0026-895X/92/040750-07$03.00/0

MOLECULAR PHARMACOLOGY, 41:750-756



# Characterization of Organophosphate Interactions at *N*-Methyl-D-aspartate Receptors in Brain Synaptic Membranes

PETER S. JOHNSON and ELIAS K. MICHAELIS

*Department of Pharmacology and Toxicology (P.S.J., E.K.M.) and the Center for Biomedical Research (E.K.M.), University of Kansas, Lawrence, Kansas 66045*

Received May 9, 1991; Accepted December 20, 1991

**SUMMARY**

Several competitive antagonists of the *N*-methyl-D-aspartate (NMDA) subtype of excitatory amino acid receptors are phosphonate analogs of L-glutamic acid. The position of the phosphonate has been shown to be important in the structure-activity relationships of these analogs. To investigate whether other phosphorous-containing compounds had activity at the NMDA receptor, several organophosphates were tested for the ability to inhibit the specific binding to brain synaptic membranes of 3-((±)-2-carboxypiperazin-4-yl)-[1,2-$^3$H]propyl-1-phosphonic acid ([$^3$H]CPP), a selective antagonist of NMDA receptors. Diisopropylfluorophosphate (DFP), dichlorvos, cyanophos, mipafox, and *o*-ethyl *o*-4-nitrophenyl phenylphosphonothioate are relatively potent inhibitors of [$^3$H]CPP binding to synaptic membranes. The inhibition produced by DFP is selective for the NMDA subtype of excitatory amino acid receptors, is irreversible, and can be prevented by preincubation with excess CPP, 2-amino-7-phosphonoheptanoic acid, or L-glutamate. Rat brain synaptic membranes have a population of phosphonate-sensitive [$^3$H]DFP binding sites that are covalently labeled by [$^3$H]DFP. Two protein bands of synaptic membrane proteins subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis are labeled by [$^3$H]DFP in a 2-amino-5-phosphonopentanoic acid-sensitive manner. These proteins have an average molecular size of 47–50 and 32 kDa. Proteins of nearly identical molecular sizes have been shown in other studies to be components of an NMDA receptor complex. These observations are indicative of an interaction between the organophosphates and the NMDA receptor protein complex and suggest that DFP may be another important pharmacological tool that can be used in the elucidation of the molecular structure of the NMDA receptor complex.

The receptors for the endogenous excitatory amino acid neurotransmitter L-glutamate have been the focus of intense research for the past several years. There are at least four subtypes of receptors activated by L-glutamate (1, 2). In the class of ion channel-forming receptors there are the receptors activated by either L-glutamate and its analog NMDA or L-glutamate and its other analogs kainic acid, AMPA, and quisqualic acid (2–4). Two additional classes of excitatory amino acid receptors, the 2-amino-4-phosphonobutyric acid-sensitive glutamate receptors and the metabotropic glutamate receptor, have been defined (2). The NMDA subtype of excitatory amino acid receptor has been analyzed very extensively because of its role in important physiological and neuropathological processes such as long term potentiation, a physiological analog of learning and memory (5, 6), the initiation of neuronal damage induced by ischemia and hypoglycemia (7, 8), and the generation of seizure activity (9–11).

The L-glutamate phosphonate analogs 2-AP5, 2-AP7, and CPP have been widely used for the characterization of NMDA receptors in neuronal membranes (12–14). These agents act as competitive antagonists of the NMDA receptor, but there is mounting evidence that there are distinct binding sites in brain neuronal membranes and isolated proteins for L-glutamate and NMDA and for these so-called "competitive" antagonists of the NMDA receptor (15–18). The evidence for this assertion includes data from autoradiographic studies that demonstrate a differential distribution of sites labeled by L-[$^3$H]glutamate and NMDA versus those labeled by [$^3$H]CPP (16), different molecular size estimates obtained by radiation-inactivation studies of the target macromolecules that bind L-[$^3$H]glutamate (NMDA-sensitive sites) and [$^3$H]CPP (17), and different protein subunits of an isolated complex to which [$^3$H]CPP binds but neither glutamate nor NMDA binds (18, 19). This has led to the conceptualization of the NMDA receptor as a complex

This work was supported by Army Research Office Grants DAAL 03-88-K0017 and DAAL 03-91-G-0167 and by National Institute of Alcohol and Alcoholism Grant AA04732 to E.K.M. P.S.J. is supported by a Training Grant from the United States Public Health Service, GM07775-12.

**ABBREVIATIONS:** NMDA, *N*-methyl-D-aspartic acid; AMPA, (±)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; 2-AP5, 2-amino-5-phosphonopentanoic acid; 2-AP7, 2-amino-7-phosphonoheptanoic acid; CPP, 3-((±)-2-carboxypiperazin-4-yl)-propylphosphonic acid; DFP, diisopropylfluorophosphate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; EPN,*o*-ethyl *o*-4-nitrophenyl phenylphosphonothioate; TCP, *N*-(1-(2-thienyl)cyclohexyl)piperidine; TOCP, triorthocresyl phosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DMSO, dimethyl sulfoxide.

of proteins with closely interacting recognition sites (15, 17–19).

Ikin *et al.* (20) have reported the purification from rat brain of an NMDA receptor complex that consists of four polypeptides of molecular sizes equal to 67, 57, 46, and 33 kDa. We have recently reported on the isolation, purification, and reconstitution of an NMDA receptor complex (19, 21) that consists of proteins of similar molecular sizes as those isolated by Ikin *et al.* (20), and we have identified the 67–70-kDa subunit in this complex as the glutamate-binding protein previously isolated and characterized in our laboratory (19, 21). Furthermore, expression of this glutamate-binding subunit in neurons grown in culture is closely related to the appearance of sensitivity to NMDA-induced neurotoxicity (22). One of the subunits of this complex has been identified, and the cDNA for this subunit was recently cloned (19).

We are currently focusing on the characterization of the second subunit of the NMDA receptor complex, a protein with the molecular size of 55–60 kDa (19). We have identified this protein as the subunit that binds CPP, 2-AP5, and 2-AP7 (19), i.e., the same protein that we previously purified and named the CPP-binding protein (18). Because it has been reported that guanosine mono-, di-, and triphosphates compete with CPP for binding to the NMDA receptor complex (23), we have explored, in this study, the possibility that organophosphates or phosphonates may also interact with the CPP-binding sites of the NMDA receptor and may label subunits of this receptor by covalent chemical bond formation. We describe the initial characterization of organophosphate interactions at the NMDA receptor in synaptic membranes, and we suggest that DFP and other organophosphorous compounds could become important pharmacological tools for the elucidation of the molecular structure of the NMDA receptor complex.

## Experimental Procedures

### Materials

Male, adult, Sprague-Dawley rats were obtained from a breeding colony housed at the animal care unit of the University of Kansas. New England Nuclear supplied the [$^3$H]CPP (33.0 Ci/mmol), [$^3$H]DFP (4.0 Ci/mmol), [$^3$H]AMPA (27.6 Ci/mmol), and [$^3$H]TCP (46.9 Ci/mmol). Amersham supplied the [$^3$H]glycine (18.7 Ci/mmol). *o*-(4-Cyanophenyl)phosphorothioate (cyanophos), 2,2-dichloroethenyl *o,o*-dimethyl phosphate (dichlorvos), and EPN were generous gifts from Dr. Paul Kitos (University of Kansas). *N,N'*-Diisopropylphosphorodiamidic fluoride (mipafox) was synthesized by Chemsyn Laboratories (Lenexa, KS) and was a generous gift from Dr. Bill Duncan (Oread Labs, Lawrence, KS). DFP, *o,o*-diethyl *o*-4-nitrophenyl phosphate (paraoxon), and phenylmethanesulfonyl fluoride were all obtained from Sigma Chemical Co. All other chemicals and reagents were obtained from sources previously described (18).

### Methods

**Isolation of synaptic plasma membranes.** All procedures were carried out at 2° unless otherwise indicated. Fresh synaptic membranes were prepared from whole-brain homogenates from male rats (250–300 g) by procedures described previously (18). Two to four brains were homogenized in 20 volumes of 0.32 M sucrose in 10 mM potassium phosphate buffer (pH 7.4) that contained the following protease inhibitors: 10 mM ε-amino caproic acid, 0.1 mM EGTA, 0.1 mM benzamidine HCl, 0.1 mM benzamide, and 0.8 μM pepstatin A. The synaptic membrane pellet was obtained by osmotic rupturing of synaptosomes isolated by Ficoll-sucrose density gradient centrifugation.

The membranes were resuspended in 50 volumes of homogenization buffer (pH 7.4) that contained 0.04% (v/v) Triton X-100 and were incubated for 15 min at 37°. The suspension was then centrifuged at 45,000 × *g* for 15 min, and the resulting pellet was resuspended in ice-cold 40 mM Tris-$SO_4$ buffer (pH 7.2). The centrifugation/resuspension process was repeated five times, to ensure removal of endogenous glutamate from the membranes. The membranes were resuspended in Tris-$SO_4$ buffer at a protein concentration of approximately 5–10 mg/ml, and this suspension was used in radioligand binding studies.

**Radioligand binding assays.** Assays of [$^3$H]CPP, [$^3$H]DFP, [$^3$H]glycine, and [$^3$H]AMPA binding to synaptic membranes were conducted under the following protocol. Aliquots of synaptic plasma membranes (250–500 μg of protein) were incubated in microfuge tubes with Tris-$SO_4$ buffer, pH 7.2, and various concentrations of radioactive ligand, as reported in Results. Various concentrations of competing agents were added to the mixture, bringing the final assay volume to 100 μl. A 30-min incubation at 25° was initiated by the addition of the radioactive ligand. The reaction was stopped by centrifugation at 23,000 × *g* for 10 min at 2°. The supernatant was carefully aspirated, and the remaining pellet was solubilized in 50 μl of 10% SDS. The retained activity was measured by liquid scintillation counting.

Estimations of nonspecific binding were obtained as follows: nonspecific [$^3$H]CPP binding was defined in the presence of 100 μM 2-AP5, 100 μM nonradioactive DFP was used to define nonspecific [$^3$H]DFP binding, and 100 μM nonradioactive glycine was used to define nonspecific glycine binding. All incubation mixtures used to determine [$^3$H]glycine binding contained 100 μM strychnine, in order to prevent glycine binding to strychnine-sensitive receptors. [$^3$H]AMPA binding was assayed in the presence of 100 mM $SCN^-$, according to the method of Nielsen *et al.* (24), using 100 μM kainate and 100 μM quisqualate to define nonspecific binding for kainate-sensitive and quisqualate-sensitive binding sites, respectively.

A rapid filtration assay was used for the measurement of [$^3$H]TCP binding to synaptic membranes. Aliquots of protein (100–120 μg of protein) were incubated in microfuge tubes with [$^3$H]TCP, Tris-$SO_4$ buffer, pH 7.2, and various concentrations of competing ligands, in a total assay volume of 200 μl. The reaction was initiated by the addition of the radioactive ligand. After a 25-min incubation at 25°, the reaction was stopped by the addition of 500 μl of ice-cold Tris-$SO_4$ buffer and was filtered through Whatman GF/C filters that had been presoaked in 0.05% polyethyleneimine. The filters were washed twice with 3 ml of potassium phosphate buffer (pH 7.4), and the retained activity was measured by liquid scintillation counting. Nonspecific binding was defined in the presence of 100 μM MK-801.

**SDS-PAGE analysis of [$^3$H]DFP-labeled polypeptides.** Rat synaptic membranes were prepared as described above, including all membrane washing steps. Aliquots of 500 μg of membrane proteins were exposed to a 15-min preincubation in microfuge tubes at room temperature, either in 40 mM Tris-$SO_4$ buffer, pH 7.2, or in the same buffer containing 10 mM 2-AP5. After this preincubation, 10 μCi of [$^3$H]DFP (final concentration, 25 μM) were added to each tube (final volume, 100 μl). The mixture was incubated at room temperature for 45 min, and the labeling reaction was stopped by centrifugation at 23,000 × *g* for 10 min. The supernatant was aspirated as described above, and the pellet was washed two times with Tris-$SO_4$ buffer. The membrane pellets were then solubilized in SDS-containing sample buffer, and the samples (after overnight incubation at 37°) were loaded onto a 16-cm 12% acrylamide slab gel, using the buffer system of Laemmli (25), at 200-mV constant potential.

The gels were then stained with Commassie blue and photographed to document the migration of the polypeptides and standard molecular size markers. The gels were subsequently sliced into 1.5-cm lanes, and each lane was cut into 2-mm slices. Each slice was incubated overnight in 1 ml of 30% (v/v) hydrogen peroxide at 37°, and the radioactivity associated with each slice was determined by liquid scintillation counting.

**Protein assay and data analysis.** The protein concentration of all samples was estimated by the bicinchoninic acid method described

by Smith *et al.* (26). Bovine serum albumin was used as the standard. The LIGAND program of Munson and Rodbard, as modified for the IBM PC by McPherson (27), was used to analyze the data of ligand binding to synaptic membranes. Data on the kinetics of ligand binding were analyzed using the computer program Enzfitter, from BIOSOFT.

## Results

**Effects of organophosphorous compounds on [$^3$H]CPP binding to synaptic membranes.** All steps in the isolation of synaptic membranes were carried out in the presence of five protease inhibitors, in order to prevent proteolytic degradation of membrane proteins. The synaptic plasma membranes were washed extensively by repeated cycles of exposure to dilute Triton X-100 solutions, centrifugation, and resuspension in fresh buffer. This was done to ensure removal of endogenous glutamate before the ligand binding studies were performed. Fresh synaptic membranes were prepared for all ligand binding studies, because freezing of the membranes caused decreases in specific [$^3$H]CPP binding activity. Stock solutions of organophosphates were prepared immediately before each binding assay, using DMSO as the solvent. The concentration of DMSO in the binding assays never exceeded 2% and, at this concentration, DMSO had no effect on control or nonspecific [$^3$H]CPP binding (data not shown).

An initial characterization of the effects of organophosphates on [$^3$H]CPP binding was performed (Fig. 1). A variety of compounds were selected on the basis of structural differences. It should be noted that all ligand binding assays were conducted at 25° for 30 min., i.e., conditions that we have determined to be appropriate for achieving equilibrium of [$^3$H]CPP binding to synaptic membranes. However, if any of the organophosphorous agents being tested produced irreversible inhibition, the incubation period of 30 min at 25° may not have been sufficient for complete reaction of each agent with all available sites. All compounds initially tested were found to be relatively potent inhibitors of [$^3$H]CPP binding. The compounds that inhibited [$^3$H]CPP binding to synaptic membranes included the fluorinated organophosphates DFP and mipafox, a nonfluorinated phosphorothioate, cyanophos, and a nonfluorinated phosphonothioate, EPN (Fig. 1). These agents inhibited [$^3$H]CPP binding to synaptic membranes with approximately the same potency as determined in these preliminary studies.

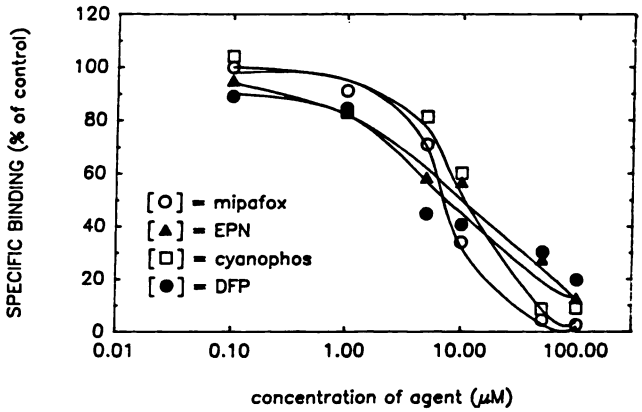


**Fig. 1.** Displacement of 100 nM [$^3$H]CPP binding to synaptic membranes by various organophosphorous agents. Each *point* is the mean of the percentage of control specific [$^3$H]CPP binding after incubation of membranes with the concentrations of competing ligand shown. The data are mean values from six to nine determinations from two or three experiments. The *curves* drawn are based on calculated values for inhibitory constants for each agent.

A few organophosphorous compounds that are thought to act on a variety of sites in the central nervous system were also tested for their effects on [$^3$H]CPP binding to synaptic membranes, in order to provide a comparison with the agents shown in Fig. 1. Most of the organophosphates tested showed near-complete inhibition of [$^3$H]CPP binding to synaptic membranes when they were introduced into the incubation medium at a concentration of 100 μM (Table 1). Interestingly, TOCP, a triaryl organophosphate that is known for its ability to induce delayed neuropathy but has little acute toxicity, was an ineffective inhibitor of [$^3$H]CPP binding. Acetylcholine, the endogenous agonist of nicotinic and muscarinic receptors and a substrate for one class of serine esterases, i.e., acetylcholinesterase, showed limited and variable inhibition of [$^3$H]CPP binding. Some nicotinic cholinergic agonists have been reported, in electrophysiological studies, to decrease the response of the NMDA receptor to its agonists (28). We do not know whether the partial inhibition of [$^3$H]CPP binding by high concentrations of acetylcholine is a reflection of such interactions with the NMDA receptor. Inorganic phosphate in ionic form had no effect on [$^3$H]CPP binding when added directly to the assay medium at a concentration of 1 mM (Table 1).

**Selectivity of organophosphate-induced inhibition of the CPP binding sites.** A series of studies were conducted to determine whether inhibition by organophosphates of [$^3$H]CPP binding to synaptic membranes was specific to the NMDA subtype of glutamate receptor and whether the organophosphates affected a multiplicity of ligand recognition sites within the NMDA receptor complex. We measured DFP-induced inhibition of quisqualate- and kainate-sensitive [$^3$H]AMPA binding to synaptic membranes, as a means of determining DFP interaction with non-NMDA receptors in these membranes. The possibility that DFP may alter the interaction of the allosteric modulator glycine or the ion channel-blocker TCP with their binding sites in the NMDA receptor complex was also explored. The results of these studies are summarized in

TABLE 1

**Effects of various compounds on 100 nM [$^3$H]CPP binding to rat synaptic membranes**

The specific binding of 100 nM [$^3$H]CPP to synaptic membranes was assayed as described in Experimental Procedures and is 0.47 ± 0.05 pmol/mg of protein (20 experiments). The values shown represent the means ± standard errors of the percentage of inhibition of specific binding caused by 100 μM levels of the compounds listed. Numbers in parentheses represent number of experimental determinations.

| Compound (100 μM) | Inhibition of [$^3$H] CPP binding |
|---|---|
| | % |
| Organophosphorus esters | |
| Cyanophos | 80 ± 8 (9) |
| Dichlorvos | 87 ± 6 (9) |
| DFP | 89 ± 4 (4) |
| EPN | 82 ± 7 (11) |
| Mipafox | 89 ± 6 (9) |
| Paraoxon | 88 ± 10 (3) |
| TOCP | 56 ± 8 (5) |
| Others | |
| Acetylcholine | 60 ± 16 (5) |
| PMSF[a] | 98 ± 9 (6) |
| $K_2HPO_4/KH_2PO_4$[b] | 7 ± 12 (12) |

[a] PMSF, phenylmethanesulfonyl fluoride.
[b] Final concentration of inorganic phosphate in the assays was 1 mM.

Table 2. Only [$^3$H]CPP binding was inhibited significantly by DFP. This agent had no effect on either kainate- or quisqualate-sensitive [$^3$H]AMPA binding, strychnine-insensitive [$^3$H]glycine binding, or [$^3$H]TCP binding to synaptic membranes.

The fact that DFP did not affect [$^3$H]TCP binding to synaptic membranes is somewhat surprising, given the previously observed inhibition of [$^3$H]TCP binding by 2-AP5 (29). This may be an indication that DFP interaction with the NMDA receptor either is occurring at a different site from the binding site for the phosphonate analogs of L-glutamate or that DFP binds to the aminophosphonocarboxylate recognition site but does not produce the same conformational change in the receptor macromolecule as the amino phosphonocarboxylic acids do.

**Irreversibility of inhibition by DFP of CPP binding to membranes.** It is well known that organophosphorous agents such as DFP are irreversible inhibitors of serine esterases. Therefore, in a separate series of experiments, the reversibility of DFP inhibition of [$^3$H]CPP binding to synaptic membranes was explored. The results of these studies are summarized in Table 3. The value of specific [$^3$H]CPP binding to membranes assayed under control conditions was arbitrarily set at 100%. Incubation of the membranes with buffer for 25 min at room temperature, followed by five washing steps with 40 mM Tris-$SO_4$ buffer, pH 7.2, produced no significant decreases in the control binding activity. A 25-min preincubation with 1 mM CPP without subsequent washing of the membranes essentially eliminated all [$^3$H]CPP binding, as would be expected; however, when the membranes that were preincubated with 1 mM CPP were subsequently washed five times with buffer, the specific [$^3$H]CPP binding activity measured after the washing steps was at the level of control values. This observation was also predictable, because the association of CPP with its binding sites is a reversible process.

A 10-min preincubation at 25° with 10 μM DFP produced approximately 50% inhibition of specific [$^3$H]CPP binding to the membranes. This inhibition was apparently irreversible, as evidenced by the fact that DFP inhibition of specific [$^3$H]CPP binding was not altered after five repeated washings and resuspensions of the membranes in fresh Tris-$SO_4$ buffer. If, on the other hand, membranes were preincubated with 1 mM CPP before the 10-min exposure to DFP and subsequently washed five times, there was complete protection of the [$^3$H]CPP binding to the membranes (Table 3). Specific binding of [$^3$H]CPP to membranes that were pretreated with 1 mM CPP before exposure to DFP and then subsequently washed five times was 98% of the control values. When either 1 mM 2-AP7 or 1 mM L-glutamate was used as the protecting agent in place of CPP in the same experimental paradigm, specific binding of [$^3$H]CPP to the washed synaptic membranes was 103% and 97% of the control values, respectively (Table 3). Exposure to these agents before the introduction of DFP, but without any membrane washing steps after such exposure, produced nearly complete inhibition of [$^3$H]CPP binding. This would be expected because of the well documented inhibition of [$^3$H]CPP binding in the presence of high concentrations of L-glutamate and 2-AP7. The fact that there is still a small amount of specific [$^3$H]CPP binding remaining in the presence of 1 mM concentrations of the competing agents is considered to be the result of variability in the assays performed across different preparations.

**Kinetic properties of [$^3$H]DFP binding to synaptic membranes.** If DFP is inhibiting the specific binding sites for [$^3$H]CPP in the NMDA receptor in synaptic membranes, then we should be able to demonstrate the converse, i.e., inhibition of at least some of the specific binding of [$^3$H]DFP by the phosphonate analogs of L-glutamate. In order to perform these studies, we first characterized the binding of [$^3$H]DFP to synaptic membranes. Specific binding of 2.5 μM [$^3$H]DFP to synaptic membranes increased linearly with increasing amounts of protein (0–540 μg) used in the assays. When 250–500 μg of protein were incubated with 2.5 μM [$^3$H]DFP, specific binding ranged from 2.0 to 5.0 pmol/mg of protein and represented approximately 50% of the total signal. The aminophosphonocarboxylic acid-sensitive component of [$^3$H]DFP binding to synaptic membranes was defined as that portion of the specific [$^3$H]DFP signal that was displaced by 100 μM 2-AP5. This component of [$^3$H]DFP binding to synaptic membranes represented 70–90% of the total specific [$^3$H]DFP binding signal (see below).

The kinetics of [$^3$H]DFP binding to sites that are also sensitive to phosphonate analogs of glutamate are shown in Fig. 2.

TABLE 2

**Effects of DFP on various glutamate receptor ligand binding recognition sites**

The specific binding of several glutamate receptor ligands to synaptic membranes was assayed as described in Experimental Procedures. Values shown are the means ± standard errors for both the specific binding of the ligand to membranes and the percentage of control binding measured in the presence of 100 μM DFP. The number of determinations is shown in parentheses.

| $^3$H Ligand | Concentration of $^3$H ligand | Binding activity | Binding in the presence of 100 μM DFP |
|---|---|---|---|
| | *nM* | *pmol/mg* | *% of control* |
| CPP | 100 | 0.43 ± 0.03 (6) | 18 ± 5 (6)[a] |
| AMPA (quisqualate sensitive) | 110 | 1.09 ± 0.07 (15) | 81 ± 17 (12)[b] |
| AMPA (kainate sensitive) | 110 | 0.89 ± 0.08 (12) | 84 ± 25 (12)[b] |
| Glycine (strychnine insensitive) | 90 | 2.29 ± 0.30 (3) | 89 ± 18 (6)[b] |
| TCP | 33 | 0.41 ± 0.01 (3) | 96 ± 4 (8)[b] |

[a] Significantly different from control, $p < 0.05$, by Student's *t* test.
[b] Not significantly different from control.

TABLE 3

**Irreversibility of DFP inhibition of CPP binding sites and protection of the sites by glutamate and phosphonate analogs**

Control specific [$^3$H]CPP (100 nM) binding, determined as described in Experimental Procedures, is 0.62 ± 0.01 pmol/mg of protein (nine experiments). This value represents 100% activity. All values are expressed as the means ± standard errors of percentage of control binding for each pretreatment condition. The number of experimental determinations is shown in parentheses.

| Initial preincubation (25 min) | 10 μM DFP preincubation (10 min) | Membrane washing | [$^3$H]CPP binding |
|---|---|---|---|
| | | | *% of control* |
| Buffer | − | 0 | 100 (9) |
| Buffer | − | 5× | 95 ± 5 (9) |
| 1 mM CPP | − | 0 | 4 ± 2 (9) |
| 1 mM CPP | − | 5× | 95 ± 4 (9) |
| Buffer | + | 0 | 47 ± 2 (15) |
| Buffer | + | 5× | 47 ± 3 (15) |
| 1 mM CPP | + | 0 | 7 ± 4 (6) |
| 1 mM CPP | + | 5× | 98 ± 6 (6) |
| 1 mM 2-AP7 | + | 0 | 5 ± 3 (6) |
| 1 mM 2-AP7 | + | 5× | 103 ± 2 (6) |
| 1 mM L-glutamate | + | 0 | 6 ± 3 (6) |
| 1 mM L-glutamate | + | 5× | 97 ± 8 (6) |

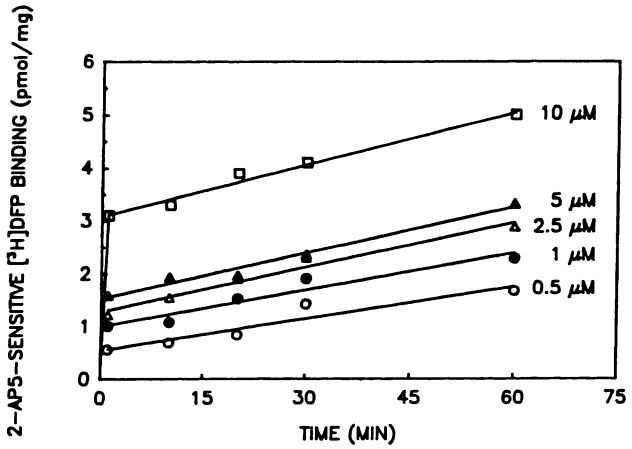


**Fig. 2.** Kinetics of the association of [$^3$H]DFP with the 2-AP5-sensitive binding sites in synaptic membranes. The reactions of synaptic membranes with [$^3$H]DFP were quenched by the addition of 300 μl of 0.1 N HCl at the times indicated. The association of [$^3$H]DFP to 2-AP5-sensitive binding sites in synaptic membranes was measured at ligand concentrations varying between 0.5 and 10 μM. Data are the mean ± standard error of values from four to six determinations from two experiments for each concentration of [$^3$H]DFP indicated. Data were analyzed by fitting to equations for psuedo-first-order kinetics.

In order to obtain an accurate termination point for the incubation of [$^3$H]DFP with the membranes, we introduced a modification to the assays used to measure ligand binding to the membranes. These assays were performed under conditions identical to those described in Experimental Procedures, with the exception that, at the time points indicated, the incubation was quenched by the addition of 300 μl of 0.1 N HCl and the bound and free ligand amounts were determined after centrifugation of the samples. The quenching step had no effect on control or nonspecific [$^3$H]DFP binding to synaptic membranes (data not shown). The data for 2-AP5-sensitive [$^3$H]DFP binding were analyzed by computer-assisted fitting to equations for pseudo-first-order kinetics. The data in Fig. 2 indicate a biphasic association of [$^3$H]DFP with phosphonate-sensitive binding sites in synaptic membranes. The initial, presumably reversible, phase of DFP binding to these sites is rapid, as indicated by the fact that it was complete within 1 min of incubation. The methodologies we used in these studies made it difficult to evaluate the kinetics of [$^3$H]DFP binding at times of incubation shorter than 1 min; therefore, it is not possible to calculate accurately any rate constants for the rapid phase of association of DFP with the macromolecules being labeled. We assume that the rapid initial binding of DFP to the phosphonate-sensitive sites leads to the formation of a DFP-protein complex, $C$, and that the rate of formation of $C$ depends on the intrinsic affinity of the protein sites for DFP, on the concentration of DFP, and on the concentration of other proteins that can bind DFP. The rate constant for the second phase of the reaction ($k_2$) was apparently independent of the concentration of [$^3$H]DFP and was determined to be equal to $1.76 \pm 0.002 \times 10^{-3}$ min$^{-1}$ (mean value of constants estimated for all concentrations tested). The rate of formation of the irreversibly labeled complex $L$ is equal to $k_2C$, i.e., the rate of formation of $L$ should remain constant after the initial formation of the complex $C$ (30). The data in Fig. 2 fit this interpretation.

**Analysis of synaptic membrane proteins labeled with [$^3$H]DFP.** Because [$^3$H]DFP was shown to bind covalently to synaptic membrane entities that are also involved in the recognition of aminophosphonocarboxylates, we used this ligand to identify the size of the labeled macromolecules after SDS-PAGE of membrane proteins. Synaptic membrane proteins were reacted with [$^3$H]DFP either in the absence or in the presence of high concentrations of 2-AP5. Membranes pretreated with 2-AP5 represent the preparations in which NMDA receptor sites are protected from labeling by [$^3$H]DFP, i.e., they represent background labeling by this organophosphate. In preliminary studies we have observed that 2-AP5 competes effectively with the binding of [$^3$H]DFP to synaptic membranes (data not shown).

The results of four different membrane-labeling and SDS-PAGE experiments are shown in Fig. 3. The histogram is a composite of the average values of radioactivity in each gel slice from the four experiments. In all experiments, two peaks of [$^3$H]DFP labeling, corresponding to polypeptides of 47–50-kDa and 32-kDa molecular size, were observed (Fig. 3). Both peaks of [$^3$H]DFP labeling were substantially diminished by pretreatment of the membranes with 2-AP5. [$^3$H]DFP labeling of the approximately 50-kDa species was completely abolished and labeling of the 32-kDa peptide was reduced by >50% by pretreatment of the membranes with 10 mM 2-AP5 (Fig. 3). The polypeptides labeled by [$^3$H]DFP in a 2-AP5-sensitive manner have molecular sizes that are very similar to those of NMDA receptor proteins purified by us (19, 21) and by Ikin *et al.* (20).

## Discussion

Several organophosphates were found to inhibit the specific binding of [$^3$H]CPP to rat synaptic membranes at physiologically relevant concentrations. The potent and irreversible inhibition by DFP of [$^3$H]CPP binding to synaptic membranes and the protection from this inhibition by high concentrations of CPP, 2-AP7, and L-glutamate indicate that organophosphates interact with the NMDA receptor and may produce some of their effects on brain neurons through these interactions with the NMDA receptor complex.

Differences in the structures of the organophosphates, in terms of ester side chains, leaving groups, or even sulfur substitution for oxygen, did not significantly alter the inhibition

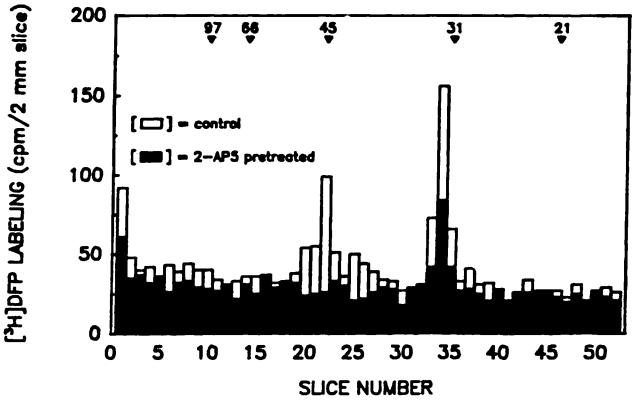


**Fig. 3.** [$^3$H]DFP labeling of synaptic membrane proteins. Synaptic membranes were pretreated with either Tris-$SO_4$ buffer (□) or 10 mM 2-AP5 in the same buffer (■) for 15 min before being exposed to 25 μM [$^3$H]DFP. After a 45-min incubation in the presence of [$^3$H]DFP, the membranes were precipitated by centrifugation, washed, and solubilized, and the labeled proteins were analyzed by SDS-PAGE, according to the methods described in Experimental Procedures. The histogram represents a composite of the mean cpm/slice from four separate gel-slicing experiments.

by the organophosphates of [$^3$H]CPP binding. However, because we did not explore the possibility of more subtle differences in inhibitory activity toward the [$^3$H]CPP binding sites in synaptic membranes, such as kinetic differences in the inhibition of [$^3$H]CPP binding produced by the various organophosphates, we are not certain that all agents have equal activity at the NMDA receptor. We can generally state, though, that all of the organophosphates that are good inhibitors of [$^3$H]CPP binding are also potent inhibitors of acetylcholinesterase. TOCP, on the other hand, is a poor cholinesterase inhibitor and a poor inhibitor of [$^3$H]CPP binding.

With respect to excitatory amino acid neurotransmitter receptors in brain synaptic membranes, the organophosphates appear to interact primarily with the NMDA receptor subtype. DFP did not significantly inhibit the specific binding of the non-NMDA receptor ligand [$^3$H]AMPA, nor did it affect significantly the binding of other ligands that have binding sites on the NMDA receptor-ion channel complex. Based on these observations, we conclude that the organophosphates are acting on either the agonist or competitive antagonist recognition sites of the complex.

The observation that DFP did not inhibit [$^3$H]TCP binding or strychnine-insensitive [$^3$H]glycine binding is somewhat puzzling, in light of the observation that phosphonate analogs of L-glutamate have been shown to modulate glycine binding or to inhibit TCP binding to their respective sites in the NMDA receptor complex (29, 31). Kloog *et al.* (29) have shown that 2-AP5 inhibits the binding of [$^3$H]TCP in membranes, and Monahan *et al.* (31) have shown that the C-5 antagonists (agents with a five-carbon atom chain between the phosphono and carboxylic acid moieties) inhibit a maximum of 55% of the strychnine-insensitive [$^3$H]glycine binding sites in brain membranes. The C-7 antagonists have no direct effect on glycine binding to its recognition sites but can reverse the inhibition produced by the C-5 antagonists. We have not examined the effect of DFP on the inhibition of [$^3$H]glycine binding sites produced by 2-AP5; therefore, it is impossible to conclude that the organophosphates did not produce some indirect effect on [$^3$H]glycine binding sites. Because we did not detect any inhibition by DFP of [$^3$H]TCP binding to synaptic membranes, it is possible that the interaction of DFP with the NMDA receptor does not produce the same conformational changes as those produced by CPP, 2-AP5, or 2-AP7.

The observation described in the present study that preincubation of membranes with either NMDA receptor agonists or antagonists protected the [$^3$H]CPP binding sites in synaptic membranes from inactivation by DFP is interesting, in light of the mounting evidence not only that there are separate recognition sites for agonists and competitive antagonists (15, 16) but also that these recognition sites are on different protein subunits of the NMDA receptor complex (17, 18). This observation would suggest a very close interaction between the proteins that make up the agonist and antagonist binding sites and would support the notion that there may be some functional overlap between the binding sites on the intact receptor complex.

Because DFP binds irreversibly to the NMDA receptor complex, we interpret this type of inhibition to be the reaction of DFP with an activated hydroxyl group in the binding site. Such an activated hydroxyl group may be part of a charge-relay system similar to that of the active site of the serine esterases. The true nature of the active site to which DFP and other organophosphates bind will have to be determined by direct sequence analysis of the proteins labeled by these agents. Our efforts to identify the proteins with which DFP interacts to produce inhibition of CPP binding to the NMDA receptor were initiated by analyzing by SDS-PAGE the proteins that are covalently labeled by [$^3$H]DFP. Two peaks of radioactively labeled peptides were detected in these electrophoretograms. These peaks of radioactivity represented labeling of proteins with molecular sizes equal to 47–50 and 32 kDa. Because a complex of proteins that we have isolated and that has NMDA receptor-like properties has subunits with molecular sizes equal to 66–70, 58–60, 41–46, and 31–36 kDa, as determined by SDS-PAGE (19, 21), it is possible that the synaptic membrane proteins labeled with [$^3$H]DFP correspond to two, or possibly three, of the proteins in the NMDA receptor complex. It is also possible that the [$^3$H]DFP-labeled polypeptides are products of proteolytic degradation of the 58-kDa CPP-binding protein. These issues can only be resolved by proceding with the use of [$^3$H]DFP to label proteins within the purified NMDA receptor complex. These studies have just been initiated.

The interactions of organophosphates with the NMDA receptor add to the complexity of excitatory amino acid neuronal transmission and that of the events leading to the induction of seizures by the organophosphates. There are some reports in the literature of a possible relationship between the effects of organophosphates on the mammalian central nervous system and changes in the activity of the excitatory amino acid neurotransmitter systems. For example, organophosphates can induce epileptiform activity in the hippocampus (32) and produce neuropathological changes that are similar to those brought about by excessive action of excitatory amino acids (33). The NMDA receptor-ion channel inhibitor MK-801 blocks the seizures induced by the organophosphorous nerve poison soman, suggesting a possible role for the NMDA receptor in the seizures induced by the organophosphates (34). Organophosphates have also been shown to activate channel currents in the locust glutamatergic neuromuscular junction (35). Although most of the evidence provided in these studies implicates only indirectly interaction of organophosphates with NMDA receptors, our observations provide a more direct assessment of such organophosphate-NMDA receptor interactions.

**Acknowledgments**

The authors wish to acknowledge the assistance provided by Nancy Harmony and Kim Bland in the preparation of this manuscript and the support provided by the Center for Biomedical Research, University of Kansas.

**References**

1. Monahan, D. T., R. J. Bridges, and C. W. Cotman. The excitatory amino acid receptors: their classes, and distinct properties in the function of the central nervous system. *Annu. Rev. Pharmacol. Toxicol.* **29**:365–402 (1989).
2. Watkins, J. C., P. Krogsgaard-Larsen, and T. Honore. Structure-activity relationships in the development of excitatory amino acid receptor agonists and competitive antagonists. *Trends Pharmacol. Sci.* **11**:25–33 (1990).
3. Cotman, C. W., and L. L. Iversen. Excitatory amino acids in the brain: focus on NMDA receptors. *Trends Neurosci.* **10**:263–265 (1987).
4. Honore, T., S. N. Davies, J. Drejer, E. J. Fletcher, P. Jacobsen, D. Lodge, and F. E. Nielsen. Quinoxalinediones: potent competitive non-NMDA glutamate receptor antagonists. *Science (Washington D. C.)* **241**:701–703 (1988).
5. Morris, R. G. M., E. Anderson, G. S. Lynch, and M. Baudry. Selective impairment of learning and blockade of long-term potentiation by an *N*-methyl-D-aspartate receptor antagonist, AP5. *Nature (Lond.)* **319**:774–776 (1986).
6. Cotman, C. W., D. T. Monahan, and A. H. Ganong. Excitatory amino acid neurotransmission: NMDA receptors and Hebb-type synaptic plasticity. *Annu. Rev. Neurosci.* **11**:61–80 (1988).
7. Simon, R. P., J. H. Swan, T. Griffiths, and B. S. Meldrum. Blockade of *N*-

methyl-D-aspartate receptors may protect against ischemic damage in the brain. *Science (Washington D. C.)* **226**:850–852 (1984).
8. Weiloch, T. Hypoglycemia-induced neuronal damage prevented by *N*-methyl-D-aspartate antagonist. *Science (Washington D. C.)* **230**:681–683 (1985).
9. Piredda, S., and K. Gale. Role of excitatory amino acid transmission in the genesis of seizures elicited from the deep piriform cortex. *Brain Res.* **377**:205–210 (1986).
10. Herron, C. E., R. Williamson, and G. L. Collingridge. A selective *N*-methyl-D-aspartate antagonist depresses epileptiform activity in rat hippocampal slices. *Neurosci. Lett.* **61**:255–260 (1985).
11. Turski, L., T. Klockgether, K.-H. Sontag, P. L. Herrling, and J. C. Watkins. Muscle relaxant and anticonvulsant activity of 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid, a novel *N*-methyl-D-aspartate antagonist, in rodents. *Neurosci. Lett.* **73**:143–148 (1987).
12. Benveniste, M., and M. L. Meyer. Kinetic analysis of antagonist action at *N*-methyl-D-aspartic acid receptors. *Biophys. J.* **59**:560–573 (1991).
13. Murphy, D. E., J. Schneider, C. Boehm, J. Lehmann, and M. Williams. Binding of [$^3$H]3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid to rat brain membranes: a selective, high affinity ligand for *N*-methyl-D-aspartate receptors. *J. Pharmacol. Exp. Ther.* **240**:778–784 (1987).
14. Olverman, H. J., D. T. Monahan, C. W. Cotman, and J. C. Watkins. [$^3$H] CPP, a new competitive ligand for NMDA receptors. *Eur. J. Pharmacol.* **131**:161–162 (1986).
15. Fagg, G. E., J. Baud, R. Hall, and J. Dingwall. Do agonists and competitive antagonists bind to distinct sites on the NMDA receptor? in *Frontiers in Excitatory Amino Acid Research* (E. A. Cavalheiro, J. Lehmann, and L. Turski, eds.). Alan R. Liss, New York, 59–88 (1988).
16. Monaghan, D. T., H. J. Olverman, L. Nguyen, J. C. Watkins, and C. W. Cotman. Two classes of *N*-methyl-D-aspartate recognition sites: differential distribution and differential regulation by glycine. *Proc. Natl. Acad. Sci. USA* **85**:9836–9840 (1988).
17. Honore, T., J. Drejer, E. O. Nielsen, J. C. Watkins, H. J. Olverman, and M. Nielsen. Molecular target size analysis of the NMDA-receptor complex in rat cortex. *Eur. J. Pharmacol.* **172**:239–247 (1989).
18. Cunningham, M. D., and E. K. Michaelis. Solubilization and partial purification of 3-((±)-2-carboxypiperizin-4-yl)-[1,2-$^3$H]propyl-1-phosphonic acid recognition proteins from rat brain synaptic membranes. *J. Biol. Chem.* **265**:7768–7778 (1990).
19. Kumar, K. N., N. T. Tilakaratne, P. S. Johnson, A. E. Allen, and E. K. Michaelis. Cloning of cDNA for the glutamate-binding subunit of an NMDA receptor complex. *Nature (Lond.)* **354**:70–73 (1991).
20. Ikin, A. F., Y. Kloog, and M. Sokolovski. *N*-Methyl-D-aspartate/phencyclidine receptor complex from rat forebrain: purification and biochemical characterization. *Biochemistry* **29**:290–295 (1990).
21. Ly, A. M., and E. K. Michaelis. Solubilization, partial purification, and reconstitution of glutamate- and *N*-methyl-D-aspartate-activated cation channels from brain synaptic membranes. *Biochemistry* **30**:4307–4316 (1991).
22. Mattson, M. P., H. Wang, and E. K. Michaelis. Developmental expression, compartmentalization, and possible role in excitotoxicity of a putative NMDA receptor protein in cultured hippocampal neurons. *Brain Res.* **565**:94–108 (1991).
23. Yoneda, Y., K. Ogita, T. Kouda, and Y. Ogawa. Radioligand labeling of *N*-methyl-D-aspartate receptors by [$^3$H](±)-3-(2-carboxypiperizinyl)propyl-1-phosphonic acid in brain synaptic membranes treated with Triton X-100. *Biochem. Pharmacol.* **39**:225–228 (1990).
24. Nielsen, E. O., J.-H. J. Cha, T. Honore, J. B. Penney, and A. B. Young. Thiocyanate stabilizes AMPA binding to the quisqualate receptor. *Eur. J. Pharmacol.* **157**:197–203 (1988).
25. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T$_4$. *Nature (Lond.)* **227**:680–685 (1970).
26. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, and D. C. Klenk. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**:76–85 (1985).
27. McPherson, G. A. Analysis of radioligand binding experiments: a collection of programs for the IBM PC. *J. Pharmacol. Methods* **14**:213–228 (1985).
28. Aizenman, E., L.-H. Tang, and I. J. Reynolds. Effects of nicotinic agonists on the NMDA receptor. *Brain Res.* **551**:355–357 (1991).
29. Kloog, Y., R. Haring, and M. Sokolovski. Kinetic characterization of the phencyclidine-*N*-methyl-D-aspartate receptor interaction: evidence for steric blockade of the channel. *Biochemistry* **27**:843–848 (1988).
30. Metzger, H., L. Wofsy, and S. J. Singer. Affinity labeling of the active sites of antibodies to the 2,4-dinitrophenyl hapten. *Biochemistry* **2**:979–988 (1963).
31. Monahan, J. B., J. P. Biesterfelt, W. F. Hood, R. P. Compton, A. L. Cordi, M. I. Vazquez, T. H. Lanthorn, and P. L. Wood. Differential modulation of the associated glycine recognition site by competitive *N*-methyl-D-aspartate receptor antagonists. *Mol. Pharmacol.* **37**:603–607 (1990).
32. Lebeda, F. J., and P. A. Rutecki. Organophosphorous anticholinesterase-induced epileptiform activity in the hippocampus, in *Neurobiology of Acetylcholine* (N. J. Dun and R. L. Perlman, eds.). Plenum Press, New York, 437–450 (1987).
33. McLeod, C. G., Jr., and H. G. Wall. Pathology of experimental nerve agent poisoning, in *Neurobiology of Acetylcholine* (N. J. Dun and R. L. Perlman, eds.). Plenum Press, New York, 427–435 (1987).
34. Braitman, D. J., and S. Sparenborg. MK-801 protects against seizures induced by the cholinesterase inhibitor soman. *Brain Res. Bull.* **23**:145–149 (1989).
35. Albuquerque, E. X., Y. Aracava, M. Idriss, B. Schonenberger, A. Brossi, and S. S. Deshpande. Activation and blockade of the nicotinic and glutamatergic synapses by reversible and irreversible cholinesterase inhibitors, in *Neurobiology of Acetylcholine* (N. J. Dun and R. L. Perlman, eds). Plenum Press, New York, 301–328 (1987).

**Send reprint requests to:** Elias K. Michaelis, M.D., Ph.D., Department of Pharmacology and Toxicology, 5064 Malott Hall, University of Kansas, Lawrence, KS 66045–2505.