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IRREVERSIBLE ORGANOPHOSPHATE EFFECTS ON NICOTINIC ACETYLCHOLINE RECEPTOR/ION CHANNEL COMPLEX

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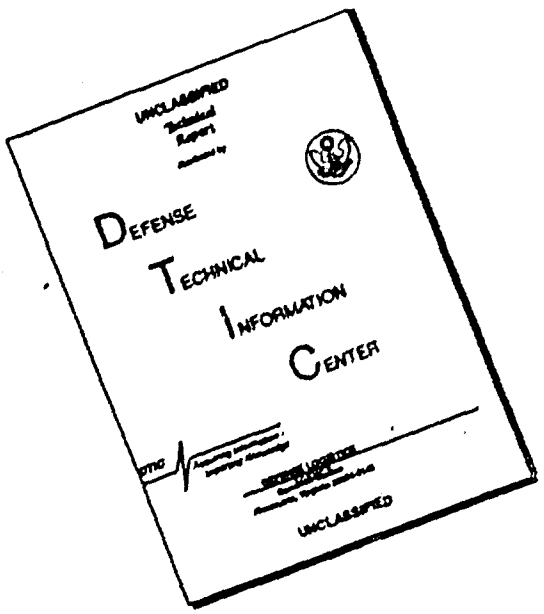

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Organophosphate toxicity is primarily due to its inhibition of acetylcholinesterase. In sublethal doses, organophosphates induce symptoms, which cannot be solely attributed to cholinesterase inhibition, with preliminary data, indicating a direct interaction with postsynaptic nicotinic acetylcholine receptors. Membrane fragments from Torpedo electric organs were used to determine these interactions using ^{3}H-phencyclidine as a probe. Highly toxic organophosphate agents were found to potentiate the binding of ^{3}H-PCP to the nonactivated acetylcholine ion channel and to inhibit carbachol-activated binding. To determine the irreversibility of the effects, membrane preparations were preincubated with organophosphates and washed several times with Tris-HCl buffer before assessing binding with ^{3}H-PCP. Results show that organophosphates irreversibly potentiate binding. These results are consistent with the hypothesis that organophosphates bind to, and irreversibly phosphorylate, an allosteric site on the ion channel associated with the nicotinic acetylcholine receptor.

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PREFACE

The work described in this report was authorized under Project No. 21083000B134. This work was started in July 1986 and completed in September 1986. The experimental data are contained in laboratory notebook No. 85-0146.

The use of trade names or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as promulgated by the committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council.

This report has been approved for release to the public.

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IRREVERSIBLE ORGANOPHOSPHATE EFFECTS ON NICOTINIC ACETYLCHOLINE RECEPTOR/ION CHANNEL COMPLEX

1. INTRODUCTION

The toxicity of organophosphorus (OP) compounds is primarily due to their irreversible inhibition of acetylcholinesterase (AChE), resulting in excess synaptic acetylcholine (ACh) accumulation. This excess results in receptor overstimulation, causing paralysis of the peripheral neuromuscular junction and inhibition of central respiratory neurons, and is thought to be responsible for the lethality of the OP's. In sublethal doses, OP's induce psychic disturbances, tremors and seizures which persist beyond the exposure period, symptoms which cannot be solely attributed to AChE inhibition.[1-3] Together these observations suggest some interaction with both cholinergic and non-cholinergic neurotransmitter systems. Recent data indicate a direct interaction of the OP compounds with postsynaptic nicotinic acetylcholine receptors (nAChR) that does not depend on the accumulation of ACh.[4]

The nAChR consists of five subunits-α_2, β, γ, and δ with an approximate molecular weight of 250,000. These subunits form a rosette-like structure with various binding sites for different drugs and toxins. These sites are the receptor active site, which binds ACh and toxins such as bungarotoxin and curare, and the allosteric, or ion channel site, which binds histrionicotoxin, batrachotoxin and phencyclidine, as well as other drugs and toxins. The binding of an agonist to the receptor site activates the ion channel, exposing the ion channel sites allowing an increase in the binding of channel binding ligands. In the resting state, the receptor site is unoccupied; the ion channel remains closed and little binding to the channel site occurs.[5-6] The electric organ of the electric ray Torpedo nobiliana as used in these studies contains a high density of nAChR that are similar to receptors in the neuromuscular junction. Receptor-rich membrane fragments were used to determine OP interactions with the nAChR-coupled ion channel. The ligand of choice to assess ion channel binding was ^{3}H-Phencyclidine (^{3}H-PCP). Its binding to Torpedo membranes is saturable and is inhibited by drugs that have been shown to interact with ion channels in muscle endplate, but not by drugs that bind to nAChR sites.[7-8]

The present studies were performed to screen a series of lethal chemical nerve agents for in vitro effects on the nAChR and the allosteric ion channel site. Activation of the receptor with subsequent ion channel opening is indicated by increased ^{3}H-PCP binding, and direct effects of OP's on the ion channel can therefore be measured as a function of ^{3}H-PCP binding. The in vivo turnover rate of nAChR is approximately 50 hours, and permanent disruption would be critical to survival during this period. These studies were therefore designed to determine the reversibility of OP effects on the ion channels.

2. METHODS

2.1 Materials

Trizma base and poly-l-lysine were obtained from Sigma Chemical Company, St. Louis, MO. GF/B glass fiber filters were obtained from Whatman International, LTD., Clifton, NJ. ^{3}H-Phencyclidine and Formula 963 aqueous counting cocktail were obtained from New England Nuclear (NEN), Boston, MA.

The four OP nerve agents used in this study were: O-ethyl S-(2-diisopropylaminoethyl)-methylphosphonothiolate (VX); ethyl-N-N-dimethyl-phosphoramidocyanidate (tabun); isopropyl methylphosphonofluoridate (sarin); and pinacolyl methylphosphonofluoridate (soman). Dilutions were made in isopropanol and ranged from 500 nM to 37.5 μM. Torpedo electric organ was obtained from Biofish Associates, Gloucester, ME.

2.2 Preparation of Torpedo Synaptosomes.

Frozen electric organ was minced in two volumes of 50 mM Tris buffer (pH 7.4) containing 154 mM NaCl, 5 mM Na_2HPO_4 and 1 mM EDTA (ethylene-diaminetetraacetic acid). This mixture was homogenized with a Brinkman polytron (setting 5 for 1 min), set on ice for 2 min and rehomogenized. The mixture was centrifuged (1000 x g, 10 min, 4^0 C) and the supernatant stored on ice. Pellets were rehomogenized, centrifuged as before, and the supernatants combined and centrifuged (30,000 x g, 65 min). The resulting pellet was suspended in one volume of the Tris buffer with five up-down strokes (Wheaton homogenizer, setting 3) and stored at 4^0 C. Protein was determined by the Bradford method using gamma globulin standard.[9]

2.3 ^{3}H-Phencyclidine Assay.

Duplicate aliquots of tissue homogenate were preincubated with each agent for either 30 or 60 min. Fifty μl of the OP-treated membrane suspension was then added to glass test tubes containing 2 nM (final conc.) ^{3}H-phencyclidine (^{3}H-PCP, 50 Ci/mmol), carbachol- 5 μM (CBC: for activation studies only) and 50mM Tris-HCl buffer (pH 7.4) to give a final volume of 1 ml. The tubes were immediately vortexed and incubated for 30 sec before aspirating the contents onto GF/B filter disks. Test tubes and filter disks were soaked in 0.1% poly-l-lysine for 30 min prior to use to minimize PCP binding to glass. The filters were washed twice with 5 ml cold Tris buffer and placed in scintillation vials containing 5 ml Formula 963 (NEN). The vials were dark and cold adapted prior to counting in a Packard Model 300-C liquid scintillation spectrometer (62% efficiency).

3. RESULTS

Results of ^{3}H-PCP binding to the nonactivated, or resting, binding sites are shown in Figure 1. OP agents activated the channel at low

concentrations as indicated by increased binding of ^{3}H-PCP. Maximal activation occurred at 1 μM for VX, 2 μM for sarin and 5 μM for soman and tabun.

Figure 2 shows the inhibition of ^{3}H-PCP binding by the OP's in the CBC-activated receptor/ion channel. At 37.5 μM, VX shows an inhibition from control of 52%; sarin, 19%; soman, 16%; and tabun, 8%. For the reversibility studies, duplicate aliquots of tissue homogenate were preincubated with each agent in a 1:9 agent-to-tissue ratio for 10 min. The membranes were pelleted by centrifugation (23,000 x g, 20 min, 4^0 C) and supernatant from one of each pair of tubes was discarded (wash set) and replaced with Tris-HCl. All pellets were resuspended and this wash procedure repeated, and the tissue suspension treated as above. The OP's tested irreversibly stimulated the binding of ^{3}H-PCP in the nonactivated receptor/ion channel as shown in Figure 3. Addition of 0.025 μg OP resulted in an increase in ^{3}H-PCP binding from the control: soman, 16%; sarin, 16%; VX, 8%; and tabun, 32%. There was an increase in binding observed even when the tissue had been washed after being exposed to the OP: soman, 44%; sarin, 40%; VX, 45%; and tabun, 43% over the washed control. This increase may be due to the removal of AChE during washing, allowing more OP to interact with the receptor.

4. DISCUSSION

The results of this study indicate that OP's interact with the ion channel associated with the nAChR in an irreversible manner. These anti-AChE's act primarily by binding irreversibly to AChE, resulting in the accumulation of ACh in the synapse, hence receptor activation. In our preparation, CBC was used to activate the receptor with subsequent channel opening. Under these conditions, OP's decreased ^{3}H-PCP binding in the channel. Since OP's stimulated ^{3}H-PCP binding in the nonactivated (ACh or CBC absent) receptor, there appears to be a direct interaction with the nAChR as well. The inhibition of CBC-activated binding suggests two possibilities: a direct interaction of OP's, possibly competing for the receptor binding sites, or the binding of OP's to allosteric sites which modulate the receptor and ion channel accordingly. These hypotheses are not mutually exclusive.

The differential effects of the OP's on ^{3}H-PCP binding may be a result of unique steric effects related to their molecular structures. In the wash experiments, OP's may form an irreversible complex with the receptor, changing its conformation and altering the dissociation rate of ^{3}H-PCP, resulting in binding of ^{3}H-PCP with a higher affinity. Tabun shows a significant decrease in ^{3}H-PCP binding when comparing the washed preparation with the unwashed preparation. This may result from the relative toxicities of the OP's. Soman, sarin, and VX, respectively, have median lethal dosages (MLD) in man of 70, 70, and 30 mg/min/m^3, whereas tabun has a MLD in man of 135 mg/min/m^3, only half as toxic as the others. Also, the rate of hydrolysis and breakdown in the pH range 7 +/- 1 is greater for tabun at 8.5 hr than the rates of soman, 45 hr; sarin, 47 hr; and VX, 40 hr.[10]

In summary, the results of the present study show the activating of the ion channel by OP's, the inhibition of CBC-activated ^{3}H-PCP binding and the irreversible stimulation of ^{3}H-PCP binding. These results are consistent with the hypothesis that OP's activate, and irreversibly phosphorylate, an allosteric site associated with the nAChR.

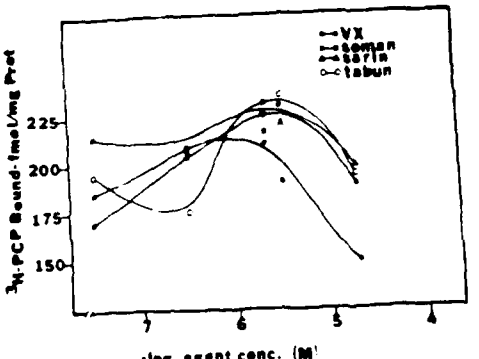

Figure 1. OPs Stimulate 3H-PCP Binding in Resting nAChR System. Results are the average of 6 experiments

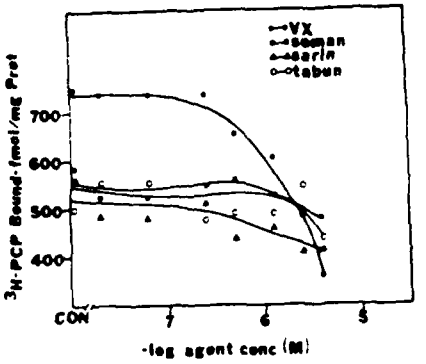

Figure 2. OPs Inhibit 3H-PCP in CBC-activated nAChR System. Results are the average of 6 experiments.

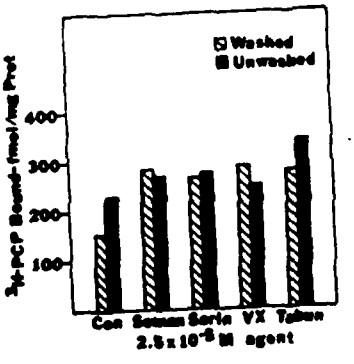

Figure 3. OPs Irreversibly Stimulate 3H-PCP Binding. Results are the average of 6 experiments.

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