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Comparison of the Actions of Carbamate Anticholinesterases on the Nicotinic Acetylcholine Receptor

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SUMMARY

Neostigmine (Neo), pyridostigmine (Pyr), and physostigmine (Phy) at low concentrations inhibited acetylcholine (ACh) esterase, thereby indirectly potentiating ACh enhancement of [^{3}H]perhydrohistrionicotoxin (H_{12}-HTX) binding to the channel sites of the nicotinic ACh receptor of *Torpedo* membranes. However, at higher concentrations, they inhibited ACh action due to their direct binding to the ACh receptor. They displaced binding of [^{3}H]ACh and ^{125}I-α-bungarotoxin (α-BGT) to the receptor sites with the following order of decreasing potency: Neo > Phy > Pyr. Furthermore, Neo and Pyr potentiated [^{3}H]H_{12}-HTX binding to the receptor's channel sites. Preincubation of ACh receptors with any of the three carbamates reduced the rate of binding of ^{125}I-α-BGT and increased the potency of carbamylcholine in inhibiting ^{125}I-α-BGT binding, suggesting that the three carbamates act as partial agonists and potentiate receptor desensitization. Although none of the three carbamates inhibited [^{3}H]H_{12}-HTX binding to the receptor's closed channel conformation, only Phy was a potent inhibitor of [^{3}H]H_{12}-HTX binding to the carbamylcholine-activated conformation. The potency of Phy was not due to the absence of positive charge since Phy methiodide acted similarly. The data suggest that the major action of the three carbamates at nicotinic cholinergic synapses is inhibition of AChesterase. Their interactions with the nicotinic ACh receptor are with its "receptor" as well as allosteric "channel" sites, but they differ in their effects. Neo and Pyr act mainly as partial agonists, while Phy is mostly an inhibitor of the channel in the activated receptor conformation.

INTRODUCTION

The toxicity of anticholinesterases is believed to be due mainly to inhibition of ACh[1]-esterase, the result of which is accumulation of ACh in cholinergic synapses that causes repeated activation of ACh receptors and their desensitization (1). However, early studies suggested that anticholinesterases have additional direct effects on the nicotinic ACh receptor, either activating or inhibiting it. Examples are tetraethylammonium (2) and *m*-hydroxyphenyltrimethylammonium (3), acting as agonists on denervated muscles, DFP, paraoxon, and echothiophate at high concentrations inhibiting ACh receptor-induced depolarization in the electric eel (4), and DFP modifying endplate currents in frog sartorius muscle (5), thus acting as a channel blocker.

Because of their anticholinesterase action, the quaternary carbamates Neo and Pyr are effective in therapy of the neuromuscular disease myasthenia gravis (6), while Pyr (7) and Phy (8) have been shown to provide some protection against soman poisoning in mammals. Binding of these carbamates to nicotinic ACh receptors was demonstrated by their competitive inhibition of specific [^{3}H]ACh binding to these receptors in the electric organ of the electric ray, *Torpedo* sp. (9), and the noncompetitive inhibition of the ^{125}I-α-BGT binding to the *Aplysia* receptors (10).

There are different sites on the nicotinic ACh receptor molecule which bind drugs, and several mechanisms by which drugs affect receptor function. They may bind to the "receptor sites" as agonists or competitive antagonists, or to the voltage-sensitive "channel sites" as noncompetitive antagonists affecting the time course of postsynaptic current and/or channel lifetime (11, 12). The drugs may also desensitize the receptor by binding to either of the two kinds of sites. The discovery of ligands that bind to the allosteric channel sites [e.g., H_{12}-HTX and phencyclidine (13, 14)] has provided new biochemical probes for studying the mechanism of interaction of drugs with this receptor. Monitoring changes in affinity

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[1] The abbreviations used are: ACh, acetylcholine; Neo, neostigmine; Pyr, pyridostigmine; Phy, physostigmine; DFP, diisopropyl fluorophosphate; H_{12}-HTX, perhydrohistrionicotoxin; α-BGT, α-bungarotoxin.

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and kinetics of drug binding to the channel sites resulting from binding of agonists or antagonists to the receptor sites provides information on the drug-induced receptor conformations (15, 16). Utilizing such interactions, several quaternary ammonium anticholinesterases were found to act as partial agonists, and increasing the chain lengths of a homologous series of symmetrically substituted tetraalkylammonium compounds from tetramethyl to tetrahexyl was found to decrease affinity for the receptor sites and increase it for the channel sites (17).

Detailed studies of the effects of Pyr on endplate currents of frog muscles (18) and single channel activity in rat myoballs (19) revealed that it acted as a weak agonist on the nicotinic ACh receptor, altered its channel conductance, and desensitized the receptor. The present investigation was initiated to compare the actions of the three carbamates Neo, Pyr, and Phy on the nicotinic ACh receptor/channel molecule, using biochemical techniques, to arrive at the mechanisms of their interactions.

MATERIALS AND METHODS

Preparation of Torpedo membranes. Frozen electric organs of *Torpedo californica* (stored at −90° for less than 6 months) were homogenized in an equal volume of 50 mM Tris-HCl buffer, pH 7.4, containing 0.1 mM phenylmethylsulfonyl fluoride, 0.02% NaN_3, and 1 mM EDTA to reduce proteolytic breakdown. The homogenate was centrifuged at 3000 rpm for 10 min in a Sorvall SS-34 rotor, and the pellet was rehomogenized in 2 volumes of the same buffer and recentrifuged under the same conditions. The two supernatants were pooled and centrifuged at 17,000 rpm for 60 min and the pellets were collected and resuspended in 50 mM Tris buffer containing 0.02% NaN_3 so that the final protein concentration was ≃1 mg/ml. DFP was present during membrane preparation at 0.1 mM, unless otherwise stated, so that all cholinesterases in the preparation were irreversibly inhibited without affecting ACh receptor binding (17). Thus, the agents tested, most of which were esters, were not hydrolyzed. This preparation was usually stable for binding measurements for up to a week of storage at 0°.

^{125}I-α-BGT binding. ^{125}I-α-BGT (specific activity > 100 Ci/mmol from New England Nuclear) binding to *Torpedo* ACh receptor was measured by cation exchange chromatography as developed by Kohanski *et al.* (20). Glass disposable Pasteur pipettes were filled with carboxymethylcellulose (Whatman-52 microgranular), preswollen, and equilibrated in phosphate buffer [1.0 mM Na_2HPO_4, 0.01% Triton X-100 (v/v), 0.03% NaN_3 (w/v)], pH 7.2. *Torpedo* membranes and ^{125}I-α-BGT were incubated in the presence or absence of drugs (at a receptor/toxin ratio of 0.1) for 40 sec at 23° and then transferred to the minicolumn and washed through with 1.0 ml buffer which took 20 sec for completion. Free ^{125}I-α-BGT bound to the resin while the eluate contained ^{125}I-α-BGT bound to ACh receptors, which was collected and counted in an Autogamma counter. Nonspecific binding was the amount of binding to *Torpedo* membranes that had been exposed for 30 min to 10 μM *Naja* α-neurotoxin.

[^{3}H]ACh binding. Binding of [^{3}H]ACh (specific activity of 90 mCi/mmol from New England Nuclear) was measured by equilibrium dialysis of the membranes (250 μl) in 10 ml of modified Krebs-Ringer phosphate solution containing 0.1 μM [^{3}H]ACh and 0.1 mM DFP as previously described (14). After 4 hr at 21°, triplicate samples (50 μl each) were taken from membrane preparation and bath and their radioactivity was counted, with the difference in counts representing amount bound. Nonspecific binding was that obtained from membranes that were preincubated with 10 μM *Naja* α-neurotoxin for 30 min.

[^{3}H]H_{12}-HTX binding. [^{3}H]H_{12}-HTX (specific activity of 21 Ci/mmol), obtained from tritiation of isodihydrohistrionicotoxin and kindly donated by Dr. John Daly (Laboratory of Bioorganic Chemistry, National Institutes of Health), was used to label the allosteric channel site of the nicotinic receptor as previously described (15). Binding of [^{3}H]H_{12}-HTX at equilibrium to resting receptors with closed ionic channels was studied by using *Torpedo* membranes that had been incubated with 10 μM α-BGT for 1 hr to ensure receptor inhibition for the duration of the experiment. These membranes were incubated with [^{3}H]H_{12}-HTX and the test drug(s) for 120 min and then filtered over a Whatman GF/B filter (pretreated with 0.01% polylysine to reduce [^{3}H]H_{12}-HTX binding to filters) and washed with 10 ml of cold buffer. The filter was then placed in a glass minivial with 5 ml of toluene-based scintillation fluid and counted after 8 hr. To determine nonspecific binding of [^{3}H]H_{12}-HTX to the tissue, control assays were run without drugs, without tissue, and with tissue plus 1 mM amantadine which totally blocks specific binding to the ionic channel sites (21).

For studying effects of drugs on the initial rate of binding of [^{3}H]H_{12}-HTX to activated receptors, untreated *Torpedo* membranes were incubated with [^{3}H]H_{12}-HTX (2 nM) in 1 ml of buffer containing 100 μM carbamylcholine; then, after 30 sec, the mixture was filtered over a treated GF/B filter and the radioactivity was counted. Similar sets of control tubes containing amantadine were assayed under these conditions to determine nonspecific binding.

ACh-esterase assay. The spectrophotometric method of Ellman *et al.* (22) was used to assay for ACh-esterase activity. Acetylthiocholine (1.5 ml, at one of five concentrations ranging from 5 to 10^{-5} to 2×10^{-3} M) in water and 1.5 ml of 5,5′-dithiobis(2-nitrobenzoic acid) (10^{-3} M) in 5×10^{-2} M Na_2HPO_4 (pH 7.4) in a 4-ml cuvette were added to control *Torpedo* membranes (0.1 ml), as well as membranes preexposed to the carbamate for 30 min. The change in absorbance at 412 nm over a 1-min interval was monitored on a double beam spectrophotometer.

RESULTS

The initial rate of binding of [^{3}H]H_{12}-HTX (i.e., binding measured after 30-sec incubation only) to *Torpedo* membranes, rich in ACh receptors and ACh-esterase, is very low, resulting in little specific binding (15). In the presence of ACh, [^{3}H]H_{12}-HTX binding in 30 sec (which does not represent equilibrium) increased dramatically and in a dose-dependent manner, reaching maximum at 10^{-3} M (● in Figs. 1 and 2). Low concentrations of ACh (i.e., 100 nM) did not increase [^{3}H]H_{12}-HTX binding, because the high ACh-esterase activity in the preparation eliminated this ACh. Preincubation of *Torpedo* membranes with 1 μM Phy, Pyr, or Neo for 30 min prior to assay inhibited ACh-esterase activity by 100, 33.5, and 98%, respectively. This treatment potentiated the effect of ACh as evidenced by the strong shift to the left of the log dose-response function (Figs. 1 and 2). It resulted from the inhibition of cholinesterases that are present in the membranes, thereby preventing ACh hydrolysis. Now, even as low an ACh concentration as 100 nM produced potentiation of [^{3}H]H_{12}-HTX binding. Phy was the most potent, followed by Neo and then Pyr, which paralleled their anticholinesterase potencies. The inhibition constant (K_i) of ACh-esterase activity 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, respectively, of carbamate at concentration I. The K_i values for Phy, Neo, and Pyr were 6, 20, and 120 nM, respectively.

Not only did the carbamates potentiate the effect of ACh on [^{3}H]H_{12}-HTX binding, but the higher concentrations of carbamates inhibited the potentiating effect of high concentrations of ACh (Figs. 1 and 2). This was least obvious with Pyr, which showed it only at 1 mM ACh and 100 μM Pyr, which inhibited 95.4% of ACh-

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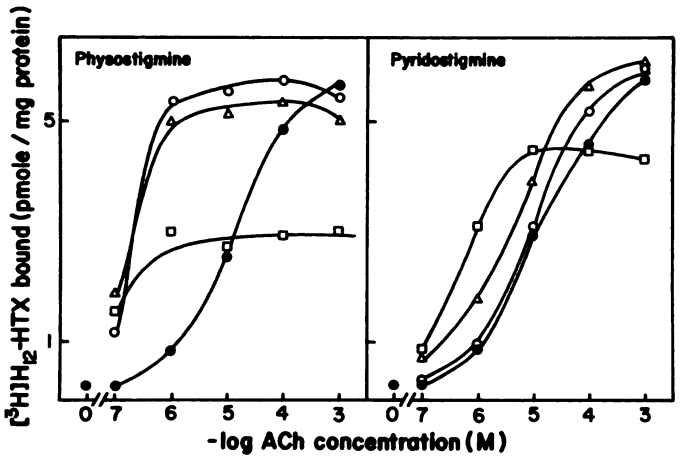

FIG. 1. *Effects of Phy and Pyr on the ACh-induced binding of [^{3}H]H_{12}-HTX to Torpedo ACh receptors*

DFP and phenylmethylsulfonyl fluoride were omitted from preparations of *Torpedo* membranes used in these experiments, and ACh-esterase activity was 5 mmol of ACh hydrolyzed/hr/mg of protein. The membranes (≃100 μg of protein containing 43 pmol of α-BGT binding sites) were preincubated for 30 min with buffer lacking (●) or containing the carbamate at 1 (○), 10 (△), or 100 μM (□); 2 nM [^{3}H]H_{12}-HTX was added and its binding was determined after 30 sec at 23°. In absence of ACh, the amount of [^{3}H]H_{12}-HTX bound at equilibrium was 7.5 pmol/mg of protein, and at 30 sec was 0.3 pmol/mg of protein. ACh and [^{3}H]H_{12}-HTX were added simultaneously. Data are means of three experiments with SD < 10%.

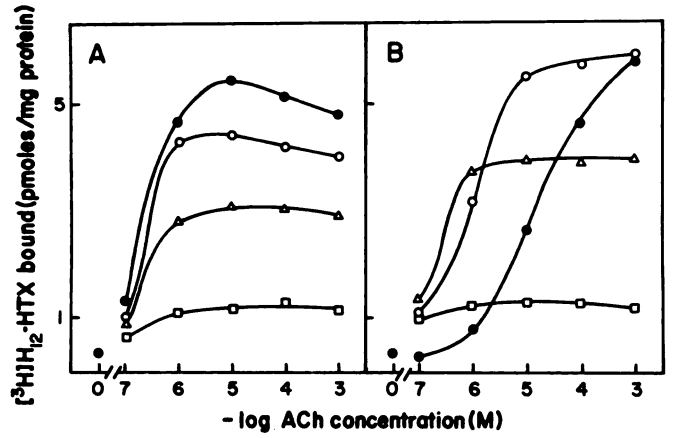

FIG. 2. *Effects of Neo on the ACh-induced binding of [^{3}H]H_{12}-HTX to Torpedo ACh receptors in membranes whose ACh-esterase activity was inhibited (i.e., prepared in the presence of 100 μM DFP) (A), and in membranes with ACh-esterase activity (i.e., prepared in the absence of DFP) (B)*

The membranes were preincubated with zero (●), 1 (○), 10 (△), or 100 μM (□) Neo for 30 min before exposure to 2 nM [^{3}H]H_{12}-HTX and ACh simultaneously for 30 sec at 23°. Data are means of three experiments with SD < 10%.

esterase activity. When *Torpedo* membranes were preexposed to 100 μM DFP for 30 min such that all ACh-esterase activity was inhibited prior to assay, low ACh concentrations (0.1 or 1 μM) were much more effective in stimulating [^{3}H]H_{12}-HTX binding. Even 1 μM Neo caused inhibition of the ACh potentiation of [^{3}H]H_{12}-HTX binding (Fig. 2A). Phy and Pyr were similarly more effective in inhibiting ACh stimulation of [^{3}H]H_{12}-HTX binding to *Torpedo* membranes that were pretreated with DFP.

The above effects of the carbamates were evidently complex, due to inhibition of cholinesterase activity and/or binding of ACh and [^{3}H]H_{12}-HTX to the ACh receptor. In order to understand the molecular mechanism of action of the carbamates on the nicotinic ACh receptor, contribution of cholinesterases was eliminated by irreversibly inhibiting them with 100 μM DFP for 30 min; then binding of [^{3}H]ACh and [^{3}H]H_{12}-HTX was studied. The three carbamates inhibited [^{3}H]ACh binding to the receptor sites competitively (Fig. 3) with the following K_i values: Neo, 25 μM; Phy, 200 μM; Pyr, 500 μM.

Since inhibition of [^{3}H]ACh binding may result from binding of agonists or antagonists to the receptor site, it was important to determine the effect of these carbamates on [^{3}H]H_{12}-HTX binding so as to distinguish between these two alternatives. If they enhance the initial rate of binding (i.e., in 30 sec), it would suggest that they act as agonists (15). The two quaternary carbamates Neo and Pyr stimulated [^{3}H]H_{12}-HTX binding in 30 sec though to a much lower degree than did the agonist carbamylcholine (Fig. 4). Phy showed no apparent stimulation of [^{3}H]H_{12}-HTX binding.

The carbamates had different effects on binding of [^{3}H]H_{12}-HTX under varying conditions. When the ACh-binding sites were preinhibited with *Naja* α-toxin (10 μM prior to the experiment and 0.1 μM after dilution during 120-min incubation with the drugs and [^{3}H]H_{12}-HTX) none of the carbamates up to 100 μM inhibited [^{3}H]H_{12}-HTX binding, unlike the channel drug amantadine (Fig. 5), which is a potent channel blocker (21). On the other hand, when free receptors (i.e., not pretreated with *Naja* α-toxin) were activated with 100 μM carbamylcholine, Phy was almost as potent as amantadine in inhibiting [^{3}H]H_{12}-HTX binding, with an IC_{50} of 40 μM, but Neo and Pyr had no significant effect up to 100 μM (Fig. 5). The inhibition by Phy was not due to the absence of a positive charge, since Phy methiodide had similar effects on [^{3}H]H_{12}-HTX binding (Fig. 6).

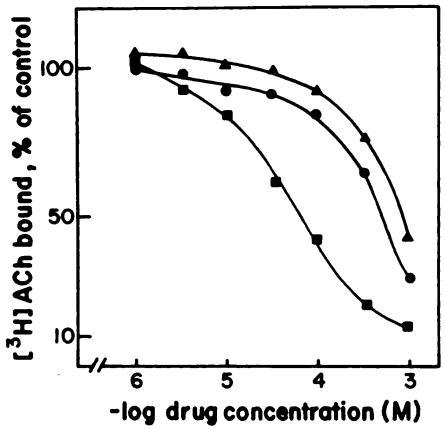

FIG. 3. *Inhibition of the binding of [^{3}H]ACh (0.1 μM) to Torpedo ACh receptors by carbamate anticholinesterases Phy (●), Pyr (▲), and Neo (■)*

Membranes were preexposed to 100 μM DFP for 30 min at 23° so as to inhibit all ACh-esterase activity, and the same concentration was present in the dialysis bath along with the carbamate and [^{3}H]ACh. Data are means of three experiments with SD < 10%.

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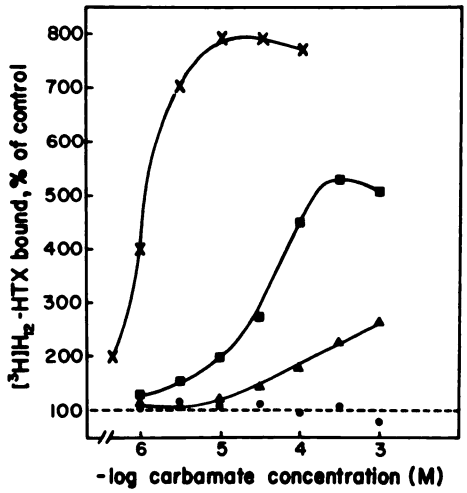

FIG. 4. *The effects of carbamylcholine (×), Neo (■), Pyr (▲), and Phy (●) on the binding of 2 nM [³H]H₁₂-HTX to nicotinic ACh receptors in Torpedo membranes*

The drug and [³H]H_{12}-HTX were added to the membranes simultaneously, and then binding was measured by filtration after 30 sec at 23°. The dashed line represents control level binding in the absence of drugs. Data are means of three experiments with SD < 10%.

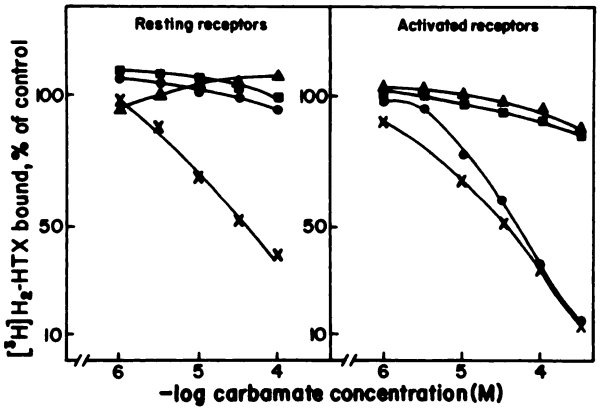

FIG. 5. *The effects of Phy (●), Pyr (▲), and Neo (■) on the binding of 2 nM [³H]H₁₂-HTX to the channel sites of the Torpedo ACh-receptor under two conditions*

Left, resting ACh receptors, where *Naja* α-neurotoxin (10 μM)-treated membranes (for 60 min) were incubated with carbamate and [³H]H_{12}-HTX for 120 min at 23° to reach equilibrium prior to measurement of binding. Right, activated ACh receptors, where membranes were incubated with 100 μM carbamylcholine, carbamate, and [³H]H_{12}-HTX for only 30 sec. Amount of [³H]H_{12}-HTX bound by control resting receptors at equilibrium was similar to that bound by control carbamylcholine-activated receptors in 30 sec. Standard deviation was <10%. The effect of the channel blocker amantadine (×) was included for comparison.

Many drugs that bind to the receptor's channel sites enhance receptor desensitization as measured by an increased affinity for agonists, which can be measured as increased potency of the agonist to inhibit ^{125}I-α-BGT binding to the receptor sites (23). Since the carbamates were suggested to bind not only to the channel site, but also to the receptor site, enhancement of inhibition of ^{125}I-α-BGT binding by the agonist could be due to their binding to either the receptor or channel site. Therefore, we compared the potencies of the carbamates in inhibiting ^{125}I-α-BGT binding when the membranes were exposed simultaneously to carbamate and ^{125}I-α-BGT for 40 sec before measurement of binding with their potencies when the membranes were preincubated for 30 min with the carbamate prior to addition of ^{125}I-α-BGT. Preincubation with the three carbamates potentiated their inhibition of ^{125}I-α-BGT binding in a manner analogous to the effect of receptor agonists (solid symbols in Fig. 7). Their IC_{50} values after 40 sec were 150, 500, and 3000 μM for Neo, Phy, and Pyr, respectively, but were reduced to 3, 70, and 600 μM when the membranes were preincubated with the carbamate for 30 min prior to exposure to ^{125}I-α-BGT and measurement of its binding after 40 sec.

Not only did the carbamates inhibit the initial rate of ^{125}I-α-BGT binding, but they also potentiated the inhibition by carbamylcholine of ^{125}I-α-BGT binding. Carbamylcholine inhibited ^{125}I-α-BGT binding with an IC_{50} of 15 μM in 40 sec and 0.5 μM when the receptors were preincubated with carbamylcholine for 30 min (data not shown). However, the presence of 1 μM Neo, 10 μM Phy, or 10 μM Pyr potentiated the effect of carbamylcholine in inhibiting ^{125}I-α-BGT binding and reduced its IC_{50} in

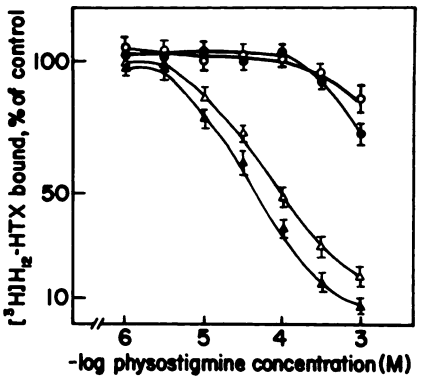

FIG. 6. *The effects of Phy (solid symbols) and Phy methiodide (open symbols) on the binding of 2 nM [³H]H₁₂-HTX to resting (●, ○) and activated (▲, △) Torpedo ACh receptors*

Conditions were as described in Fig. 5.

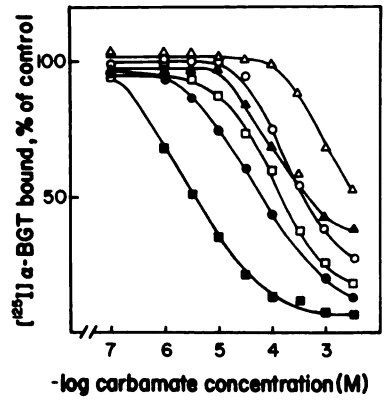

FIG. 7. *Inhibition of the binding of ^{125}I-α-BGT (5 nM) to Torpedo ACh receptors by Phy (○, ●), Pyr (△, ▲), and Neo (□, ■)*

Membranes were exposed to the carbamate either simultaneously with ^{125}I-α-BGT for 40 sec (open symbols) or preincubated with the carbamate for 30 min before exposure to ^{125}I-α-BGT for 40 sec at 23° (solid symbols) and then binding was measured. Preincubation increased the affinity of the ACh receptor for the three carbamates.

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40 sec from 15 μM to 1.5, 3, and 5 μM, respectively. Thus, the potencies of the three carbamates, alone or in the presence of carbamylcholine, in affecting the initial rate of α-BGT binding were Neo > Phy > Pyr.

DISCUSSION

The anticholinesterase action of the three carbamates causes potentiation of the stimulation of [^{3}H]H_{12}-HTX binding to the receptor's channel sites by low concentrations of ACh, which otherwise would be all hydrolyzed (Figs. 1 and 2). Phy is the most potent of the three carbamates in this respect. In addition, the carbamates interact directly with the nicotinic ACh receptor in different ways. They inhibit binding of [^{3}H]ACh (Fig. 3) and ^{125}I-α-BGT (Fig. 7) to the receptor sites with the following decreasing potencies: Neo > Phy > Pyr. Their binding to the receptor sites is also reflected indirectly in the inhibition by high concentrations (100 μM) of the stimulation of [^{3}H]H_{12}-HTX binding by ACh concentrations from 10 μM to 1 mM, depending on the carbamate (Figs. 1 and 2). It is also shown by the noncompetitive inhibition by Neo of ACh stimulation of [^{3}H]H_{12}-HTX binding, when all ACh-esterase is already inhibited (Fig. 2A), which is due to direct action on the ACh receptor molecule. Binding of Neo and Pyr to the receptor sites results in agonist-like stimulation of [^{3}H]H_{12}-HTX binding to the channel sites (Fig. 4), but they are less potent than carbamylcholine. Single nicotinic receptor channels opened by agonistic action of Pyr and Phy were shown in patch clamp studies of tissue-cultured muscle cells of neonatal rats (19, 24). The absence of effect of Phy in stimulating [^{3}H]H_{12}-HTX binding (Fig. 4) is not due to lack of binding to the receptor sites, for it does so at 100 μM and above (Fig. 3). Rather, stimulation of [^{3}H]H_{12}-HTX binding is probably there but is outweighed by Phy's strong direct inhibition of [^{3}H]H_{12}-HTX binding (Fig. 5), which is similar to that caused by amantadine.

An interesting characteristic of the action of these carbamates on the ACh receptor's channel is their inhibition of [^{3}H]H_{12}-HTX binding only to the receptor's activated[2] channel conformation with no effect up to 100 μM on the closed channel conformation in which the receptor is inhibited with *Naja* α-toxin (Fig. 5). Voltage and patch clamp studies clearly demonstrate that Phy acts as a blocker of the nicotinic receptor's open channel conformation (24), similar to the actions of gephyrotoxin (25) and depentylhistrionicotoxin (26). The higher potency of Phy compared to Neo or Pyr as activated channel blockers is not because Phy is a tertiary carbamate, but rather because of its structure since its quaternary analog Phy methiodide is similarly potent on binding of [^{3}H]H_{12}-HTX to the channel sites (Fig. 6). At 1 mM, they both cause little inhibition of binding of [^{3}H]H_{12}-HTX to the resting channel conformation, but significant inhibition at lower concentrations in the presence of carbamylcholine.

Binding of the carbamates to the receptor molecule induces time-dependent conformational changes, which are reflected in increased potency in inhibiting ^{125}I-α-BGT binding in 30 min (Fig. 7). This change is an accepted biochemical correlate of receptor desensitization, which is induced either by binding of agonists to the receptor sites (23) or by binding of most allosteric inhibitors to the channel sites (27, 28). The desensitizing action of the three carbamates is probably due to their binding to the receptor sites and action as agonists since this effect is produced by the three carbamates with IC_{50} between 3 and 600 μM, while only Phy inhibits [^{3}H]H_{12}-HTX binding to the activated receptor conformation (Fig. 5).

In comparing the effect of the three carbamates on the nicotinic ACh receptor, we find that they all bind to the receptor sites as shown by their inhibition of [^{3}H]ACh binding competitively (K_i = 25–500 μM) (Fig. 3) as well as ^{125}I-α-BGT binding (IC_{50} = 3–600 μM after preincubation) (Fig. 7), with the following order of decreasing potency: Neo > Phy > Pyr. Neo is more potent than Pyr as a partial agonist, and this is reflected in its more potent stimulation of [^{3}H]H_{12}-HTX binding to the channel sites (Fig. 4) and induction of receptor desensitization (Fig. 7). The agonist-like effect of Phy is overshadowed by its channel-blocking action (IC_{50} of 40 μM on [^{3}H]H_{12}-HTX binding). The use of high concentrations of these carbamates, particularly Phy, to inhibit ACh-esterase in studies of binding of [^{3}H]ACh to the ACh receptor (29) would influence the data obtained because of this direct effect of the carbamate on the ACh receptor. The three carbamates are much more potent ACh-esterase inhibitors (K_i = 6–120 nM; Phy > Neo > Pyr) than they are effectors of ACh receptor function. Pyr is the least potent of the three in its inhibition of ACh-esterase activity or [^{3}H]ACh binding, while Phy is the most potent in inhibiting the enzyme (K_i = 6 nM) and the channel site of the ACh receptor.

Neo and Pyr are used in therapy of myasthenia gravis, which is an autoimmune neuromuscular disease in which there are reduced numbers of ACh receptors in skeletal muscle (30). Their effectiveness is presumably due to their anticholinesterase action that increases the levels of ACh at the neuromuscular junction. The concentrations in blood of patients taking these two drugs may reach 0.29 and 1.34 μM, respectively (9, 31). At these concentrations, Neo and Pyr would be inhibiting a great deal of ACh-esterase since their estimated K_i values on *Torpedo* electric organ ACh-esterase are 20 and 120 nM, respectively (9). At <1 μM, Neo and Pyr do not bind significantly to the receptor sites (Fig. 3) or channel sites (Fig. 5) of the ACh receptor. But 30-min exposure to 1 μM Neo results in significant binding and induction of a desensitized receptor conformation (Fig. 7). Pyr has this effect at 100 μM, which may explain its preferred use as a therapeutic drug for myasthenia, even though its therapeutic dose is about 4 times that of Neo and it is about 4 times less potent in inhibiting ACh-esterase. However, recent findings in our laboratories indicate that Pyr

[2] The term "activated conformation" is used to denote the receptor conformations (i.e., open channel and desensitized) that occur when agonist is present and are dependent upon agonist concentration and length of exposure. Since our earliest measurement is after 30-sec incubation of the receptor with agonist, [^{3}H]H_{12}-HTX, and carbamate, the predominant conformation is probably a desensitized one.

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interacts with the receptor at concentrations as low as 1 μM.[3]

In summary, it is evident that the three carbamates in addition to being potent ACh-esterase inhibitors are also effectors of the nicotinic ACh receptor. They bind to the receptor, activate it as weak agonists, and cause receptor desensitization. Furthermore, Phy is a potent blocker of the receptor's activated channel conformation.

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[3] Unpublished results.

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