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Interactions of Quaternary Ammonium Drugs with Acetylcholinesterase and Acetylcholine Receptor of *Torpedo* Electric Organ

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SUMMARY

The structure-activity relationship of 16 quaternary ammonium compounds was studied on the activity of acetylcholinesterase (ACh-esterase; EC 3.1.1.7), on the binding of [^{3}H] acetylcholine ([^{3}H]ACh) and [^{3}H]phencyclidine ([^{3}H]PCP) to the "receptor" and "channel" sites, respectively, of the nicotinic ACh receptor, and on the receptor-regulated $^{22}Na^+$ influx into microsacs made from the electric organ membranes of the electric ray, *Torpedo* species. The potencies of 12 compounds in inhibiting ACh-esterase activities varied a great deal more (6667-fold) than their potencies in inhibiting [^{3}H]ACh binding to the receptor sites (916-fold), and no correlation was found between these potencies. Methyl-β-dimethylammonium propionate methiodide was the most potent inhibitor of binding to the receptor site and the least potent inhibitor of ACh-esterase activity, which was inhibited most effectively by hydroxyphenolic compounds. Introduction of an *m*-hydroxy group on the phenyltrimethylammonium had little effect on binding to the receptor site, but dramatically increased its anti-ACh-esterase potency. Increasing steric hindrance reduced the affinity of these drugs for the receptor sites, while attachment of an aromatic ring to the onium group dramatically increased it. Most of these compounds behaved like agonists, inducing $^{22}Na^+$ influx and stimulating binding of [^{3}H]PCP to the channel sites, although to much lower degrees than carbamylcholine. At high concentrations several of these compounds, including edrophonium, inhibited [^{3}H]PCP binding to the channel sites. Increasing the chain length of a homologous series of symmetrically substituted tetraalkylammonium compounds from tetramethyl to tetrahexyl decreased affinity for the receptor sites and increased it for the channel sites.

INTRODUCTION

Anticholinesterases inhibit the catalytic activity of cholinesterases and allow ACh[2] to accumulate in the synaptic cleft, thus potentiating its effect on the ACh receptor in the postsynaptic membrane of skeletal muscles (1). However, several such drugs have been shown to interact directly with the ACh receptor, causing either its activation or inhibition. Tetramethylammonium (2) and *m*-hydroxyphenyltrialkylammonium drugs (3, 4) have been found to act as agonists by directly activating denervated muscles. Also, DFP, diethyl-*p*-nitrophenyl phosphate (Paraoxon), and (2-mercaptoethyl)trimethylammonium iodide *O,O*-diethyl phosphorothioate (echothiopate) at micromolar concentrations potentiate the effect of ACh on the electric organ of the electric eel, *Electrophorus electricus*, causing depolarization of the electroplax, but at millimolar concentrations they reversibly repolarize the membrane owing to their inhibiting action on the ACh receptor (5). On the other hand, the depolarizing action of pinacolyl methylphosphonofluoridate (soman) on the electroplax has been found to be irreversible, an action that is prevented by the ACh receptor antagonist *d*-tubocurarine, which led to the suggestion that it activates the ACh receptor (6). Interactions of organophosphates with ACh receptors have also been detected biochemically by *in vitro* binding studies. Low concentrations of the organophosphate *O,O*-diethyl *S*-(β-diethylamino)ethyl phosphorothiolate (Tetram) totally inhibit activity of ACh-esterase (EC 3.1.1.7) in the electric organ of the electric ray, *Torpedo*, thereby allowing detection of [^{3}H]ACh binding, whereas concentrations higher than 0.5 mM reversibly inhibit [^{3}H]ACh binding to the ACh receptor sites (7). Similar effects are observed with the carbamate anticholinesterases, neostigmine and pyridostigmine (8). Such binding data do

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[2] The abbreviations used are: ACh, acetylcholine; DFP, diisopropyl fluorophosphate; ACh-esterase, acetylcholinesterase; PCP, phencyclidine; carb, carbamylcholine.

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not distinguish between drugs that act as agonists and those that act as antagonists, but this may be accomplished by studies on their effects on receptor-induced ionic fluxes or on the receptor's molecular conformations.

The nicotinic ACh receptor molecule is a glycoprotein that traverses the membrane and extends outside it by about 55 Å and inside it by about 15 Å (9). It carries binding sites for ACh and other agonists as well as antagonists such as α-bungarotoxin, and these are designated as the "receptor" sites. The major portion of the molecule forms a cation-selective channel, and there are sites on the molecule whose binding of ligands [e.g., perhydrohistrionicotoxin (10) and PCP (11)] alters channels properties and the time course of end-plate currents. These sites are designated as "channel" or allosteric sites. Binding of ligands to the latter sites affects binding to the receptor sites (12), and the reverse is also true (13). Binding of both agonists and antagonists increases the rate of binding of channel drugs, but the effect of agonists is much greater and faster (10, 13).

The present study was initiated to probe into the ACh receptor and ACh-esterase sites by means of studying the interactions of quaternary ammonium anticholinesterase compounds with the two proteins. The electric organ of *Torpedo* was the tissue used because of the similarity of drug specificity of its ACh receptor to that of skeletal muscles and the high concentrations of the receptor and esterase proteins, which are 4–5 orders of magnitude greater than those in skeletal muscles (14). In addition, the esterase and receptor of the electric organ have been purified and are well-characterized (14, 15). The receptor and esterase proteins were studied herein in their membrane-bound states so as to preserve their natural configurations. Drugs which interacted with the ACh receptor were identified by several means: their competition with [^{3}H]ACh for the "receptor" binding sites and with [^{3}H]PCP for the "channel" sites, as well as by their effects on receptor-induced specific $^{22}Na^+$ flux into microsacs made from electric organ membranes.

MATERIALS AND METHODS

Membrane preparation. The tissue used was the electric organ of *Torpedo ocellata*, obtained fresh from Alexandria, Egypt, and stored frozen at −90° for not more than 6 months. Tris-HCl (10 mM, pH 7.4, 0.02% NaN_3) was used to homogenize the electric organ membranes (1 g/ml), with different additives depending on the purpose of the experiment. For ACh-esterase assays, the Tris-HCl solution contained 1 mM EDTA; for binding assays, it contained 1 mM EDTA, 0.1 mM DFP, and 0.1 mM phenylmethylsulfonyl fluoride; and for $^{22}Na^+$ flux assays, it contained 0.1 mM phenylmethylsulfonyl fluoride and 0.02% NaN_3. The homgenate was centrifuged at 3,000 × *g* for 10 min, and the pellets were rehomogenized and centrifuged as before. The supernatants of these two centrifugations were combined and centrifuged at 30,000 × *g* for 60 min and the pellets were rinsed, lyophilized, and stored at −20° until use. The membranes were reconstituted in 10 mM Tris buffer for the binding (with 0.1 mM DFP and 0.02% NaN_3, at a concentration of 2 mg of protein per milliliter) and ion flux (at a concentration of 10 mg of protein per milliliter) assays. For the ACh-esterase assay, membranes were reconstituted in Krebs' original Ringer phosphate buffer [concentrations (millimolar): NaCl, 107; KCl, 4.8; $CaCl_2$, 0.65; $MgSO_4$, 1.23; Na_2HPO_4, 15.7; pH 7.4) at a concentration of 10 μg of protein per milliliter (≃2 units of ACh-esterase activity).

Binding of radioactive ligands. Equilibrium dialysis was used to determine the amount of [*acetyl*-^{3}H]acetylcholine ([^{3}H]ACh; 49.5 mCi/mmole; obtained from New England Nuclear Corporation, Boston, Mass.) bound to its receptor. Dialysis was carried out in modified Krebs' original Ringer phosphate solution as previously described (13). The membrane preparation was incubated with DFP at a concentration of 10^{-3} M for 10 min prior to the start of equilibrium dialysis. Each 0.3 ml of membranes was pipetted into dialysis tubing (5.6 mm, Union Carbide, pretreated to remove contaminants), which was tied at both ends and placed in a flask containing 50 ml of Ringer's solution containing [^{3}H]ACh at different concentrations (ranging from 0.02 to 0.1 μM) and 10^{-4} DFP. After 4 hr of shaking at 21°, triplicate samples of 50 μl were taken from each dialysis bag contents and bath, and each was placed in 5 ml of toluene scintillation solution (4.75 g of 2,5-diphenyloxazole, 0.32 g of dimethyl 1,4-bis[2-(5-phenyloxazolyl]benzene, and 40 ml of Beckman BBS 3 solubilizer for every liter of toluene) and counted in a Beckman LS-3133P liquid scintillation spectrometer. Radioactivity in the bag samples in excess of that in the bath samples represented bound ligand. When the effect of drugs on binding was determined, the drug was placed in the dialysis medium along with the [^{3}H]ACh.

Binding of the ionic channel probe PCP was measured as described (11) by incubating [*piperidyl*-3,4-^{3}H(N)] PCP (48 Ci/mmole; New England Nuclear Corporation; 1 Ci = 3.7 × 10^{10} Bq) with *Torpedo* membranes (2–10 μg of protein) in 50 mM Tris-HCl buffer (pH 7.4) for different times at room temperature; the mixture was then filtered with suction through Whatman GF/B glass-fiber filters that had been dipped in 1% organosilane concentrate (Prosil-28). The filters were washed with 7 ml of the Tris buffer, and their radioactivity was measured in a toluene-based solution containing 4% BioSolv (Beckman Instruments, Palo Alto, Calif.) after at least 8 hr. Specific [^{3}H]PCP binding was the portion of total binding that was inhibited with a 5 mM concentration of the channel drug amantadine. Each ammonium drug was added to [^{3}H]PCP and its effect in stimulating its binding was determined; each drug was also added with carb and [^{3}H]PCP to determine whether it inhibited the carb-stimulated [^{3}H]PCP binding.

ACh-esterase assay. The spectrophotometric method of Ellman *et al.* (16) was used to assay for ACh-esterase activity. Acetylthiocholine (1.5 ml, at one of five concentrations ranging from 5 × 10^{-5} M to 2 × 10^{-3} M) in water was added to 1.5 ml of 5,5′-dithiobis(2-nitrobenzoic acid) (10^{-3} M) in 5 × 10^{-2} M Na_2HPO_4 (pH 7.4); up to 100 μl of the drug at the desired concentration or water were then added followed by 0.1 ml of membrane preparation. The change in absorbance at 412 nm was monitored. The weighted linear regression of Wilkinson (17) was used to analyze the data.

^{22}Na flux measurements. The method used was essentially that of Epstein and Racker (18) on reconstituted membranes, with some modifications (10). Dowex 50W-X8, 100–200 mesh hydrogen (from Bio-Rad Laboratories,

Richmond, Calif.), was converted to the Tris form, pH 7 (19), and a 2-ml suspension was equilibrated in 0.2 M sucrose and 10 mM Tris, pH 7, then packed into disposable pipette droppers (Dynatech Laboratories, Inc., Alexandria, Va.). The *Torpedo* microsacs (200 μl) were rapidly added to 200 μl of ^{22}Na (3 μCi/ml, carrier-free, from New England Nuclear Corporation) in 20 μl of original buffer and 50 μM carb. After 0.8 min, 300 μl of the mixture were filtered through the Dowex column to remove the free $^{22}Na^+$, and the column was then washed with 2 ml of 0.2 M sucrose and 10 mM Tris, pH 7. Air pressure was used to speed the passage of sample and buffer (each taking about 1 sec). The effluent containing the microsacs with their sequestered ^{22}Na was collected and counted in a Packard Autogamma scintillation spectrometer. Nonspecific $^{22}Na^+$ influx averaged 2100 dpm/mg of protein, whereas receptor-regulated (i.e., carb-induced) $^{22}Na^+$ influx averaged 5800 dpm/mg of protein. From the dose-response function of the effect of carb on $^{22}Na^+$ influx, the K_d of carb was calculated as ≃3 μM (10).

RESULTS

Inhibition of the activity of *Torpedo* electric organ ACh-esterase by the 12 drugs tested was competitive (Fig. 1). All compounds were initially screened at 1, 10, 100, and 1000 μM for their anticholinesterase potencies. Two concentrations were then selected for each drug within its effective range, and the least-squares lines were obtained. The inhibition constant (K_i) was calculated from the expression $K_i = [I]/(K_p/K_m) - 1$, where K_m and K_p are the effective Michaelis constants in the absence and presence of inhibitor at concentration I, respectively. One line for each drug was selected to be presented in a composite figure so as to show their relative potencies (Fig. 1). An example of the detailed data obtained is presented in Fig. 2, where the points and standard deviations are shown. The drugs varied 6667-fold in their K_i values, which ranged from 0.4 to 2667 μM (Table 1).

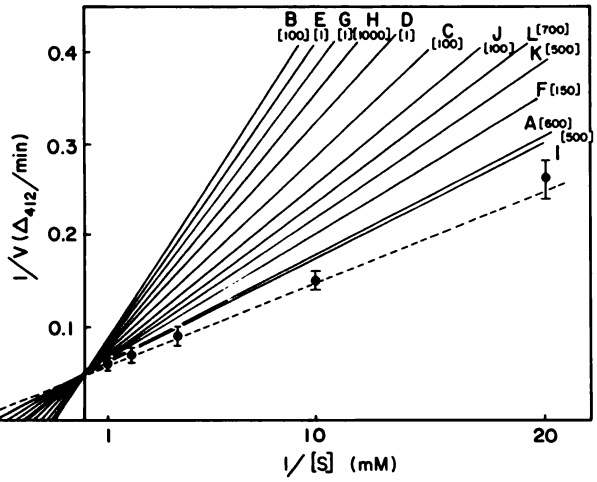

FIG. 1. *Double-reciprocal plot of ACh-esterase activity of Torpedo membranes in absence (- - -) and presence (——) of various ammonium drugs*

Symbols represent the means of triplicate assays, and bars representing standard deviations of measurements are shown only for the control line. Standard deviations for the experimental lines are <10%. Each straight line is drawn by weighted regression analysis of the data points from three experiments. The letters represent the compounds listed in Table 1. Each drug was tested at two concentrations in triplicate to determine its K_i value, but only one line representing one concentration (micromolar, given in brackets) is illustrated.

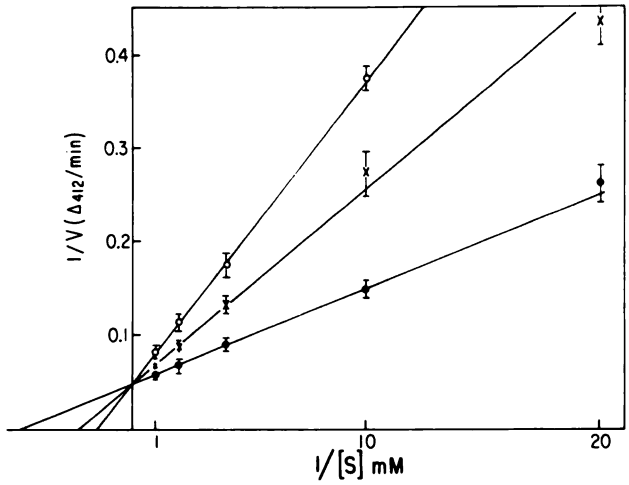

FIG. 2. *Double-reciprocal plot of ACh-esterase activity of Torpedo membranes in absence (●) and presence of m-hydroxybenzyl trimethylammonium iodide (Compound C), 100 μM (×) and 200 μM (○)*

Symbols and bars represent the means of triplicate assays and standard deviations.

The nicotinic ACh receptor in *Torpedo* membranes was identified *in vitro* by its specific binding of [^{3}H]ACh. Binding of [^{3}H]ACh was inhibited by all of the drugs tested with K_i values that differed 916-fold, ranging from 0.12 to 110 μM (Table 1). The concentration that inhibited 50% (IC_{50}) of [^{3}H]ACh binding for each ammonium compound was determined from the dose-response curve of its effect at 10^{-8}–10^{-3} M on binding of [^{3}H]ACh at 0.1 μM to the receptor sites (Fig. 3). The K_i value was calculated from the expression $K_i = IC_{50} \cdot (1 - f)$, where f, the fractional occupancy of the ACh-binding sites at 0.1 μM [^{3}H]ACh, was 0.85. The most effective drug in

TABLE 1

Inhibition constants (K_i) of the quaternary ammonium compounds on binding of [^{3}H]ACh (0.1 μM) to its ACh receptor and ACh-esterase activity in membranes from Torpedo electric organ

Compound	Inhibition constant		K_i ratio: receptor/esterase
	[^{3}H]ACh binding	ACh-esterase activity	
	μM		
A. Methyl-β-dimethylaminopropionate methiodide	0.12 ± 0.01	2667 ± 40	4.5×10^{-5}
B. Phenyl trimethylammonium	0.35 × 0.02	45 ± 1.5	7.7×10^{-3}
C. *m*-Hydroxybenzyl trimethylammonium	0.34 ± 0.03	85 ± 2.3	4×10^{-3}
D. *m*-Hydroxyphenyl trimethylammonium	0.64 ± 0.05	0.7 ± 0.02	0.91
E. *m*-Hydroxyphenyl ethyldimethylammonium	0.99 ± 0.07	0.4 ± 0.01	2.47
F. *n*-Amyl trimethylammonium	0.90 ± 0.06	1162 ± 26	7.7×10^{-4}
G. *m*-Hydroxyphenyl triethylammonium	8.60 ± 0.55	0.4 ± 0.01	21.5
H. *m*-Hydroxycyclohexyl trimethylammonium	24 ± 1.1	470 ± 5.1	5.1×10^{-2}
I. Tetramethylammonium	38 ± 2.1	1062 ± 18	3.5×10^{-3}
J. *m*-Hydroxymethylphenyl trimethylammonium	7.5 ± 0.46	81 ± 1.2	9.3×10^{-2}
K. Ethyltrimethylammorium	56 ± 3.5	789 ± 11	7.1×10^{-3}
L. Tetraethylammonium	110 ± 6.8	800 ± 9.6	1.4×10^{-2}

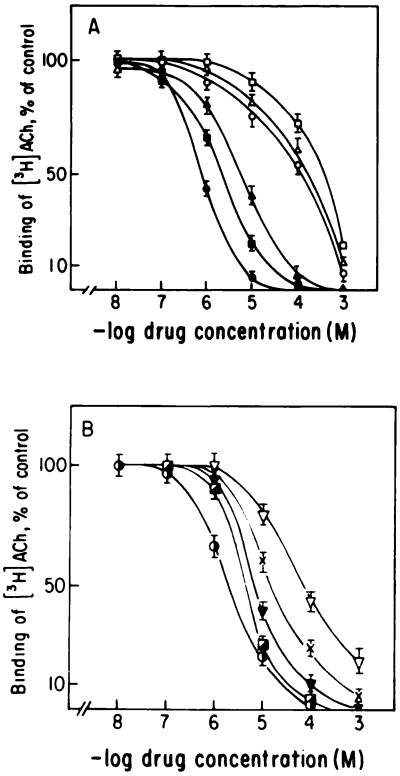

FIG. 3. *Log dose-response relationships for the effect of the various ammonium compounds on the binding of [^{3}H]ACh (0.1 μM) to Torpedo membranes*

A, Compounds A (●), C (■), E (▲), I (○), L (□) and K (△); B, Compounds B (◑), D (◪), F (▼), J (×), and G (▽). Letter designations are the same as in Table 1. Symbols and bars represent means and standard deviations of three experiments.

inhibiting [^{3}H]ACh binding to its receptor was Compound A (methyl-β-dimethylaminopropionate methiodide), with a K_i of 0.12 μM (Table 1). On the other hand, it was the least effective in inhibiting ACh-esterase activity, with a K_i of 2.7 mM.

Binding of [^{3}H]ACh was inhibited by agonists as well as antagonists. Therefore, in order to determine biochemically whether any of the quaternary ammonium drugs tested had an agonist-like activity, their effects in stimulating receptor-activated $^{22}Na^+$ influx into *Torpedo* microsacs were studied. For comparison, the effect of carb was first determined and found to be dose-dependent, giving maximal stimulation at 50–100 μM carb (13). All of the quaternary ammonium drugs were tested at a single concentration (0.25 mM) and found to cause $^{22}Na^+$ influx to various degrees (Fig. 4). The preaddition of *d*-tubocurarine (0.1 mM) inhibited these drug-induced $^{22}Na^+$ influxes. This assay, which measured flux extents rather than rates, suggested that all of the drugs tested acted like agonists.

Another biochemical method for distinguishing receptor agonists from antagonists is to study their allosteric effects on the specific binding of channel blockers (e.g., [^{3}H]perhydrohistrionicotoxin or [^{3}H]PCP). These are drugs which have voltage-dependent effects on channel lifetime, and the kinetics of their binding is conformation-dependent (10, 11, 13). Accordingly, the effects of quaternary ammonium drugs were tested on the binding of the channel probe [^{3}H]PCP. The ability of carb to displace [^{3}H]ACh binding at a 1 μM concentration (i.e., >99% occupancy) correlated well with its fractional occupancy of the receptors [which equals $x/K_d + x$, where x is carb concentration and the K_d of carb is 3 μM (10)] and its ability to stimulate the binding of [^{3}H]PCP at 2 nM (i.e., <1% saturation) (Fig. 5). Also, the carb-stimulated [^{3}H]PCP binding was blocked partially by *d*-tubocurarine (100 μM) and totally by prior incubation of *Torpedo* membranes with 10 μM α-bungarotoxin (Fig. 6). This confirmed that the stimulation of [^{3}H]PCP binding was due to binding of carb to the receptor sites (11). Tests using a 0.25 mM concentration of the ammonium compounds on the initial rate (30 sec) of binding of [^{3}H]PCP showed stimulation by all compounds, similar to their effects on $^{22}Na^+$ uptake. Therefore, we studied the effect of each drug in the concentration range of 10^{-8}–10^{-3} M

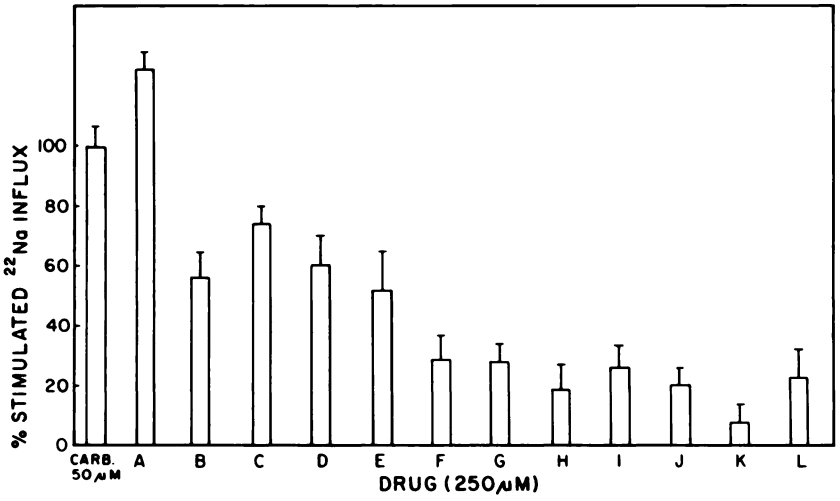

FIG. 4. *ACh receptor-regulated ^{22}Na uptake by Torpedo microsacs*

The amount of ^{22}Na uptake stimulated by 50 μM carb (*far left column*) is used as a reference for maximal stimulation (100%). The stimulated ^{22}Na uptake by the various ammonium drugs (Compounds A–L) is presented as percentage of maximal. The bar on *top* of each column represents the standard deviation of triplicate measurements.

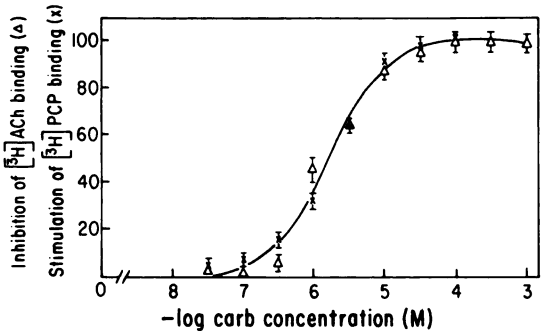

FIG. 5. *Effect of carb on the binding of 1 μM [^{3}H]ACh (Δ) (measured by equilibrium dialysis) and 2 nM [^{3}H]PCP (×) (measured by filtration, 30-sec incubation) to Torpedo membranes*

The line was drawn according to fractional occupancy of the receptors by carb. Symbols and bars represent means and standard deviations of three experiments.

on the initial rate of binding of [^{3}H]PCP (2 nM). The dose-response functions for the different compounds are presented in Fig. 7. The stimulating effects of Compounds A, I, H, and K were not reduced at higher concentrations, whereas the effects of the other compounds were reduced, especially those of Compounds B, C, E, and F. The reduction in [^{3}H]PCP binding by high concentrations of these compounds may result from increased receptor desensitization by agonists or from direct binding to the channel sites (13). Not only agonists but also antagonists were previously found to stimulate the binding of blockers to the ACh-receptor's ionic channel sites (13), although agonists stimulated the binding up to 100-fold, and nonpolypeptide antagonists like curare were slower in stimulating it and did so to a much lower extent (20). Thus, the stimulating effects of the quaternary ammonium compounds on [^{3}H]PCP binding were, with the

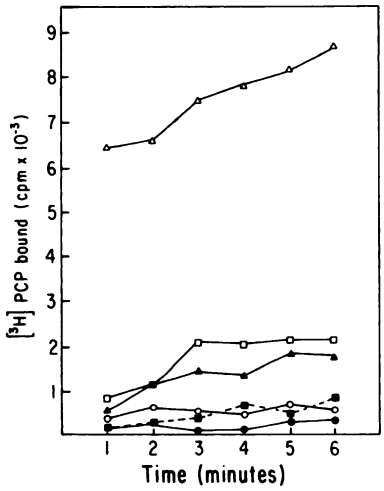

FIG. 6. *Binding of [^{3}H]PCP (2 nM) to Torpedo membranes as a function of time in absence (▲) and presence of 10 μM ACh (Δ), 10 μM ACh and 10 μM d-tubocurarine (□), 10 μM ACh and tissue pretreated with 10 μM α-bungarotoxin for 60 min (●), 10 μM ACh and 5 mM amantadine (○), or 10 μM ACh and 5 mM amantadine and tissue pretreated with 10 μM α-bungarotoxin for 60 min (●)*

Symbols represent means of three experiments with standard deviation <10%.

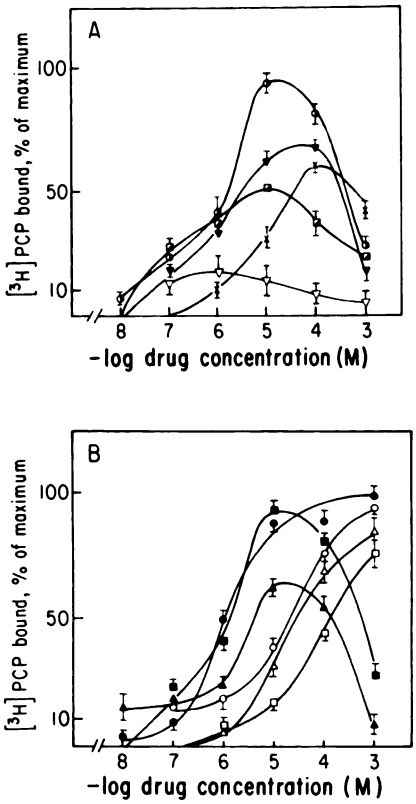

FIG. 7. *Stimulating effect of different concentrations of quaternary ammonium drugs on the binding of [^{3}H]PCP (2 nM) to the channel sites of the ACh receptor in Torpedo membranes*

The control nonspecific binding (i.e., binding in presence of 5 mM amantadine) value was subtracted from all experimental points. In A the symbols stand for Compounds B (◑), D (◪), F (▼), J (×), and G (▽); in B, for Compounds A (●), C (■), E (▲), I (○), H (△), and K (□). Letter designations are the same as in Table 1. The 100% level is the highest level of counts established for Compound A. Symbols and bars represent means and standard deviations for triplicate experiments.

probable exception of Compound G, more like those of agonists than antagonists.

The higher apparent potency of tetramethylammonium (Compound I) in stimulating [^{3}H]PCP binding as compared with ethyltrimethylammonium (Compound K) (Fig. 7B) suggested that increased size of the tetraalkylammonium group reduced the agonistic activity of the drug. When we tested a homologous series of symmetrically substituted tetraalkylammonium drugs (C_1–C_6), all but tetramethylammonium inhibited binding of [^{3}H]PCP (Fig. 8); thus they acted like channel blockers that reduce the binding of [^{3}H]PCP rather than increase it. The C_2–C_6 compounds were more potent inhibitors of [^{3}H]PCP binding when 100 μM carb was present (Fig. 9). Since the concentration of carb used (100 μM) produced maximal stimulation of [^{3}H]PCP binding, the addition of another agonist like tetramethylammonium, as expected, did not increase that response. It was also the only drug that did not inhibit the carb-stimulated response. When the effects of these tetraalkylammonium drugs on [^{3}H]ACh (0.1 μM) binding were studied, tetramethylammonium was a relatively potent inhibitor (K_i = 38 μM), tetraethylammonium was less so, and the other com-

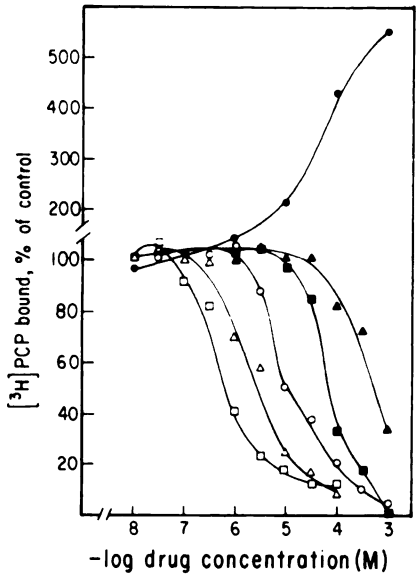

FIG. 8. *Log dose-response relationships of the effect of six symmetrically substituted tetraalkylammonium compounds on the binding of [^{3}H]PCP (2 nM) to the channel sites of the ACh receptor in Torpedo membranes*

The drugs used were tetramethylammonium (●), tetraethylammonium (▲), tetrapropylammonium (■), tetrabutylammonium (○), tetrapentylammonium (△), and tetrahexylammonium (□). Incubation time was 30 sec, and nonspecific binding (i.e., binding in presence of 5 mM amantadine) was subtracted. Symbols represent means of three experiments; the standard deviation was <10%.

pounds at concentrations up to 1 mM stimulated [^{3}H]ACh binding (Table 2). This increased binding is a feature of drugs that bind to the channel sites.

The dose-response function of the effects of Compounds B, C, D, E, F, and J (Fig. 7A and B), i.e., stimulating [^{3}H]PCP binding at low concentrations through allosteric action at the receptor site and reducing this stimulation at higher concentrations (e.g., 1 mM), suggested that at the higher concentrations they might be inhibiting [^{3}H]PCP binding directly. Therefore, we tested their effects on [^{3}H]PCP binding in the presence

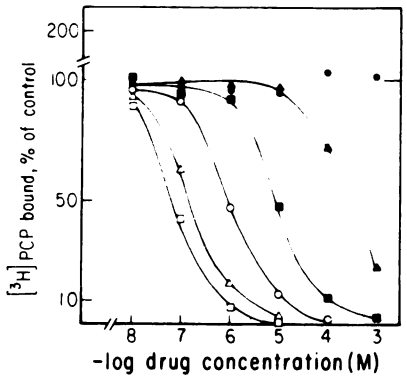

FIG. 9. *Effect of the same six tetraalkylammonium drugs as in Fig. 8 on the binding of [^{3}H]PCP (2 nM) to the channel sites of the ACh receptor in Torpedo membranes, in presence of 100 μM carb and an incubation interval of 30 sec*

Symbol designations are the same as in Fig. 8. Standard deviations were <10%.

TABLE 2

Effect of tetraalkylammonium compounds on the binding of [^{3}H]ACh (0.1 μM) to Torpedo membranes

Drug	Binding of [^{3}H]ACh as % of control in presence of four drug concentrations[a]			
	10^{-6} M	10^{-5} M	10^{-4} M	10^{-3} M
Tetramethylammonium (Compound I)	76	61	31	5
Tetraethylammonium (Compound L)	97	77	58	15
Tetrapropylammonium	125	121	109	110
Tetrabutylammonium	124	114	119	96
Tetrapentylammonium	98	103	133	102
Tetrahexylammonium	99	99	136	116

[a] These values represent means of triplicate experiments with standard deviations <10%.

of 100 μM carb, which stimulated [^{3}H]PCP binding greatly. All of these compounds reduced the binding to values that were far below that of the control of [^{3}H]PCP binding in the absence of carb (data not shown). Thus, at 1 mM concentration, they behaved like channel blockers. We subjected one of these compounds, edrophonium (Compound E), to a more definitive test to study its interaction with the channel sites without interference from the receptor sites. First we treated *Torpedo* membranes for 1 hr with a 10 μM concentration of the α-neurotoxin from the venom of the cobra *Naja naja siamensis*, a treatment which inhibited irreversibly for the duration of the experiment all [^{3}H]ACh and [^{125}I]α-bungarotoxin binding to the receptor sites without inhibiting [^{3}H]PCP binding to the channel sites, thus canceling any agonist-like action for the ammonium compound. Edrophonium stimulated [^{3}H]PCP binding to the control membranes with an EC_{50} of 10 μM and a maximum at 100 μM (Fig. 10A). The maximal stimulation of [^{3}H]PCP binding produced by edrophonium was only 60% of that produced by carb (Fig. 7B). However, edrophonium inhibited [^{3}H]PCP binding to the toxin-treated membranes with a K_i of 150 μM (Fig. 10B). The channel blocker amantadine also inhibited these toxin-treated membranes, but carb did not.

DISCUSSION

It is evident that the series of quaternary ammonium compounds tested interact not only with ACh-esterase (Figs. 1 and 2), but also with the nicotinic ACh receptor channel molecule at the "receptor" (Table 1) and/or the "channel" sites (Fig. 7). Their action on ACh-esterase is competitive at the concentrations, and under the conditions, used (Fig. 1), which does not exclude action at a peripheral site under different conditions as previously shown for quaternary compounds (21). Their action on the receptor is agonist-like for most of the drugs, as shown by their induction of $^{22}Na^+$ flux (Fig. 4) and stimulation of [^{3}H]PCP binding to the channel sites of the ACh-receptor (Fig. 7). However, the actions of the homologous symmetrically substituted series of tetraalkylammonium drugs, with the exception of tetramethylammonium, are antagonist-like as shown by their inhibition of [^{3}H]PCP binding (Figs. 8 and 9). Interestingly, edrophonium (Compound E) (and possibly other drugs

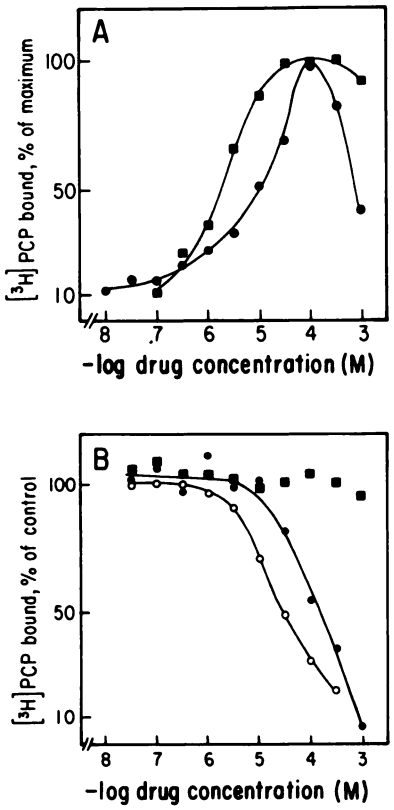

FIG. 10. *Comparison of the effects of carb (■), edrophonium (●), and amantadine (○) on the binding of 2 nM [^{3}H]PCP to the channel sites of the nicotinic ACh-receptor in Torpedo membranes after a 30-sec incubation (A) and in Torpedo membranes that had been preincubated for 2 hr with a 10 μM concentration of the α-neurotoxin from the venom of the cobra Naja naja siamensis (B)*

The 100% level of [^{3}H]PCP binding represents the maximal binding obtained for each of the two drugs separately. Symbols represent means of triplicate experiments with standard deviation <10%.

such as Compounds B, C, D, F, and J) acts like an agonist by stimulating [^{3}H]PCP binding, although less than does carb (Fig. 10A); but at higher concentrations it also interacts with the ionic channel sites as shown by its inhibition of [^{3}H]PCP binding to membranes whose receptor sites were inhibited with *Naja* α-toxin (Fig. 10B).

The structure-activity data obtained with the 12 quaternary ammonium compounds listed in Table 1 indicate that the ACh-binding sites of *Torpedo* receptor and of ACh-esterase differ significantly. The K_i values of these ions on [^{3}H]ACh binding to receptor and on ACh-esterase activity do not correlate ($0.4 < p < 0.5$ according to Spearman's rank test). This difference can also be appreciated from the greater stringency of ligand structural requirements for interaction with ACh-esterase than with receptor. The range of potencies of these quaternary ammonium drugs in inhibiting ACh-esterase activity (ratio of the largest to the smallest K_i value = 6667) is much higher than the range of their potencies in inhibiting binding of [^{3}H]ACh to the ACh receptor sites (ratio = 916).

The importance of the quaternary nitrogen group in the nicotinic action of these organic ions has long been recognized from structure-activity relationship studies with neuromuscular preparations *in vivo* and *in vitro* (22, 23), as well as with eel electroplax *in vitro* (24, 25). This was reaffirmed by the present findings. The homologous 3-hydroxyphenyltriethylammonium (Compound G) and 3-hydroxyphenyltrimethylammonium (Compound D) ions represent an $-N^+Et_3$ for an $-N^+Me_3$ exchange. The increased onium radius in Compound G results in a 14-fold reduction of its affinity for the receptor sites (Table 1; Fig. 3B) and potentiation of its binding of [^{3}H]PCP (Fig. 7B). A single exchange of *N*-ethyl (Compound E) for *N*-methyl (Compound D) in the *m*-hydroxyphenyl dialkylammonium compound causes a slight reduction in the affinity of the compound for the receptor site, as shown by the small increase in the K_i value on [^{3}H]ACh binding (Table 1). However, Compound E (edrophonium) has been shown to be a less potent agonist on neuromuscular transmission (4, 26) and *Electrophorus* electroplax (24). The decreased potency may result from channel blockade, which apparently occurs at higher concentrations (Figs. 7B and 10A and B). The methonium center evidently provides the optimal steric fit to the recognition site of the receptor. Exchange of the trimethyl (Compound D) with a larger trialkyl group (Compound G) results in loss of agonist-like potency (Figs. 4 and 7). This is more evident in the series of symmetrically substituted tetraalkylammonium compounds. Increasing the chain length from tetramethyl to tetraethyl converts the drug from a receptor agonist to possibly a channel blocker (Figs. 8 and 9). The longer the chain length the stronger is the inhibition of [^{3}H]PCP binding. The channel-blocking property of tetraethylammonium has been studied in detail on the ACh receptor/channel of muscle end-plate using electrophysiological methods (27).

Triggle (23) cited indirect evidence to show that interactions of a methonium center, with a specific anionic site of the receptor, are aided by an optimal steric fit of the *N*-methyl groups; thus relatively strong Van der Waals forces serve to bind the methyl groups to the hydrophobic receptor. The notion of an "esterophilic" receptor site analogous to the esteratic site of ACh-esterase has been considered by some. Although the high receptor affinity of the "reversed carbonyl" analogue of ACh (Compound A) and its strong agonist-like activity, triggering $^{22}Na^+$ influx and stimulating [^{3}H]PCP binding, appear to support this view (as do the comparable properties of carb), it is noteworthy that both are poor substrates for ACh-esterase (28). Thus, a reaction of a carbonyl group with the receptor would likely differ from that with the esteratic site of ACh-esterase. Furthermore, the depolarizing effect of phenyltrimethylammonium ion on electroplax (25) and its binding to *Torpedo* receptor sites also refute the idea of comparable "esterophilic" sites in ACh receptor and ACh-esterase. Therefore, the high receptor affinity of the "reversed carbonyl" analogue of ACh (Compound A) and its strong potency in activating [^{3}H]PCP binding and in triggering $^{22}Na^+$ influx are viewed as consequences of a primary methonium anionic site interaction and an important subsidiary electron donor reaction at a receptor region neighboring the anionic locus.

This idea is further illustrated by Compounds B, C, D, E, and F, which have a high order of affinity for receptor sites (K_i values < 1 μM) and stimulate $^{22}Na^+$ influx and

[^{3}H]PCP binding. Presumably, the conjugated ring system in Compounds B, C, D, and E could, like the carbonyl group of ACh and its "reversed" analogue, act as an electron donor promoting a charge transfer in amino acids neighboring the anionic site. In contrast, the poor agonist activity of *n*-amyltrimethylammonium (Compound F) might attribute to its apolar methylene chain. Accordingly, its relatively high receptor affinity can be related to a hydrophobic interaction of the hydrocarbon chain at a region neighboring the anionic site. In view of the present results showing binding of an alkyl chain such as this to the ion channel sites, its poor agonist activity might be explained by a channel blockade concurrent with its binding to the receptor sites, similar to decamethonium (13, 29); thus it would react as a depolarizing blocker.

The foregoing indicates that such a subsidiary reaction mechanism, occurring at a locus proximate to the anionic site, is important to channel activation by classical agonists such as ACh and carb. Wasserman *et al.* (30) reported on the highly specific interaction of the *Electrophorus* ACh receptor with the structurally constrained aromatic bis-quaternary ammonium compound [*trans*-3,3′-bis-α-(trimethylammonium)methylazobenzene dibromide]. Since this compound is a potent depolarizer with distinctive physicochemical characteristics, Wasserman *et al.* were able to decipher both conformational and binding site properties of the receptor. Our findings accord with the conclusions of Wasserman *et al.* (30): (a) inflexible quaternaries can depolarize (Compounds B, D, and E); (b) the binding site has a hydrophobic region interactive with methylene groups (Compounds A, C, and F); (c) there is also a site reactive with electron-donating groups, such as in the aryl compounds and those having a carbonyl group (Compounds A, B, C, D, E, and G); and (d) a trimethylammonium moiety best fits the anionic site of the receptor.

In conclusion, it is evident that the molecular requirements are different for drug interactions with the active sites of ACh-esterase and the receptor and channel sites of the nicotinic ACh receptor. A quaternary ammonium center, when present, serves to orient the functional group to the enzyme's esteratic site and perhaps regulate this site's activity (31). The receptor site, on the other hand, has less stringent requirements. In order to trigger a sequence of conformational changes that lead to opening of its ionic channel, the receptor reacts optimally with a methonium center. The quaternary ammonium compounds interact with both ACh-esterase and the nicotinic ACh receptor. Most bind to the receptor site and act as agonists. A few bind at high concentrations to sites on the ionic channel moiety of the ACh receptor. A drug like edrophonium may potentiate neuromuscular transmission not only by inhibiting ACh-esterase, but also by a direct agonist-like action on the receptor. Furthermore, the interaction of edrophonium with the channel sites suggests that, like decamethonium, it may act as a depolarizing blocker of motor end-plates. It appears that the affinity of the ammonium drugs for the receptor sites is reduced by steric hindrance. Increasing the chain length of the symmetrically substituted tetraalkylammonium compounds increases their affinity for the ion channel sites and decreases their affinity for the receptor sites of the ACh receptor.

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