions were terminated at the indicated times and ^{3}H-inositol phosphates released were analyzed (see Materials and Methods). Buffer (■) or 1 mM carbachol (●) was added to the slices at time 0. NMDA (100 μM) (△) was added to the carbachol-treated slices at 0, 15, or 30 min after carbachol addition. The *dashed lines* represent the time courses of PI hydrolysis after addition of the NMDA. Each *point* represents the mean ± standard error of three experiments.

blocked only 82 ± 11% of the NMDA response at 3 mM. The nonspecific glutamate antagonist kynurenic acid blocked the NMDA inhibition of carbachol-stimulated PI hydrolysis at 3 mM (98%) but was only 15% effective at 1 mM). Glutamic acid diethylester did not antagonize the inhibitory effect of 100 μM NMDA at concentrations up to 10 mM. It is interesting to note that most of the antagonists shown in Fig. 5 were able to completely reverse the inhibition of carbachol-stimulated PI hydrolysis by 100 μM NMDA and to cause a slight enhancement of the response beyond that seen with carbachol alone. The blockade of the NMDA response by MK-801 was stereoselective, with (+)-MK-801 approximately 4.5-fold more potent than its stereoisomer (−)-MK-801 (Fig. 5).

Fig. 6 shows that other drugs that have been linked to the NMDA receptor through an interaction with the σ opiate receptor subtype also are effective antagonists for NMDA inhibition of carbachol-stimulation of PI hydrolysis. (−)-Cyclazocine was the most potent of the opiates tested, with an IC_{50} of 6.2 μM. (+)-Cyclazocine was about 4.5-fold times less potent than its stereoisomer. Both of these drugs exhibited inverted U-shaped concentration-effect curves. In contrast, the difference in potency for the stereoisomers (+)-*N*-allylnormetazocine and (−)-*N*-allylnormetazocine was less than 2-fold (Fig. 6; table 1). Nalorphine failed to antagonize the NMDA inhibitory effect on carbachol-stimulated PI hydrolysis at concentrations up to 1 mM. Haloperidol, which has recently been postulated to interact with σ/phencyclidine binding sites (17), also had no effect on the NMDA modulation of PI hydrolysis at concentrations up to 10 μM. The dissociative anesthetic etoxadrol was an effective antagonist of NMDA inhibition of carbachol-stimulated PI hydrolysis, with a potency similar to that of ketamine and (+)-cyclazocine. Increasing the etoxadrol concentration above 30 μM caused a reduction of the accumulation of inositol phosphates similar to that observed with cyclazocine. An analog of etoxadrol, dexoxadrol, failed to affect the NMDA of carbachol-stimulated PI hydrolysis below 10 μM but antagonized

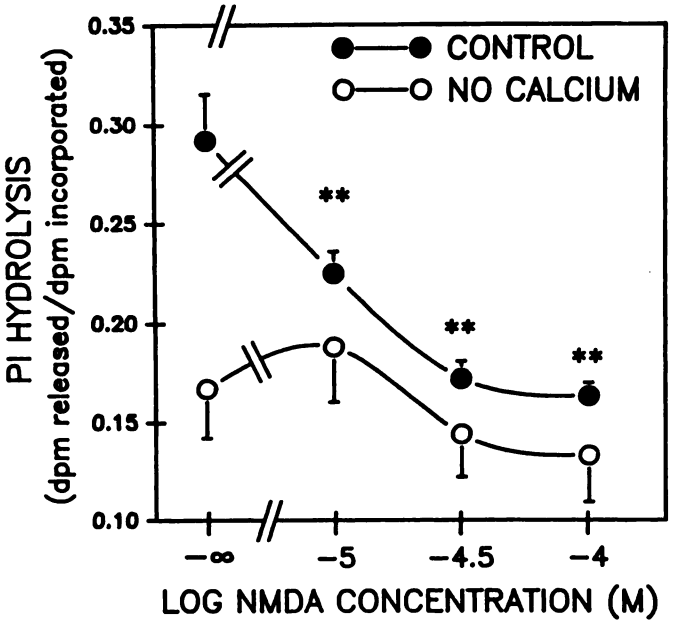

Fig. 3. Concentration-response curve for the effect of NMDA on carbachol-stimulated PI hydrolysis in the presence and absence of extracellular calcium. Prelabeled cortical slices were washed for 10 min with Krebs-Ringer bicarbonate buffer or calcium-free Krebs-Ringer bicarbonate buffer before incubation with 1 mM carbachol and the indicated concentrations of NMDA in the presence or absence of calcium. After 60 min, the accumulation of ^{3}H-inositol phosphates was measured as described in Materials and Methods. Each *point* represents the mean ± standard error of five experiments $^{**}p < 0.01$ compared with control, by analysis of variance and Newman-Keuls test.

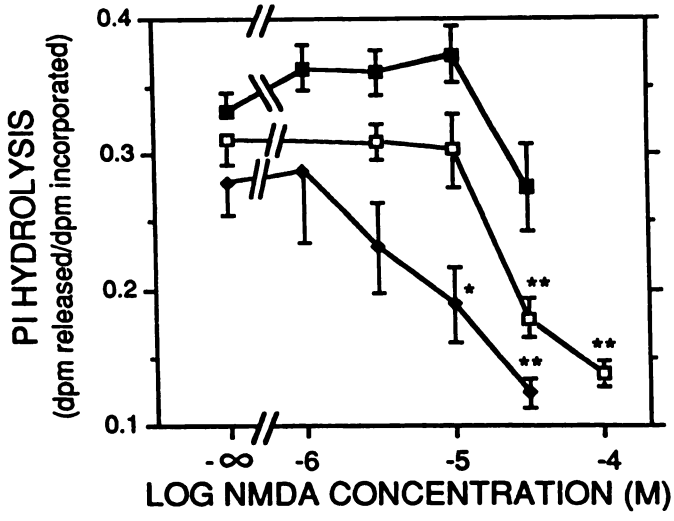

Fig. 4. Mg^{2+} sensitivity of NMDA inhibition of carbachol-stimulated PI hydrolysis in cortical slices. Labeled slices were washed with Mg^{2+}-free buffer and incubated with various concentrations of NMDA or buffer in medium that contained 1.2 mM (□), 10 mM (■), or no added Mg^{2+} (♦). Slices were washed three times with 4 volumes of normal Krebs-Ringer bicarbonate buffer and were then stimulated with 1 mM carbachol or carbachol plus NMDA for 30 min. Release of ^{3}H-inositol phosphates was determined as in Fig. 1. Each *point* represents the mean ± standard error of four to six experiments. $^*p < 0.05$; $^{**}p < 0.01$ compared with its respective value with carbachol alone by analysis of variance and Newman-Keuls test. Basal values were 0.05 ± 0.01, 0.07 ± 0.02, and 0.11 ± 0.01 dpm released/dpm incorporated for slices preincubated in buffer containing 1.18 mM, none, and 10 mM Mg^{2+}, respectively (mean ± standard error; five or six experiments).

the stimulation of PI hydrolysis by carbachol alone at concentrations above 3 μM (Fig. 7). (+)-Cyclazocine was also able to inhibit carbachol-stimulated PI hydrolysis at 300 μm (45%), whereas (−)-cyclazocine was not as effective at blocking muscarinic receptor-stimulated PI hydrolysis (28% inhibition at 300 μM).

The type of inhibition of carbachol-stimulated PI hydrolysis by NMDA in cortical slices was assessed using AP-5 and (+)-MK-801. The concentration-effect curve for NMDA inhibition of PI hydrolysis was shifted to the right by 15-fold in a parallel manner in the presence of 300 μM AP-5, indicating a competitive type of inhibition (Fig. 8). The inhibition of the NMDA effect by 1 μM (+)-MK-801 was not overcome by increasing the NMDA concentration to 10 mM. Thus, (+)-MK-801 antagonizes the NMDA response in a noncompetitive manner.

Discussion

Receptor-stimulated phosphoinositide hydrolysis has, in recent years, become widely recognized as an important signal transduction mechanism for the process of synaptic transmission. Thus, neurotransmitters such as acetylcholine and norepinephrine activate a specific phosphoinositidase C through an interaction with muscarinic or α_1-adrenergic receptors (13, 14, 18). The subsequent hydrolysis of inositol phospholipids may release second messengers inositol 1,4,5-trisphosphate and diacylglycerol (19, 20). Recently, interest has focused on the regulatory aspects of this signaling system, including the temporal and spatial integration of these second messenger signals within the cell (21). Although initial experiments concentrated on the characterization of the stimulation of PI hydrolysis by various neurotransmitters, it is now clear that inhibitory influences are also operative and may be important for regulation of the system. Baudry *et al.* (9) first reported the inhibition of carbachol-stimulated PI hydrolysis in hippocampal slices by *N*-methylaspartic acid, an excitatory amino acid. In this study we have further characterized the receptor specificity of the inhibitory effect of NMDA and explored some possible mechanisms for this negative modulation of PI hydrolysis in rat cortical slices.

Our studies clearly demonstrate that the inhibitory interaction of NMDA with muscarinic receptor-stimulated PI hydrolysis occurs in rat brain cerebral cortex, in agreement with previous reports of this effect in hippocampal and striatal preparations (8, 9). A previous study had reported that NMDA did not inhibit carbachol-stimulated PI hydrolysis in cortical tissue (22). We do not have an explanation for the lack of effect of NMDA observed by these other investigators. However, more recently, Godfrey *et al.* (12) reported that *N*-methyl-DL-aspartic acid inhibited carbachol-stimulated PI hydrolysis in a rat cerebral cortical slice preparation. It thus appears that the negative modulatory influence of NMDA on muscarinic-stimulated PI hydrolysis occurs throughout the brain, and this suggests that many different brain functions may be subserved, at least in part, by this mechanism.

Several experiments were carried out to probe some of the possible mechanisms for this inhibitory effect of NMDA on carbachol-stimulated PI hydrolysis. To determine whether NMDA may be accelerating the breakdown of the inositol phosphates that are formed after muscarinic receptor activation, NMDA was added to cortical slices at various times after carbachol stimulation. The results of these experiments suggest

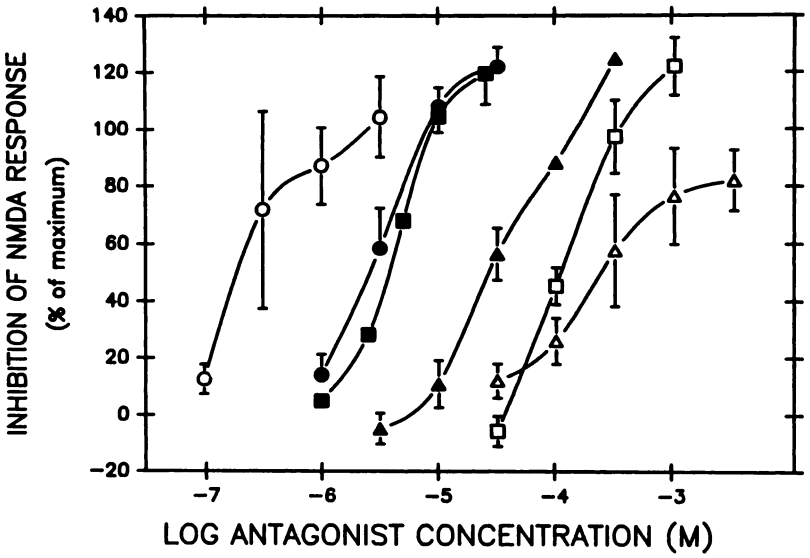

Fig. 5. Reversal of NMDA inhibition of carbachol-stimulated PI hydrolysis in cortical slices by NMDA antagonists. Prelabeled tissue was incubated with the antagonist for 10 min before addition of 1 mM carbachol and 100 μM NMDA. Reactions were stopped after 60 min and ^{3}H-inositol phosphates release was measured ○, (+)-MK-801; ●, (−)-MK-801; ■, phencyclidine; □, AP-5, ▲, ketamine; △, D-α-aminoadipic acid. Each *point* represents the mean ± standard error of at least three experiments. Control values (mean ± standard error; 16 experiments) for buffer, carbachol, and carbachol plus NMDA for these experiments were 0.069 ± 0.004, 0.328 ± 0.014, and 0.185 ± 0.006 dpm released/dpm incorporated, respectively.

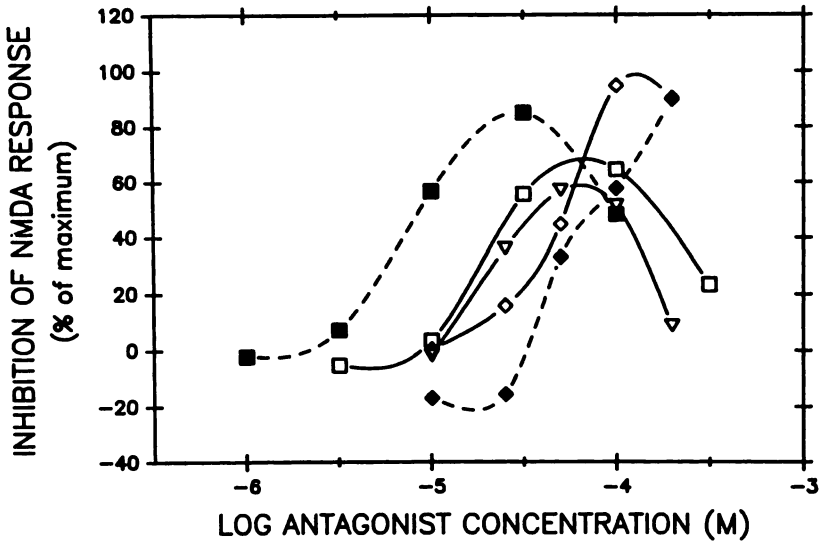

Fig. 6. Effects of σ opiate ligands on NMDA inhibition of carbachol-stimulated PI hydrolysis. Experiments were performed as described in the legend to Fig. 5. ■, (−)-cyclazocine; (□, (+)-cyclazocine; ◇, (+)-*N*-allylnormetazocine; ◆, (−)-*N*-allylnormetazocine; ▽, etoxadrol. Each *point* represents the mean of at least three experiments. Standard error values are omitted for clarity but were less than 12% of the mean. Control values (mean ± standard error; 12 experiments) for buffer, carbachol, and carbachol plus NMDA for these experiments were 0.069 ± 0.003, 0.327 ± 0.010, and 0.188 ± 0.008 dpm released/dpm incorporated, respectively.

TABLE 1

Potencies of NMDA antagonists for reversal of NMDA inhibition of carbachol-stimulated PI hydrolysis

IC_{50} values were determined from the concentration-effect curves shown in Figs. 5 and 6.

Drug	No. of experiments	IC_{50}
		−log M
(+)-MK-801	4	6.08 ± 0.22
(−)-MK-801	3	5.43 ± 0.04
Phencyclidine	4	5.37 ± 0.06
(−)-Cyclazocine	3	5.20 ± 0.06
Ketamine	4	4.63 ± 0.16
Etoxadrol	2	4.61 ± 0.01
(+)-Cyclazocine	2	4.58 ± 0.06
(+)-*N*-Allylnormetazocine	3	4.39 ± 0.04
(−)-*N*-Allylnormetazocine	3	4.22 ± 0.04
AP-5	4	3.92 ± 0.05
D-α-Aminoadipic acid	5	3.59 ± 0.11

that activation of the NMDA receptor does not increase the rate of hydrolysis of accumulated inositol phosphates that have already accumulated as a result of muscarinic receptor stimulation. Instead, stimulation of the slices with NMDA slows the rate of accumulation of the inositol phosphates in the presence of the muscarinic receptor agonist, suggesting that the interaction is not occurring at a level distal to receptor activation. In view of the lack of effect of NMDA on muscarinic antagonist binding reported by Schmidt *et al.* (8), it is likely that NMDA is interfering with the coupling of the muscarinic receptor to the phosphoinositidase C. Greater than 90% of the inositol phosphates we recover in these experiments are in the form of inositol monophosphate (14, 18) and, therefore, we conclude that NMDA stimulation does not alter the activity of inositol monophosphatase in the presence of 8 mM lithium, at least under the conditions used here. More experiments are necessary to determine whether NMDA alters the production or distribution of other inositol phosphates that may be formed after muscarinic receptor stimulation.

Calcium may be involved in the inhibition of carbachol stimulation of PI hydrolysis by NMDA in cortical slices. Fig. 3 illustrates that the inhibitory effect of NMDA is very much attenuated in medium in which calcium has been omitted. Recently, Palmer *et al.* (23) found that NMDA inhibition of quisqualate-stimulated PI hydrolysis in neonatal hippocampal slices is reduced in a low calcium medium. Our results are at variance with the report by Baudry *et al.* (9) that *N*-methylas-

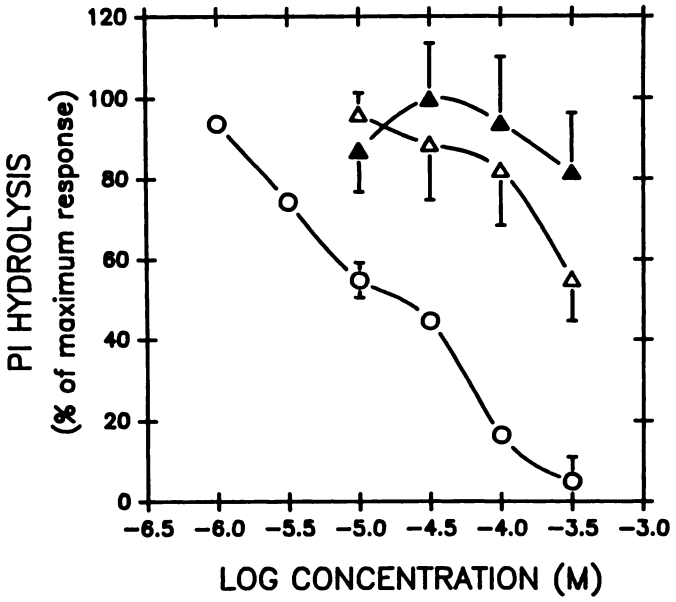

Fig. 7. Inhibition of carbachol-stimulated PI hydrolysis in cortical slices by cyclazocine and dexoxadrol. Experiments were performed as in Fig. 5 except that only 1 mM carbachol was used as the stimulant. ▲, (−)-cyclazocine (two experiments), △, (+)-cyclazocine (two experiments); ○, dexoxadrol (two experiments). Control values (mean ± standard error; four experiments) for buffer and carbachol-stimulated samples for these experiments were 0.068 ± 0.003 and 0.35 ± 0.03 dpm released/dpm incorporated, respectively.

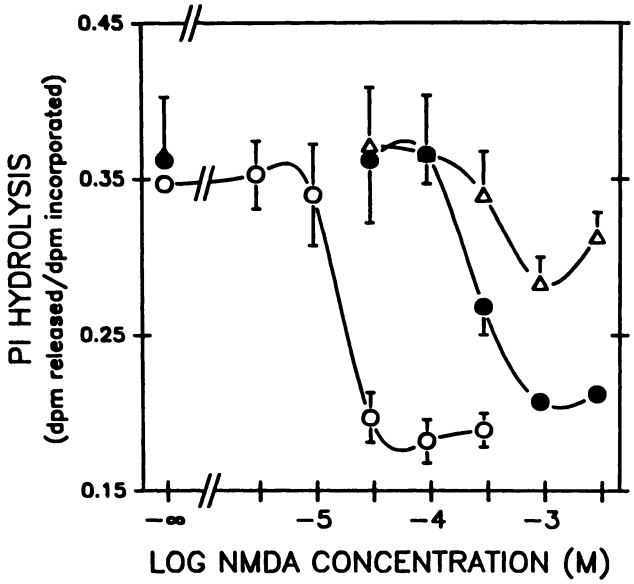

Fig. 8. Competitive and noncompetitive inhibition of the negative modulation of carbachol-stimulated PI hydrolysis in cortical slices by AP-5 and (+)-MK-801. NMDA concentration-effect curves for the inhibition of carbachol-stimulated PI hydrolysis were determined in the absence (○) or presence of 300 μM AP-5 (●) or 1 μM (+)-MK-801 (△). Experiments were performed as described in the legend to Figure 5. Each *point* is the mean ± standard error of three experiments. When not shown, the *standard error bar* is within the *symbol* for that *point*.

partic acid inhibition of carbachol-stimulated PI hydrolysis in hippocampal slices is unaltered in calcium-free medium. It is possible that this reflects differences in the mechanism of the modulatory influence of NMDA on muscarinic receptor function in cerebral cortex compared with hippocampus. However, other experimental variables such as temperature or time of incubation may also contribute to the different findings. The data here suggest that NMDA-associated calcium channels may play a role in the modulation of muscarinic receptor-stimulated PI hydrolysis in the cerebral cortex. One possible explanation for our findings may be that the effect of NMDA is due to consequences of cation channel activation including calcium influx and not due to a primary receptor-linked mechanism. A recent paper by Vallar *et al.* (24) reported that D_2 receptor-activated inhibition of thyrotropin-releasing hormone-stimulated PI hydrolysis in pituitary cells is due to calcium influx. Further studies are needed to elucidate the precise role that NMDA-coupled calcium channels may play in the modulation of receptor-coupled PI hydrolysis.

Magnesium is thought to block electrophysiological responses of NMDA by interacting with a binding site within the cation channel that is gated by the NMDA receptor (16, 25). Our initial observation of NMDA inhibition of carbachol-stimulated PI hydrolysis in Krebs-Ringer bicarbonate buffer that contained 1.18 mM Mg^{2+} suggested that the NMDA binding site linked to muscarinic receptor-coupled PI hydrolysis is separate from the Mg^{2+}-dependent cation channel. Further studies showed that preincubation of cortical slices with NMDA also caused inhibition of the carbachol-stimulated response, even when NMDA was not present during the stimulation of muscarinic receptors. Using this technique of preincubating the slices with NMDA, we were able to observe a small but significant increase in potency of NMDA in the absence of Mg^{2+}. Thus, the inhibitory effect of NMDA on carbachol-stimulated PI hydrolysis is slightly sensitive to the presence of Mg^{2+} in the medium, although it is not completely blocked by Mg^{2+}.

The demonstration that preincubation of slices with NMDA is able to cause a reduction in the muscarinic-stimulated PI hydrolysis response further suggests that the effect of NMDA may be due to events that are subsequent to activation of the cation channel. These data are not consistent with the idea that NMDA inhibition of carbachol-stimulated PI hydrolysis is a primary receptor-triggered event. Further experiments investigating shorter time courses and separation of inositol phosphates may be helpful in further elucidating the mechanism of NMDA inhibition of carbachol-stimulated PI hydrolysis.

The characterization of the NMDA type of glutamate receptors by radioligand binding techniques has been difficult because of the lack of selective antagonists for this site. AP-5 blocks the NMDA recognition site and is a competitive antagonist in functional assays (17, 28). However, several compounds have recently been discovered that inhibit NMDA-induced excitations through an interaction with a cation channel that is gated by this NMDA receptor. Phencyclidine and other dissociative anesthetics are potent inhibitors of NMDA-gated cation conductance and are thought to bind to a site inside the ionophore (25, 26). MK-801, a potential anticonvulsant, has recently been demonstrated to be a potent ligand that can block NMDA-evoked depolarizations (1). In addition, ligands that have a high affinity for the σ opiate receptor also bind to the phencyclidine site (17). Thus, the rank order of potencies of a series of these compounds has been used to distinguish between putative phencyclidine and σ receptor sites (29). The phencyclidine site is defined by the rank order MK-801 > phencyclidine > (−)-cyclazocine > *N*-allylnormetazocine > ketamine > haloperidol. We have determined the potencies of these com-

pounds and others as antagonists of the inhibition of carbachol-stimulated PI hydrolysis by NMDA. The order of antagonist potencies for the reversal of NMDA inhibition of carbachol-stimulated PI hydrolysis in cortical slices is (+)-MK-801 > phencyclidine = (−)-cyclazocine > ketamine = (+)-cyclazocine > *N*-allylnormetazocine > AP-5 ≥ D-α-aminoadipic acid. This order of potency matches that proposed for the phencyclidine site. In contrast, the order of potencies for inhibition of NMDA-modulated PI hydrolysis does not agree with the order of potencies for the σ site. The stereoselectivity that we observed for the isomers of MK-801, cyclazocine, and *N*-allylnormetazocine agrees with published potencies of these compounds in phencyclidine binding assays or behavioral tests (17). We conclude that the antagonist binding site for NMDA effects on PI hydrolysis has characteristics similar to those of the phencyclidine binding site, which has previously been shown to be associated with the NMDA-gated cation channel.

The dissociative anesthetics etoxadrol and dexoxadrol have been used as ligands to characterize the phencyclidine site, with affinities for this site similar to that of phencyclidine (26, 30). However, in our studies etoxadrol was clearly not as potent as phencyclidine for antagonizing the effect of NMDA on carbachol-stimulated PI hydrolysis (Table 1). Furthermore, our studies revealed significant antimuscarinic properties for both etoxadrol and dexoxadrol. The σ ligand (+)-cyclazocine also inhibited muscarinic-stimulated PI hydrolysis at concentrations above 100 μM. The inverted U-shaped concentration-effect curves for (−)-cyclazocine, (+)-cyclazocine, and etoxadrol are probably due to these antimuscarinic properties. We are not aware of any previous studies that have reported antimuscarinic effects of σ ligands. It is possible that some of the behavioral effects of these drugs may be due to blockade of muscarinic receptor-coupled PI hydrolysis.

Characterization of the type of inhibition of the NMDA effects on carbachol-stimulated PI hydrolysis by AP-5 and (+)-MK-801 agrees with previous studies of the effects of these drugs on NMDA-stimulated responses (26, 33). AP-5 caused a parallel shift to the right of the NMDA concentration-effect curve, consistent with competitive antagonism. A dose ratio analysis of the data in Fig. 8 provides an estimate of 4.8 for the pA_2 value for AP-5, which agrees with previous studies using electrophysiological responses (31, 32). Our data with (+)-MK-801 show that its type of inhibition of NMDA modulation of carbachol-stimulated PI hydrolysis is insurmountable, similar to findings with neurophysiological measures (1). In view of binding data that suggest that MK-801 does not directly interact with the NMDA recognition site, we conclude that MK-801 is probably acting at an allosteric site (33).

Wroblewski *et al.* (34) reported the inhibitory potencies of phencyclidine congeners for antagonism of basal PI hydrolysis in cerebellar cultures, which was probably due to stimulation of NMDA receptors by endogenous glutamate. This potency series also agrees well with that proposed for the phencyclidine site. It thus appears that the proposed GP_1 receptor for stimulation of PI hydrolysis in cerebellar cultures has structural features similar to those of the inhibitory NMDA receptor that modulates muscarinic-stimulated PI hydrolysis in cortical slices.

The bulk of the data we have presented here strongly suggest that the effect of NMDA on carbachol-stimulated PI hydrolysis is mediated by a receptor similar to the well characterized NMDA receptor that controls cation conductance. These data include the rank order of potencies of a series of antagonists, antagonist efficacy of channel-blocking drugs, and dependence on extracellular calcium. Less strong evidence is the low sensitivity of the potency of NMDA to extracellular Mg^{2+}. Other workers using chopped brain tissue have similarly reported a lack of effect of Mg^{2+} on NMDA mediated $^{22}Na^{+}$ efflux (35, 37). It is possible that the chopped brain slice preparation used here has become become sufficiently depolarized to relieve the Mg^{2+} blockade of the NMDA-gated cation channel. Another discrepancy concerns the relative lower potency of etoxadrol as an antagonist, compared with phencyclidine, which does not agree with the proposed order of potencies for definition of the phencyclidine recognition site (29). Presumably, these antagonists are acting as channel blockers, and their action is dependent upon the opening of the channel by endogenous agonists. Because we used a 10-min preincubation time, this may not have been long enought to allow equilibration of the antagonist with its binding site inside the channel. This and other unknown factors may have influenced our determination of potencies, so small differences such as this are not surprising. However, the suggestion of a phencyclidine site separate from the Mg^{2+}-sensitive site that is located in the NMDA-gated cation channel cannot be absolutely excluded at this time (36).

Schmidt *et al.* (8) have suggested that the neurotoxic properties of NMDA may be responsible for inhibition of muscarinic-stimulated PI hydrolysis in striatal primary cultures. Certainly, long term incubations of primary cultures with glutamate or NMDA cause release of lactate dehydrogenase, indicating loss of plasma membrane viability (8, 36). Under these conditions, the calcium gradient would be expected to collapse, causing a large increase in intracellular calcium concentrations. This would then lead to an activation of calcium-dependent phosphoinositidase C, resulting in an increase in levels of inositol phosphates instead of a reduction (38). In view of the inhibitory efficacy of NMDA against carbachol-stimulated PI hydrolysis when tissue is preincubated with NMDA, we speculate that the cells have undergone drastic changes in function, which lead to an uncoupling of the muscarinic-stimulated PI response. However, we think it is unlikely that neuronal death itself is the sole underlying mechanism for the inhibitory effect of NMDA.

In summary, we have shown that NMDA can negatively modulate PI hydrolysis stimulated by muscarinic receptor activation in rat cortex. This interaction is not due to increased metabolism of accumulated inositol phosphates and probably occurs as a result of activation of the NMDA-gated cation channel. The effect of NMDA is reduced in the absence of extracellular calcium and is only slightly affected by changes in magnesium concentration. Antagonists of the NMDA-gated cation channel are also effective inhibitors of the effects of NMDA on carbachol-stimulated PI hydrolysis. The rank order of potencies of the antagonists suggests that a phencyclidine binding site is associated with NMDA modulation of PI hydrolysis. Although there are minor differences between the characteristics of NMDA inhibition of muscarinic-stimulated PI hydrolysis we describe and the NMDA-gated cation channel, such as the magnitude of blockade in the presence of Mg^{2+} and relative potency of etoxadrol versus phencyclidine, the data taken together suggest that activation of the NMDA-gated cation channel is the underlying mechanism for the effects on

PI hydrolysis. Further studies are in progress to determine the mechanism of the interaction between the NMDA receptor and muscarinic receptors that are coupled to phosphoinositidase C in the rat cortex.

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