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[^{3}H]Phencyclidine Interactions with the Nicotinic Acetylcholine Receptor Channel and Its Inhibition by Psychotropic, Antipsychotic, Opiate, Antidepressant, Antibiotic, Antiviral, and Antiarrhythmic Drugs

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SUMMARY

The binding of [^{3}H]phencyclidine ([^{3}H]PCP) to the ionic channel sites of the nicotinic acetylcholine (ACh) receptor of *Torpedo* electroplax was similar to, although not identical with, that of [^{3}H]perhydrohistrionicotoxin ([^{3}H]H_{12}-HTX). The stimulating effects of agonists and antagonists on [^{3}H]PCP binding and the inhibiting effects of desensitizing conditions, sulfhydryl reagents, disulfide bond reduction, and several adamantane derivatives are similar to those observed for binding of [^{3}H]H_{12}-HTX to the same channel sites. Although the affinity of [^{3}H]PCP and [^{3}H]H_{12}-HTX for the channel sites increased at higher temperatures, when carbamylcholine was present the affinity of the former became almost temperature-insensitive, whereas that of the latter was still sensitive. A variety of drugs inhibited [^{3}H]PCP binding to these nicotinic channel sites; the most potent (having K_i < 2 μM) were the antipsychotics (chlorprothixene > thiothixene > chlorpromazine > thioridazine > fluphenazine > trifluoperazine > mesoridazine > piperacetazine), antidepressants (amitriptyline > nortriptyline > desimipramine > imipramine > protriptyline > doxepin), psychotropic opiate antagonists (SKF 10047 > cyclazocine > levallorphan), the antiarrhythmic propranolol, and the antibiotic polymyxin B. Less potent drugs (K_i 2 μM–80 μM) were quinidine and several antibiotics (neomycin > gentamicin > amikacin > clindamycin); while poor inhibitors (K_i > 80 μM) were morphine, naloxone, naltrexone, nalorphine, procainamide, kanamycin, and lincomycin. Twelve PCP analogues were potent inhibitors of [^{3}H]PCP binding to the channel sites of the nicotinic ACh receptor, but there was poor correlation with published potencies in modifying animal behavior, suggesting that a similar nicotinic ACh receptor was not the target for these modifications. Similarly, there was poor correlation between the antiviral potencies of tilorone analogues and their potencies in inhibiting [^{3}H]PCP binding. The high affinities that several kinds of drugs have for these nicotinic channel sites point to their relative nonspecificity and suggest that at therapeutic doses some of these drugs may have adverse effects on motor end-plate function.

INTRODUCTION

The nicotinic ACh[1] receptor of muscle end-plates and fish electric organs is a regulatory glycoprotein that carries binding sites for different drugs and toxins. These are the "receptor" sites that bind ACh, agonists, and antagonists in a voltage-independent manner, and the "allosteric" or "ionic channel" sites whose action is voltage-dependent (1, 2). Binding to the latter sites usually causes nonlinearity in the peak current-voltage relationship and affects the time constant of decay of end-plate currents. This ACh receptor/channel molecule undergoes ligand-induced conformational changes, and binding of a drug to the receptor or channel sites allosterically affects binding to the other (3). A variety of drugs with different modes of action was found to interact with these ionic channel sites, such as the antiviral amantadine (4), the local anesthetic procaine (5), the ganglion-blocker tetraethylammonium (6), the antimalarial quinacrine (7), the tricyclic antidepressant imipramine (8), and the general anesthetic and hallucinogen PCP (9). Examples of radiolabeled probes that have been used to identify the receptor sites are ACh (10), α-BGT (11), and *d*-tubocurarine (12); those for the channel sites are H_{12}-HTX (3), PCP (13), trimethisoquin (14), meproadiphen (12), and imipramine (8).

The data obtained on the effect of PCP on neuromus-

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[1] The abbreviations used are: ACh, acetylcholine; PCP, phencyclidine; α-BGT, α-bungarotoxin; H_{12}-HTX, perhydrohistrionicotoxin; carb, carbamylcholine; SKF 10047, *N*-allylnocyclazocine.

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cular transmission and the binding of [^{3}H]PCP to the nicotinic ACh receptor, which is found in high concentrations in the electric organ of the electric ray *Torpedo ocellata*, suggested that PCP interacted with the ionic channel sites of this ACh receptor/channel molecule (9, 13). PCP decreased the peak amplitude of the end-plate current in a voltage- and time-dependent manner, caused nonlinearity in the current-voltage relationship, accelerated the decay time constant of end-plate currents and miniature end-plate currents, and shortened the mean lifetime of a single ionic channel. PCP, which did not inhibit the binding of [^{3}H]ACh or [^{125}I]α-BGT to the ACh receptor of *Torpedo* electric organ, inhibited the binding of [^{3}H]H_{12}-HTX. In a preliminary study we reported that the binding of [^{3}H]PCP to *Torpedo* membranes was saturable and was inhibited by drugs that bound to the channel sites of ACh receptors and not by those that bound to the receptor sites (13). The affinity of this [^{3}H]PCP binding and the initial rate of its binding were increased by the binding of carb to the ACh receptor sites. Thus, we concluded that [^{3}H]PCP was indeed binding to the channel sites of the ACh receptor. We also found that although PCP inhibited [^{3}H]H_{12}-HTX binding competitively, the potencies of many drugs in inhibiting binding of the two probes varied enough to suggest some differences in their binding properties.

The present study was initiated to examine in greater detail the binding of [^{3}H]PCP to *Torpedo* membranes and the effect of several cholinergic agonists and antagonists on this binding, with emphasis on comparing the binding of [^{3}H]PCP with that of [^{3}H]H_{12}-HTX. Furthermore, [^{3}H]PCP was used as a probe to study the interactions of several groups of drugs with the nicotinic ACh receptor/channel molecule, including PCP analogues, antipsychotics, tricyclic antidepressants, opiate agonists and antagonists, antiarrhythmic drugs, antibiotics, the antiviral tilorone and analogues, and adamantane derivatives. These drugs were selected on the basis of reports on their postsynaptic inhibition of neuromuscular transmission, their membrane-stabilizing properties, or the similarity of their structures to drugs shown to inhibit this ionic channel. Potencies of these drugs were compared with their *in vivo* actions and therapeutic doses in hope of identifying the drugs that might act on this receptor/channel protein as a primary or secondary target.

MATERIALS AND METHODS

Tissue preparation. Electric organs of *T. ocellata* obtained from the Mediterranean and stored at −90° were homogenized in an equal volume of ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM disodium ethylenediaminetetraacetate, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM diisopropyl fluorophosphate. The homogenate was centrifuged at 5,000 rpm for 10 min in a Sorvall centrifuge, the supernatant fraction was recentrifuged at 17,000 rpm for 60 min, and the pellets were resuspended in the Tris buffer containing 0.1 mM diisopropyl fluorophosphate and 0.02% sodium azide. The protein concentration, determined by the method of Lowry *et al.* (15), was about 1 mg/ml.

Binding assay. [^{3}H]PCP (specific activity 48 Ci/mmole; New England Nuclear Corporation, Boston, Mass.) was used at 2 nM final concentration. The membrane preparation (0.05 ml) was incubated with 2 nM [^{3}H]PCP in a final volume of 1 ml of 50 mM Tris buffer (pH 7.4). The contents were mixed, and, after incubation for an appropriate time at room temperature, the mixture was filtered over a Whatman GF/B glass-fiber filter that had been rinsed with organosilate solution (1% Prosil-28; PCR Research Chemicals, Inc., Gainesville, Fla.) to reduce nonspecific binding. The latter never exceeded 0.5% of the total radioactivity added in the assay. The sample was washed on the filter with 10 ml of 50 mM Tris buffer, and the filter was placed in 5 ml of toluene solution (containing 4.75 g of 2,5-diphenyloxazole, 0.32 g of dimethyl 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene, and 40 ml of Beckman BBS-3 solubilizer per liter); radioactivity was counted in a scintillation spectrometer after 10 hr. Binding of [^{3}H]H_{12}-HTX [specific activity 21 Ci/mmole obtained by tritiation of the natural alkaloid isodihydrohistrionicotoxin (16)] was measured by the same filter assay used for [^{3}H]PCP. Specific binding of either probe was calculated as the difference in binding obtained in the presence and absence of 5 mM amantadine, a concentration which inhibited all specific binding (4). For measuring the initial rate of binding the incubation was carried out for only 30 sec. Drugs to be tested for their effects on the binding of [^{3}H]PCP were placed in the buffer with [^{3}H]PCP prior to addition of the membrane preparation unless otherwise indicated.

Drugs. Cholinergic drugs and antiarrhythmic drugs were purchased from Sigma Chemical Company (St. Louis, Mo.); adamantane and derivatives from Aldrich Chemical Company (Milwaukee, Wisc.); and chlorpromazine from Sigma Chemical Company. PCP and its analogues were kindly supplied by the National Institute of Drug Abuse (Bethesda, Md.); fluphenazine by Schering Corporation (Kenilworth, N. J.); chlorprothixene by Hoffmann-La Roche (Nutley, N. J.); piperacetazine by Dow Chemical Company (Indianapolis, Ind.); mesoridazine by Boehringer Ingelheim (Ridgefield, Conn.); thiothixene and doxepin by Pfizer Laboratories (New York, N. Y.); thioridazine by Sandoz Pharmaceuticals (East Hanover, N. J.); imipramine by Geigy Pharmaceuticals (Ardsley, N. Y.); amitriptyline by Merck Sharp & Dohme (West Point, Pa.); lincomycin and clindamycin by The Upjohn Company (Kalamazoo, Mich.); neomycin and polymyxin B by Burroughs Wellcome (Research Triangle Park, N. C.); gentamicin by Schering Corporation; and tilorone and analogues by the Merrell Research Center (Cincinnati, Ohio). Dr. E. X. Albuquerque (University of Maryland) kindly provided us with desimipramine, protriptyline, nortriptyline, morphine, naloxone, naltrexone, SKF 10047, cyclazocine, levallorphan, amikacin, and kanamycin.

RESULTS

Characteristics of [^{3}H]PCP binding to Torpedo membranes. The binding of [^{3}H]PCP (1 nM) to *Torpedo* membranes was time-dependent (Fig. 1). In the presence of carb (i.e., stimulated binding) equilibrium was reached faster and was at a higher level than that of the unstimulated binding. The dissociation of [^{3}H]PCP was fit by a

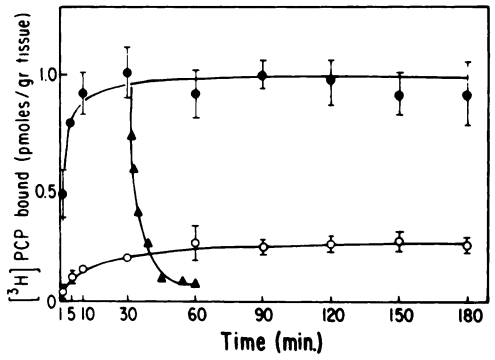

FIG. 1. *Binding of 1 nM [^{3}H]PCP to Torpedo membranes in the presence (●) and absence (○) of 20 μM carb as a function of time and its dissociation (▲) upon the addition of 100 μM of nonradioactive PCP (arrow)*

single rate constant (4.6×10^{-3} sec^{-1}). We had previously shown that binding of [^{3}H]H_{12}-HTX to the channel sites of the *Torpedo* ACh receptor was temperature-dependent in the presence and absence of carb, with higher affinity at the higher temperatures (3). This contrasted with the binding of receptor site ligands such as ACh or carb. When we studied binding of [^{3}H]PCP to the channel sites, it was temperature-sensitive in the absence of carb, increasing in affinity with higher temperatures, but the carb-stimulated binding was temperature-insensitive (Fig. 2). Binding of [^{3}H]PCP (1 nM) to the channel sites at different temperatures was inhibited by increasing concentrations of PCP (10 nM–100 μM) and the IC_{50} values determined. K_i values were calculated according to the relationship $K_i = IC_{50}/(1 + [F]/K_d)$, where $[F]$ is the concentration of free [^{3}H]PCP (1 nM) and K_d is the dissociation constant for [^{3}H]PCP [0.25 μM in the presence of carb, but in its absence it has two affinities: 0.1 and 3.1 μM (13)]. Since F is so small (1 nM) as compared with the K_d values, the difference in K_i values obtained by using either of the two [^{3}H]PCP affinities is negligible. To obtain the K_A values in Fig. 2, the K_d of 3.1 μM was used.

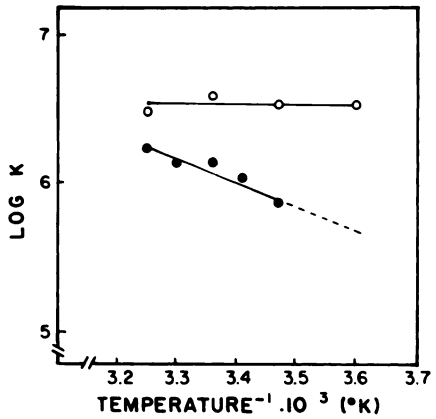

FIG. 2. *Arrhenius plot of the effect of temperature on the affinities (K) of [^{3}H]PCP to the channel sites of the nicotinic ACh receptor in Torpedo membranes in the absence (●) and presence (○) of 100 μM carb*

Each point represents values obtained from a complete binding curve at a certain temperature.

Effects of nicotinic receptor agonists and antagonists on the binding of [^{3}H]PCP. Four agonists increased the initial rate of binding of [^{3}H]PCP similar to their relative potencies in increasing the initial rate of binding of [^{3}H]H_{12}-HTX (Fig. 3). The agonist doses (micromolar) that caused a 50-fold activation in [^{3}H]PCP binding were 0.52, 3, 0.25, and 1 for ACh, carb, decamethonium, and succinylcholine, respectively. Their values in increasing [^{3}H]H_{12}-HTX binding were 0.25, 2.5, 0.35, and 0.8, respectively. In addition, the biphasic dose-response function seen for the effect of decamethonium on the binding of [^{3}H]H_{12}-HTX (3) was also seen with [^{3}H]PCP. These *Torpedo* membranes were pretreated with 0.1 mM diisopropyl fluorophosphate, so that their acetylcholinesterase activity was inhibited; otherwise the stimulating effect of ACh would reach maximal values only at millimolar concentrations. Preincubation of the tissue with agonists for 30 min resulted in a reduction of the 100 μM carb-stimulated binding of 2 nM [^{3}H]PCP and [^{3}H]H_{12}-HTX by 66.1% and 69.2%, respectively. This is possibly due to desensitization of the receptor/channel molecule. The binding of [^{3}H]PCP to *Torpedo* membranes was stimulated not only by agonists but also by receptor antagonists. *d*-Tubocurarine, gallamine, and hexamethonium, which are known for their nondepolarizing blockade of ACh responses, increased [^{3}H]PCP binding but to a much smaller extent (20–40%) than did carb (Fig. 4). α-BGT was the only antagonist that did not stimulate the binding of [^{3}H]PCP. Pretreatment of *Torpedo* membranes with 10 μM α-BGT to inhibit all receptor sites blocked the stimulating effect of carb and antagonists on the binding of [^{3}H]PCP.

Effects of treatments with sulfhydryl and disulfide reagents on the binding of [^{3}H]PCP and [^{3}H]H_{12}-HTX. The effects of two disulfide bond-reducing reagents (1, 4-dithiothreitol and mercaptoethanol) and two mercuric sulfhydryl reagents (mercuric chloride and *p*-chloromercuribenzoate) on the binding of [^{3}H]PCP and [^{3}H]H_{12}-HTX to the channel sites of the ACh receptor in *Torpedo* membranes were compared as follows. Aliquots from stock solutions of the reagent were added to 1 ml of *Torpedo* membranes (≃ 1 mg of protein) to yield the desired concentration, and the mixture was stirred and

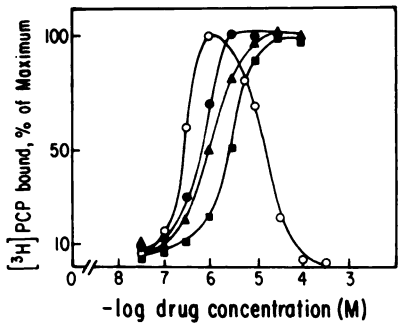

FIG. 3. *Effects of four receptor agonists [ACh (●), succinylcholine (▲), carb (■), and decamethonium (○)] on the binding of [^{3}H]PCP (2 nM) to Torpedo membranes*

The incubation period was 30 sec, and the incubation volume was 1 ml. Maximal binding (100%) was similar for all drugs with the exception of decamethonium, whose maximal binding was 60% of that obtained for the other drugs.

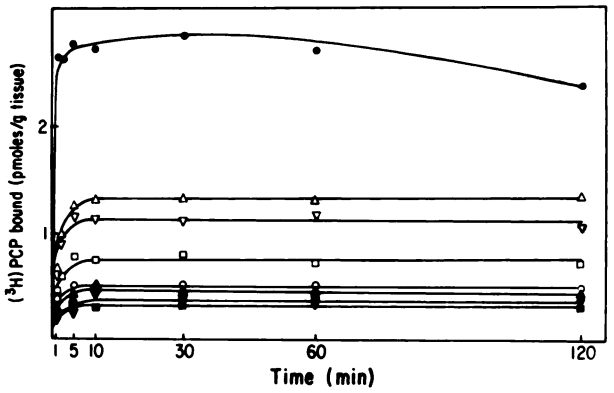

FIG. 4. *Effects of three receptor antagonists (20 μM) [d-tubocurarine (△), gallamine (▽), and hexamethonium (□)] on the kinetics of [^{3}H]PCP binding to Torpedo membranes*

The solid symbols (▲, ▼, ■) represent the effect of the three drugs on 2 nM [^{3}H]PCP binding to *Torpedo* membranes pretreated with 10 μM α-BGT for 60 min. The *circles* represent the effect of carb (100 μM) on the binding of [^{3}H]PCP to untreated (●) and α-BGT-treated *Torpedo* membranes (○). Each *symbol* represents the mean of three experiments; standard deviations were less than 10% in all replicates.

incubated at 21° for 1 hr. The mixture was then centrifuged at 30,000 × *g* for 60 min, and the pellets were rinsed with 50 mM Tris-HCl buffer (pH 7.4) and resuspended in a final volume of 1 ml. Aliquots of 50 μl of the treated membranes were added to 1 ml of Tris buffer containing 2 nM [^{3}H]PCP or [^{3}H]H_{12}-HTX and incubated for 120 min to reach equilibrium; the contents were then filtered and the radioactivity was counted. Binding of either radioactive ligand to untreated *Torpedo* membranes was used to represent the 100% control level. Treatment with either disulfide bond-reducing reagents or sulfhydryl reagents inhibited binding of both [^{3}H]H_{12}-HTX (Fig. 5A) and [^{3}H]PCP (Fig. 5B) similarly. The most effective agent was mercuric chloride, and the least effective was mercaptoethanol. The inorganic mercuric chloride was more effective than the organic *p*-chloromercuribenzoate, and the reducing reagent 1,4-dithiothreitol was much more effective than mercaptoethanol.

Effects of adamantane derivatives on the binding of [^{3}H]PCP and [^{3}H]H_{12}-HTX. Adamantane and several of its derivatives inhibited the stimulated binding (i.e., in presence of 20 μM carb) of 2 nM [^{3}H]H_{12}-HTX (Fig. 6A) or [^{3}H]PCP (Fig. 6B) to the channel sites of the ACh receptor in *Torpedo* membranes, but were more potent on the latter. Adamantanamine (amantadine) and its hydrochloride salt were the most potent inhibitors, with approximate K_i values of 10 and 20 μM for displacing [^{3}H]PCP and [^{3}H]H_{12}-HTX, respectively. The K_i was calculated from the IC_{50} (the concentration that inhibits 50% of binding of a certain concentration) by using the formula $K_i = IC_{50}(1 - f)$, where f = fractional saturation which is less than 1% for 2 nM [^{3}H]PCP. It is important to note that the primary amine adamantanamine, when placed in the 50 mM Tris-HCl buffer (pH 7.4), must exist mainly in the ionized form, the same as adamantanamine hydrochloride. Next in potency were 1-adamantyl methyl ketone and 2-bromo-adamantane. The methyl ketone derivative was much more potent in inhibiting [^{3}H]PCP than [^{3}H]H_{12}-HTX binding, with K_i values of 30 μM and 200 μM, respectively. Adamantane, adamantol, and 1-adamantane carboxylic acid at concentrations up to 300 μM had no effect on [^{3}H]H_{12}-HTX binding but inhibited [^{3}H]PCP binding slightly.

Effect of opiates on [^{3}H]PCP binding. Naltrexone (up to 100 μM) had no effect on the binding of [^{3}H]ACh (1 μM) to *Torpedo* membrane (data not shown), but displaced the carb-stimulated [^{3}H]PCP binding in a dose-dependent manner (Fig. 7). However, the affinity of naltrexone was rather low, with a K_i of 100 μM (Fig. 7, *inset*). This effect of naltrexone prompted us to test a few other opiate antagonists; therefore, the carb-stimulated binding of [^{3}H]PCP (2 nM) to *Torpedo* membranes was titrated with varying concentrations of naloxone, levallorphan, cyclazocine, and SKF 10047 (Fig. 8). Morphine, the only agonist studied, had little effect, causing 25% inhibition at 100 μM. Naloxone and nalorphine were only slightly more effective than morphine, producing 40% and 60% inhibition, respectively, at 100 μM. The psychotropic opiate antagonists, levallorphan, cyclazocine, and SKF 10047, were much more effective in displacing [^{3}H]PCP, with K_i values of 2, 1.2, and 0.9 μM, respectively.

Effects of tricyclic antidepressants on [^{3}H]PCP binding. Our recent studies indicated that imipramine inhibited neuromuscular transmission through its action

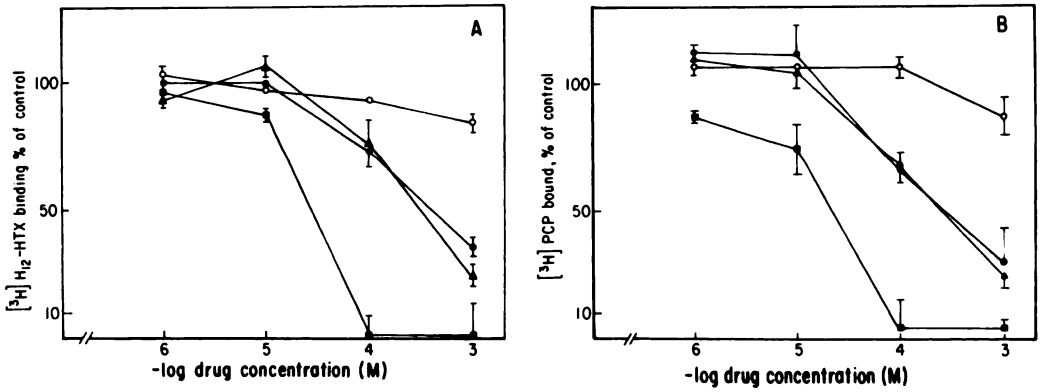

FIG. 5. *Effects of four sulfhydryl reagents [mercaptoethanol (○), dithiothreitol (●), p-chloromercuribenzoate (▲), and mercuric chloride (■)] on the binding of 2 nM [^{3}H]H_{12}-HTX (A) and 2 nM [^{3}H]PCP (B) to Torpedo membranes*

All measurements were made after 120-min incubation. Each *symbol* and *bar* represents the mean ± standard deviation of three experiments.

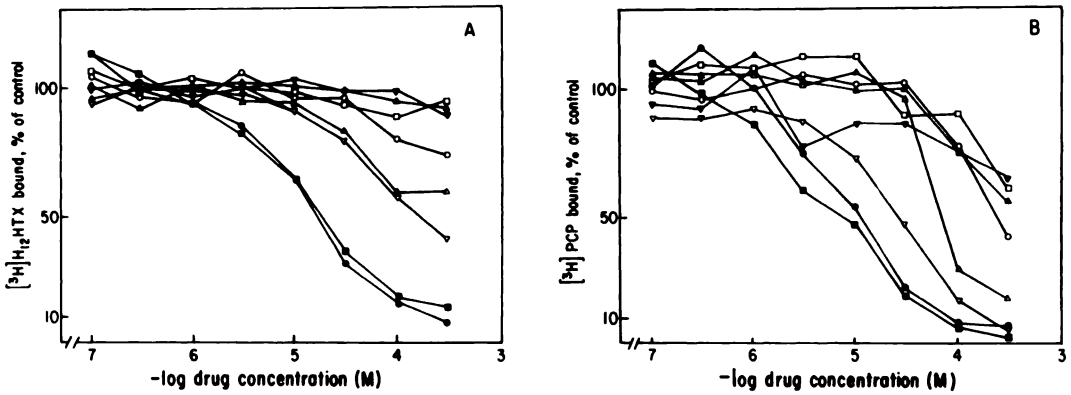

FIG. 6. *Effects of adamantane derivatives on carb-stimulated binding of 2 nM [^{3}H]H_{12}-HTX (A) and 2 nM [^{3}H]PCP (B)*

Adamantane (□), adamantamine (■), adamantamine-HCl (●), 1-adamantyl methyl ketone (▽), 2-bromo-adamantane (△), 1-bromo-adamantane (○), adamantol (▲), and 1-adamantane carboxylic acid (▼). All measurements were made in the presence of 100 μM carb. Each *symbol* represents the mean of three experiments.

on the ionic channel of the ACh receptor (3). It had no effect on the binding of [^{3}H]ACh or [^{125}I]α-BGT but inhibited the binding of [^{3}H]H_{12}-HTX and [^{3}H]PCP to *Torpedo* membranes noncompetitively (8). Imipramine and other tricyclic antidepressants inhibited the binding of 2 nM[^{3}H]PCP in the presence of 100 μM carb in a dose-dependent manner. The following K_i values (micromolar) were calculated from dose-response curves: imipramine = 0.13, desimipramine = 0.1, amitriptyline = 0.063, nortriptyline = 0.065, protriptyline = 0.17, and doxepin = 0.26. Dixon plots of the effect of several concentrations of imipramine on the specific binding of [^{3}H]PCP at two concentrations (2 and 4 nM) to *Torpedo* membranes indicated a common intercept on the abscissa indicative of noncompetitive interactions (data not shown), similar to its effect on the binding of [^{3}H]H_{12}-HTX (8).

Effects of antipsychotic drugs on [^{3}H]PCP and [^{3}H] ACh binding. Chlorpromazine and seven other antipsychotics were found to be potent inhibitors of carb-stimulated [^{3}H]PCP binding to the ionic channel sites of the ACh receptor in *Torpedo* membranes (Fig. 9). The two thioxanthene drugs, thiothixene and chlorprothixene, were the most potent (K_i values of 0.058 and 0.034 μM, respectively). Chlorpromazine, thioridazine, fluphenazine, mesoridazine, piperacetazine, and trifluoperazine had K_i values (micromolar) of 0.22, 0.26, 0.38, 0.65, 1.0, and 0.45, respectively. None of these drugs at concentrations ranging from 0.1 to 100 μM inhibited the binding of [^{3}H]ACh (0.1 μM) to *Torpedo* membranes. In fact, most of them increased the binding of [^{3}H]ACh to the receptor sites (by up to 62%), possibly through allosteric effects.

Effect of the antiviral tilorone and analogues on [^{3}H] PCP and [^{3}H]ACh binding. Tilorone (2,7-bis[2-(diethylamino)ethoxy]-9*H*-fluoren-9-one) and the following bis-substituted polycyclic aromatic compounds, known for their antiviral activity (17, 18), were tested for their effects on the binding of [^{3}H]PCP and [^{3}H]ACh to *Torpedo* membranes: RMI 12,358 [3,6-bis(diethylaminoacetyl)acenaphthene], RMI 11,567 [2,8-bis(dimethylaminoacetyl)dibenzofuran, RMI 11,645 [3,9-bis(dimethylaminoacetyl)fluoranthene], RMI 11,877 [2,7-bis(dimethylaminoacetyl)dibenzothiophene], RMI 11,002 [2,7-bis(diethylaminoacetyl)fluorene], and RMI 11,513 [2,7-bis(dimethylaminoacetyl)xanthene]. All were effec-

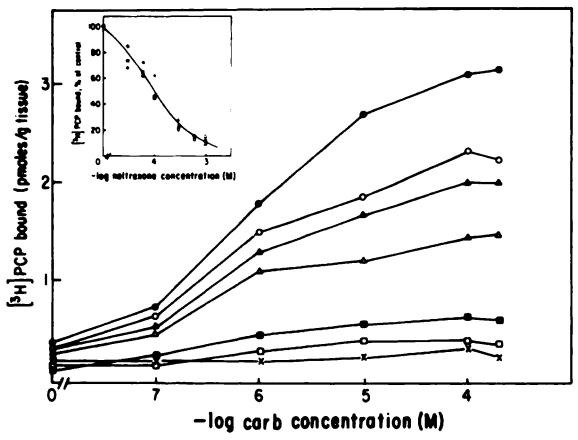

FIG. 7. *Effects of naltrexone on carb-stimulated binding of [^{3}H] PCP to Torpedo membranes*

Control—no naltrexone (●); naltrexone 30 μM (○), 60 μM (▲), 100 μM (△), 300 μM (■), 600 μM (□), and 1 mM (×). *Inset* represents the dose-response function of the inhibition of the binding of [^{3}H]PCP (2 nM) in the presence of 100 μM carb; *symbols* represent 30 μM (○), 100 μM (△), and 600 μM (□) naltrexone. All measurements were made after a 30-sec incubation.

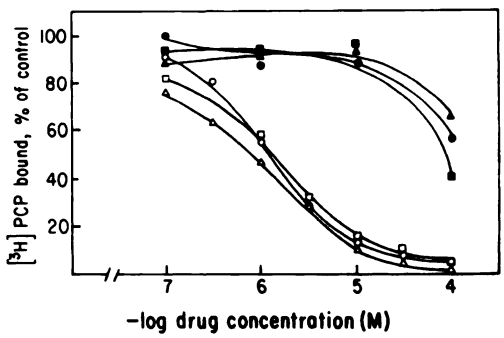

FIG. 8. *Effects of opiates on carb-stimulated binding of 2 nM* [^{3}H] PCP to Torpedo membranes

Morphine (▲), nalorphine (■), naloxone (●), cyclazocine (○), SKF 10047 (△), and levallorphan (□). All measurements were made in the presence of 100 μM carb. The incubation time was 30 sec and the incubation volume was 1 ml. Each *symbol* represents the mean of three experiments; standard deviations were less than 10%.

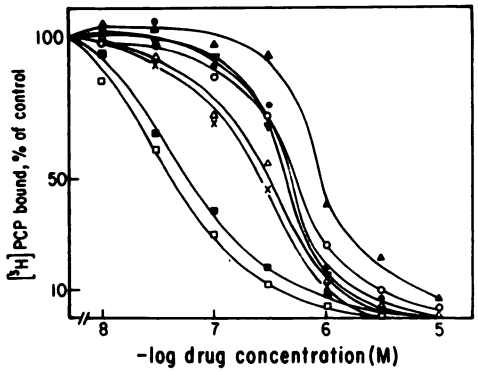

FIG. 9. *Effects of seven hydrochloride salts of phenothiazine antipsychotic drugs on the binding of 2 nM [³H]PCP to Torpedo membranes in the presence of 100 μM carb*

Chlorpromazine (×), fluphenazine (●), piperacetazine (○), mesoridazine (▽), thioridazine (△), thiothixene (■), chlorprothixene (□), and trifluoperazine (▼). All measurements were made in the presence of 100 μM carb. The incubation time was 30 sec and the incubation volume was 1 ml. Each *symbol* represents the mean of three experiments; standard deviations were less than 10%.

tive inhibitors of 2 nM [³H]PCP binding in the presence of 100 μM carb to the channel sites, with K_i values ranging from 1.2 to 22 μM (Table 1). None of these drugs at concentrations up to 100 μM inhibited [³H]ACh (0.1 μM) binding to the receptor sites with the exception of tilorone, and only at 100 μM concentration did it inhibit 26%. Actually, all of the drugs, including tilorone (0.1–100 μM), increased binding of 0.1 μM[³H]ACh up to 49%. This is similar to the effects of the antipsychotic drugs, which are also polycyclic aromatic compounds.

Effects of antiarrhythmic drugs on [³H]PCP binding. Three antiarrhythmic drugs, propranolol, quinidine, and procainamide, were tested for their effects on the binding of [³H]PCP to the channel site of the ACh receptor in *Torpedo* in the presence and absence of 20 μM carb (Fig. 10). Propranolol was the most potent of the three, with a K_i on carb-stimulated [³H]PCP binding of 2 μM, followed by quinidine (K_i = 30 μM). Procainamide (K_i > 1 mM) was almost ineffective, especially in the absence of receptor stimulation. These three drugs at concentration up to 100 μM did not inhibit the binding of [³H]ACh to the receptor sites in *Torpedo* membranes.

TABLE 1

Comparison of the relative potencies of tilorone and analogues in inhibiting [³]PCP binding (2 nM) to Torpedo membranes in the presence of 100 μM carb with data on their antiviral activities

Drug	Relative antiviral potency[a]		Inhibition of [³H]PCP binding	
	RNA virus	DNA virus	K_i	Relative potency
			μM	
Tilorone	M	M	2.4	H
RMI 11,567	M	H	20.0	L
RMI 12,358	H	H	1.2	H
RMI 11,513	M	L	22.0	L
RMI 11,645	H	H	2.5	H
RMI 11,002	H	Inactive	3.4	H
RMI 11,877	M	M	6.8	M

[a] Data from ref. 17. H, High; M, medium; L, low potencies.

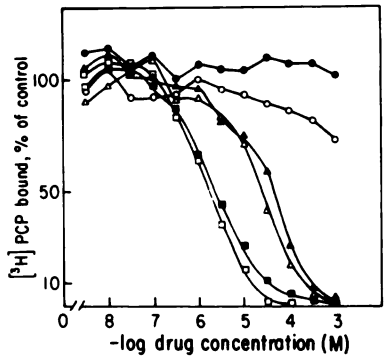

FIG. 10. *Effects of three antiarrhythmic drugs [propranolol (□, ■), quinidine (△, ▲), and procainamide (○, ●) on the binding of 2 nM [³H]PCP to Torpedo membranes*

Solid symbols represents unstimulated binding; *open symbols* represent carb-stimulated binding (100 μM carb). Each *symbol* represents the mean of three experiments; standard deviations were less than 10%.

Effects of antibiotics on [³H]ACh and [³H]PCP binding. Several antibiotics known to produce neuromuscular blockade (19) were tested for their possible interaction with the receptor and ionic channel sites of the *Torpedo* ACh receptor/channel. None (0.1–100 μM) inhibited 0.1 μM [³H]ACh binding to the receptor sites. Clindamycin and polymyxin B at 10 μM increased binding of [³H]ACh (0.1 μM) by 13 and 50%, respectively, possibly through allosteric effects. However, all of the antibiotics interacted with channel sites and inhibited the binding of [³H]PCP in the presence of 100 μM carb (Fig. 11). The affinities (K_i values, micromolar) of the seven antibiotics were polymyxin B, 0.87; neomycin, 4.5; gentamicin, 5.8; amikacin, 35; clindamycin, 78; and both kanamycin and lincomycin, > 100.

Effects of PCP and analogues on [³H]PCP binding and relationship to its mode of action. The effects of several PCP analogues, which had been assessed in two animal behavior tests (the rat discriminative stimulus test and the mouse rotarod test) (20), were studied on

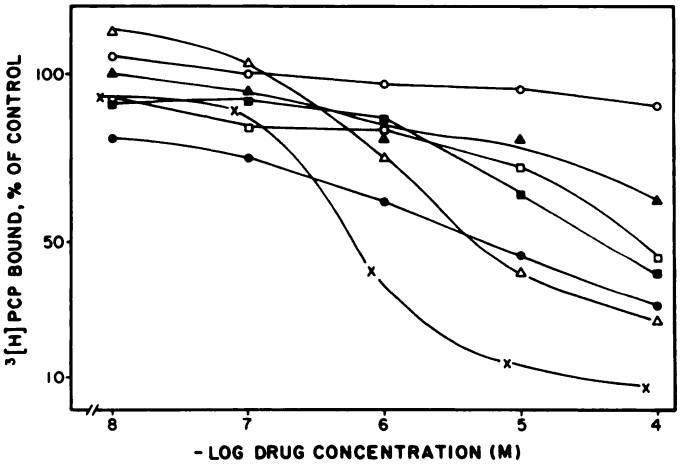

FIG. 11. *Inhibition by seven antibiotics of the binding of 2 nM [³H] PCP to Torpedo membranes in the presence of 100 μM carb*

Polymyxin B (×), gentamicin (●), neomycin (△), amikacin (■), clindamycin (□), kanamycin (▲), and lincomycin (○). Each *symbol* represents the mean of triplicate experiments; standard deviations were less than 10%.

TABLE 2

Relative potencies of selected phencyclidine analogues in inhibiting binding of [^{3}H]PCP (2 nM) in the presence of 100 μM carb to Torpedo membranes and in modifying animal behavior

Drug	Name	Inhibition of [^{3}H]PCP binding		Relative behavioral potencies[a]	
		K_i	Relative potency	Rat discriminative stimulus test	Mouse rotarod test
		μM			
PCP	Phencyclidine	0.18	1.00	1.00	1.00
NMPCA	*N*-Methyl-1-phenylcyclohexylamine	0.37	0.49	1.02	1.02
PCDMA	*N,N*-Dimethyl-1-phenylcyclohexylamine	0.20	0.90	0.60	—
PCE	*N*-Ethyl-1-phenylcyclohexylamine	0.37	0.49	5.79	2.15
PCDEA	*N,N*-Diethyl-1-phenylcyclohexylamine	0.13	1.38	0.83	1.52
NIPPCA	*N*-(Isopropyl)-1-phenylcyclohexylamine	0.14	1.29	2.86	—
NNBPCA	*N*-(*n*-Butyl)-1-phenylcyclohexylamine	0.08	2.25	0.30	0.39
NSBPCA	*N*-(*s*-Butyl)-1-phenylcyclohexylamine	0.03	6.00	0.58	0.29
PCHHMI	1-(1-Phenylcyclohexyl)hexamethyleneimine	0.09	2.00	0.49	—
TCPY	1-[1-(2-Thienyl)cyclohexyl]pyrrolidine	0.18	1.00	0.87	1.54
TCM	1-[1-(2-Thienyl)cyclohexyl]morpholine	0.85	0.21	0.07	0.17
PCM	1-(1-Phenylcyclohexyl)morpholine	0.78	0.13	0.10	0.18
PCP-MeI	Phencyclidine methiodide	0.90	0.20	—	—

[a] From Jasinski *et al.* (20).

carb-stimulated [^{3}H]PCP binding to the channel sites of the ACh receptor in *Torpedo* membranes, and their potencies were correlated with published data on the effects of these drugs on animal behavior. All of the derivatives were quite effective, and the higher the lipid solubility of the derivative the higher was its potency (Table 2). The *s*-butyl derivative (i.e., NSBPCA) was the most potent (K_i = 0.03 μM), whereas the positively charged PCP-MeI was the least potent (K_i = 0.9 μM). Increasing the aliphatic chain length of the phenylcyclohexylamine increased the potency of the compound on the channel site, yet had mixed potencies in modifying animal behavior (Table 2). It is obvious that the rank order of potency of these compounds on the two animal behaviors was not correlated to their effects on binding of [^{3}H]PCP to the nicotinic ACh receptor/channel.

DISCUSSION

Comparison of [^{3}H]PCP binding to the nicotinic ACh receptor with that of [^{3}H]H_{12}-HTX. The binding of [^{3}H]PCP to the nicotinic ACh receptor/channel molecule is remarkably similar to that of [^{3}H]H_{12}-HTX. Both ligands bind with high affinity and reversibly to the ionic channel sites and not to the receptor sites (3, 13). Several agonists increase the affinities and initial rates of binding of both radiolabeled probes (3) (Fig. 3) with similar potencies, which are in the same rank order as their potency in depolarizing the postsynaptic membranes of *Torpedo* electric organ (21). Also, the depolarizing blocker decamethonium activates at low concentrations and then inhibits at higher concentrations [^{3}H]PCP binding to *Torpedo* membranes (Fig. 4), as it does that of [^{3}H]H_{12}-HTX (3). This is possibly due to binding to the receptor sites and then to channel sites, since high concentrations also block ACh receptor-regulated end-plate conductances whereas lower concentrations depolarize the membrane (22). Preincubation of membranes with agonists similarly reduces the stimulation of binding of [^{3}H]PCP and [^{3}H]H_{12}-HTX (3). In addition, binding of [^{3}H]PCP and [^{3}H]H_{12}-HTX to these channel sites is equally sensitive to blockade by drugs (3) (Fig. 6) as well as to reduction of disulfide bond(s) or alkylation of —SH group(s) (2) (Fig. 5). Pretreatment of *Torpedo* membranes with α-BGT abolishes the stimulating effects of agonists and antagonists, indicating that the stimulation results from drug/receptor-induced effects. Again, as with [^{3}H]H_{12}-HTX binding (3), antagonists stimulate [^{3}H]PCP binding to a much smaller degree and at a slower rate than the increase induced by agonists. Also, the specific binding of [^{3}H]PCP, like that of [^{3}H]H_{12}-HTX (3), is time-dependent and slowly reversible (Fig. 1), which matches their effects on skeletal muscles (13, 16).

There are only a few differences between [^{3}H]PCP and [^{3}H]H_{12}-HTX binding to these ionic channel sites. Adamantane and derivatives are more potent inhibitors of [^{3}H]PCP binding (Fig. 6). This is particularly true for the methyl ketone derivative, which has K_i values of 30 and 200 μM for [^{3}H]PCP and [^{3}H]H_{12}-HTX binding, respectively. Finally, we find that the binding of [^{3}H]H_{12}-HTX to these channel sites is much more sensitive to changes in temperature than is binding of [^{3}H]PCP, particularly in the presence of agonists (2) (Fig. 2).

Interactions of antidepressant and antipsychotic drugs with the ionic channel sites. The interactions of tricyclic antidepressants with the ionic channel of ACh receptors have been shown by their inhibition of [^{3}H]H_{12}-HTX (23) as well as [^{3}H]PCP binding to the channel sites of the *Torpedo* ACh receptor. These antidepressants as well as the psychotomimetic PCP inhibit biogenic amine uptake (24, 25) and bind with similarly high affinities to the ionic channel sites of the nicotinic ACh receptor. This raises the possibility that these ionic channel sites and the carriers involved in biogenic amine uptake share some structural features that affect their drug-binding properties. However, it is difficult to correlate the affinities of tricyclic antidepressants for the ACh receptor/channel sites with their antidepressant actions,

the reason being their varied potencies against different depressions. In our study of their potencies in inhibiting [^{3}H]H_{12}-HTX binding to the nicotinic ACh receptor/channel, there was no correlation with their therapeutic success index (26), and the most potent antidepressant in producing sedation (doxepin) (26) was the least potent in inhibiting [^{3}H]PCP binding. In any case, the upper limit of the plasma concentration (0.3–0.8 μM for amitriptyline and imipramine, respectively) during clinical use and their high affinities for the nicotinic ACh receptor/channel suggest that this receptor may be a secondary target for antidepressants.

The strong inhibition by chlorpromazine and the other antipsychotics of specific [^{3}H]PCP binding to *Torpedo* membranes (Fig. 9) (K_i 0.034–1.0 μM) suggests that they interact with the channel sites of the nicotinic ACh receptor, but they do not bind to receptor sites since they do not inhibit (and in fact enhance) [^{3}H]ACh binding at concentrations up to 100 μM. This supports the finding of inhibition of ACh responses in muscle caused by chlorpromazine (27) and suggests that the observed time-dependent and desensitization-like inhibition is due to an interaction with the channel sites. It also supports the use of chlorpromazine to label subunit(s) of the nicotinic ACh receptor that contribute(s) to its ionic channel (28). The potency of trifluoperazine in inhibiting binding of [^{3}H]PCP to the channel sites of the nicotinic ACh receptor may explain its inhibition of neuromuscular transmission (29). It was attributed to antagonism of calmodulin, which was proposed to regulate receptor function (29), but it may also be due to inhibition of the ACh receptor through its channel sites.

Interactions of opiates and antiarrhythmic drugs with the ionic channel sites. Certain psychotomimetic opiates (e.g., SKF 10047), which produce discriminative stimuli in the rat and ataxia on the rotarod test, like PCP (18), displace specific binding of [^{3}H]PCP from *Torpedo* membranes (Fig. 8). These are the benzomorphan derivative SKF 10047 (K_i = 0.9 μM) and cyclazocine (K_i = 1.2 μM), which have *sigma* receptor activities (30), and levallorphan (K_i = 2.0 μM), which is psychotomimetic in humans. The other opiates, morphine, nalorphine, and naloxone, are least 2 orders of magnitude less effective in displacing [^{3}H]PCP from *Torpedo* membranes, with K_i values of > 100 μM. Naltrexone has a voltage-dependent inhibitory action on neuromuscular transmission,[2] and inhibits the binding of [^{3}H]PCP (Fig. 7) to the *Torpedo* ACh receptor but not that of [^{3}H]ACh. However, its low affinity (K_i = 100 μM) for this receptor is at least 3–4 orders of magnitude lower than the concentration required for its opiate antagonistic action (31).

Another group of drugs that inhibit binding of [^{3}H]PCP to the channel sites of the nicotinic ACh receptor comprises the antiarrhythmic drugs (Fig. 10), including the *beta*-adrenergic blocker propranolol. This drug has been shown to inhibit neuromuscular transmission (32), perhaps because of its inhibition of the channel sites of the nicotinic ACh receptor as shown in Fig. 10. However, we used a racemic mixture, whereas the (−)- and (+)-isomers had the same effects on neuromuscular transmission. Quinidine is better known for its myocardial membrane-stabilizing properties. However, its more toxic (−)-isomer, quinine, antagonizes the action of ACh and produces a curare-like effect on skeletal muscles, and it has been used in treatment of myotonia congenita (33). This raises interesting questions as to whether quinidine and other drugs that bind to the channel sites of the ACh receptor are helpful in the treatment of myotonic disorders.

The ionic channel of the ACh receptor as the site for end-plate postsynaptic action of antibiotics and antiviral drugs. Several antibiotics have been shown to inhibit neuromuscular transmission through presynaptic inhibition of transmitter release as well as postsynaptic inhibition (19, 34). These include aminoglycosides; the lincosamides, lincomycin and clindamycin; and polymyxins. The presently observed binding of these antibiotics to the channel sites of the nicotinic ACh receptor, shown by inhibition of [^{3}H]PCP binding (Fig. 10), and the lack of inhibition of [^{3}H]ACh binding to the receptor sites, suggest that their postsynaptic inhibition of neuromuscular transmission (34) is due to inhibition of the ionic channel component of the ACh receptor. The potent inhibition of [^{3}H]PCP binding by certain of the antibiotics tested occurs at concentrations that are well within those that may be established in circulation during administration of therapeutic doses of the drugs. After intramuscular injection of 80 mg and 50 mg of gentamicin (35) and polymyxin B (36), respectively, serum levels can reach peaks of 7 and 8 mg/liter, equaling about 9 and 7 μM, respectively. These values are higher than their K_i values (5.8 and 0.87 μM, respectively) for inhibition of [^{3}H]PCP binding.

We had previously reported on the interaction of the antiviral amantadine with the channel sites of the nicotinic ACh receptor and not with the ACh-binding sites (4); the same was found in the present study for other adamantane derivatives. The amino group on the adamantane nucleus is important for its binding to the ionic channel sites, as shown by the potency of adamantanamine as compared with that of adamantane and other derivatives (Fig. 6). The amino group is also important for the antiviral potency of the adamantane nucleus, since its replacement by H, OH, SH, CN, COOH, Cl, or Br produced inactive compounds (37). Another group of antiviral compounds, tilorone and analogues (17, 18), inhibits the channel sites of the nicotinic ACh receptor. However, we found no correlation between their potencies in inhibiting [^{3}H]PCP binding and their antiviral activities (Table 1). It is evident that the ionic channel of the nicotinic ACh receptor is not a primary target for the antiviral action of these drugs, but it may be a secondary site of action for antiviral drugs.

Relationship of the potencies of PCP analogues on the channel sites to their psychotropic action. PCP was introduced as a clinical anesthetic, but the psychoses it produced, which resemble schizophrenia (38), prevented its clinical use, while the hallucinations it caused and ease of synthesis made it a very popular drug of abuse. PCP has been found to interact with several vital macromolecules in the nervous system, but its primary target involved in the psychotic effect is not yet known. PCP is a potent inhibitor of butyrylcholinesterase (39), muscarinic ACh receptors (40), reuptake carriers for catechol-

[2] E. X. Albuquerque, personal communication.

amines (24, 25), and the axonal potassium channel (41). PCP and analogues are also potent inhibitors of the channel sites of the nicotinic ACh receptor (Table 2). It is interesting that PCP binds with a similarly high affinity to specific binding sites in rat brain (42). PCP produces a variety of behavioral actions, not only those detected by the rotarod test (which examines motor capability) and the discriminative stimulus test (which examines whether an animal differentiates between two drugs on the basis of their pharmacological actions). If a nicotinic receptor channel with similar drug specificities is found in the central nervous system, the lack of correlation of the potencies of PCP drugs in inhibiting this receptor channel with their behavior-modifying potencies (Table 2) rules out such ACh receptors as primary targets for these actions of PCP.

Conclusions. The channel sites on the nicotinic ACh receptor that bind [^{3}H]PCP appear to be the same as those that bind [^{3}H]H_{12}-HTX. They are coupled to the ACh-binding sites, as evidenced by the reciprocal allosteric stimulating effects of binding to one site on binding of the other. These interactions allow the detection of four conformations of the nicotinic ACh receptor: resting, active, desensitized, and nonconducting antagonist-induced (3).

It is evident that a variety of drugs that have different modes of action bind to the channel sites of the nicotinic ACh receptor, suggesting the relative nonspecificity of these sites. Several of the drugs that inhibit this nicotinic channel have been found to be cardiotoxic owing to inhibition of other cation channels (43). Although none of these drugs tested appear to exert their primary effects through interactions with the nicotinic channel sites, the high affinities that some have (e.g., K_i values of 0.03–0.9 μM for PCPs, 0.9–2 μM for psychotropic opiates, 0.04–1 μM for chlorpromazine and other antipsychotics, 0.08–0.9 μM for tricyclic antidepressants, and 0.87 μM for the antibiotic polymyxin) suggest that the nicotinic ACh receptor may be a secondary target for their actions. This is not so for the majority of nonpsychotropic opiate antagonists (K_i 90–>100 μM), antiarrhythmic drugs (K_i = 2–100 μM), and most antibiotics (K_i 4.5–>100 μM). On the basis of the poor correlation found between the potencies of this series of PCP analogues in inhibiting [^{3}H]PCP binding to these nicotinic channel sites and their behavior-modifying potencies, it is suggested that the unique psychotomimetic and behavioral effects of PCP are apparently mediated via interactions with targets other than nicotinic channel sites.

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