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The Muscarinic Antagonists Aprophen and Benactyzine Are Noncompetitive Inhibitors of the Nicotinic Acetylcholine Receptor

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SUMMARY

Certain muscarinic antagonists (e.g., atropine, aprophen, and benactyzine) are used as antidotes for the treatment of organophosphate poisoning. We have studied the interaction of aprophen and benactyzine, both aromatic esters of diethylaminoethanol, with nicotinic acetylcholine receptor (AChR) in BC3H-1 intact muscle cells and with receptor-enriched membranes of *Torpedo californica*. Aprophen and benactyzine diminish the maximal carbamylcholine-elicited sodium influx into muscle cells without shifting K_{act} (carbamylcholine concentration eliciting 50% of the maximal $^{22}Na^+$ influx). The concentration dependence for the inhibition of the initial rate of $^{22}Na^+$ influx by aprophen and benactyzine occurs at lower concentrations (K_{ant} = 3 and 50 μM, respectively) than those needed to inhibit the initial rate of [^{125}I]-α-bungarotoxin binding to the agonist/antagonist sites of the AChR (K_p = 83 and 800 μM, respectively). The effective concentration for atropine inhibition of AChR response (K_{ant} = 150 μM in BC3H-1 cells) is significantly higher than those obtained for aprophen and benactyzine. Both aprophen and benactyzine interact with the AChR in its desensitized state in BC3H-1 cells without further enhancing agonist affinity. Furthermore, these ligands do not alter the value of K_{des} (equilibrium concentration of agonist which diminishes 50% of the maximal receptor response) in BC3H-1 muscle cells. The affinity of aprophen and benactyzine for the allosterically coupled noncompetitive inhibitor site of the AChR in *Torpedo* was determined using [^{3}H]phencyclidine as a probe. Both compounds were found to preferentially associate with the high affinity (desensitized) state rather than the resting state of *Torpedo* AChR. There is a 14- to 23-fold increase in the affinity of aprophen and benactyzine for the AChR (K_D = 0.7 and 28.0 μM in the desensitized state compared to 16.4 and 384 μM in the resting state, respectively). These data indicate that aprophen and benactyzine binding are allosterically regulated by the agonist sites of *Torpedo* AChR. Thus, aprophen and benactyzine are effective noncompetitive inhibitors of the AChR at concentrations of 1–50 μM, in either *Torpedo* or mammalian AChR. These concentrations correspond very well with the blood level of these drugs found *in vivo* to produce a therapeutic response against organophosphate poisoning.

Certain muscarinic antagonists (e.g., *N*-methyl 4-piperidyl phenylcyclohexylglycolate) are capable of preventing or reversing OP- or carbamate-induced twitch augmentation in cat soleus muscle (1). However, these muscarinic antagonists have only limited or no effect on the twitch response of normal muscle. This protective action against carbamates and OP compounds was dose dependent but unrelated to their known antagonist action on muscarinic receptors (1). It was further noted that the action of antimuscarinic drugs on OP- or carbamate-induced effects at the neuromuscular junction is very similar to the action of some local anesthetics (e.g., lignocaine and procaine) and putative membrane-stabilizing drugs (e.g., quinine and triflupromazine). However, no definite mechanism was suggested because certain anticholinesterase agents have also been shown to decrease Ca^{2+} permeability in the nerve terminals (2). Furthermore, presynaptic muscarinic receptors may also be involved (2). More recently, it was shown that certain muscarinic antagonists (e.g., diphenyldioxolane and hyoscyamine) bind directly, at μM concentrations, to the ion channel of *Torpedo* AChR (3).

Aprophen (2′-(*N*,*N*-dietylaminoethyl)-2,2-diphenylpropionate) and benactyzine (2′-(*N*,*N*-diethylaminoethyl-2,2-diphenylglycolate) are two structurally related drugs (Fig. 1) exerting several pharmacological actions. Both drugs have been used as antidotes against poisoning by OP cholinesterase inhibitors (4, 5). Aprophen has been used clinically in humans as a spasmolytic drug (6) and also causes a transient hypotensive response in cats (7). Aprophen and benactyzine bind to muscarinic cholinergic receptors in various tissues with K_D values ranging from 1 nM (8) to 100 nM (9). However, these muscarinic

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ABBREVIATIONS: OP, organophosphate; ACh, acetylcholine; AChR acetylcholine receptor; nAChR, nicotinic acetylcholine receptor; dTC: *d*-tubocurarine hydrochloride; PCP phencyclidine [1-(1-phenylcyclohexyl)piperidine]; EDTA, ethylenediaminetetraacetate; HEPES, 4-(hydroxyethyl)-1-piperazineethanesulfonic acid; α-Bgt, α-bungarotoxin.

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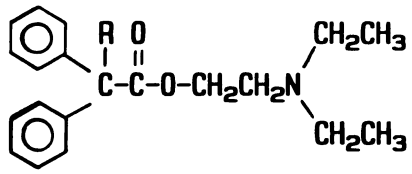

R = H, adiphenine; CH_3, aprophen;

OH, benactyzine; C_3H_7, proadifen

Fig. 1. Molecular structure of diethylaminoethanol esters of 1-alkyl 1,1-diphenylacetic acid.

antagonists also bind to the nAChR in rat brain and other tissues with K_i values of 0.1–1 mM (8). The K_i value for aprophen in rat brain (0.19 mM) was obtained by measuring its competitive effect on specific [^{3}H]ACh binding to AChR (8). It is likely, however, that aprophen and benactyzine also bind to a noncompetitive site on the AChR at much lower concentrations than those reported for their direct competition with ACh, as, indeed, was shown to occur with their congeners adiphenine (3) and proadifen (10).

A combination of a muscarinic antagonist (e.g., atropine), together with a quaternary oxime (e.g., 2-PAM) is commonly used as an antidotal treatment of OP poisoning (5). In especially severe OP poisoning the use of either aprophen or benactyzine as antidote has been shown to be remarkably efficient (11, 12). The role of the muscarinic antagonist is to prevent excess ACh from binding to and activating the muscarinic cholinergic receptor. During acute OP poisoning, however, high concentrations of ACh are also present in the synaptic cleft of the neuromuscular junction, which activate the AChR and cause continuous depolarization of the postjunctional membrane. It is conceivable, therefore, that addition of a nicotinic antagonist to the therapeutic mixture will have a palliative action during the acute phase of poisoning. Unfortunately, most classical nicotinic antagonists, such as dTC and gallamine, are extremely toxic and their use as antidotes is, therefore, limited (5).

In the present study, we have investigated the interaction of aromatic esters of diethylaminoethanol with AChR, in BC3H-1 intact muscle cells and in AChR-enriched membranes of *Torpedo californica*, focusing mainly on aprophen and benactyzine. The BC3H-1 muscle cells grow in monolayer culture and elaborate a homgeneous population of AChR at high density (1.5–2.5 pmol of α-toxin sites/mg of protein) (13); they thus permit parallel measurement of ligand binding- and agonist-induced ion permeability changes (14). Furthermore, we have measured the affinity of these diethylaminoethanol esters for the noncompetitive inhibitor site of *Torpedo* AChR using [^{3}H]PCP as a probe for equilibrium binding. Some of these data have been presented previously in abstract form (15).

Experimental Procedures

Materials. Carbamylcholine chloride, dTC, benactyzine hydrochloride, adiphenine hydrochloride, atropine sulfate, and dimethisoquin were obtained from Sigma. Aprophen hydrochloride was synthesized at the Israel Institute for Biological Research, Ness Ziona, Israel, according to the method of Zuagg and Horrom (16). [^{3}H]PCP (49.9 Ci/mmol) was purchased from New England Nuclear. Purified *Naja naja siamensis* toxin was a gift from Dr. David Johnson of the University of California, Riverside. The BC3H-1 cell line was subcloned by Ms. Karen Berger of the Department of Pharmacology, University of California, San Diego, from the original clone isolated at the Salk Institute (13). Dulbecco's modified Eagle's medium was obtained from Gibco. Trypsin and fetal calf serum were purchased either from Gibco or from Biological Industries, Kibbutz Beth Haemek, Israel. Plastic tissue culture flasks and dishes were manufactured by Nunc (Denmark) and Corning (U. S. A.), respectively. The radionuclides, iodine-125 and sodium-22, were purchased from the Radiochemical Centre, Amersham, England.

Cell cultures. BC3H-1 cells were propagated as described previously (14), except that the stock cells were dissociated in the presence of 0.1% trypsin (trypsin-EDTA solution B, Biological Industries). Petri dishes (35-mm) were seeded at a density of 4×10^3 cells/ml in 2 ml of medium containing 10% fetal calf serum in Dulbecco's modified Eagle's medium. Fresh medium was supplied on days 4 and 10, and experiments were usually performed at days 12–14.

Purification of Torpedo AChR. Receptor-enriched membrane fragments were isolated from the electric organ of *T. californica* by established procedures (17) with the following modification. Membranes were base extracted according to the method of Neubig *et al.* (18) in order to remove peripheral membrane proteins. Specific activities of the receptor preparations were determined by measuring the specific binding of [^{125}I]-α-toxin receptor to DEAE-cellulose filters (19). Specific activities for the receptor ranged from 1.0 to 2.2 nmol of α-toxin-binding sites/mg of protein.

Assays of agonist occupation and the agonist-stimulated permeability response. Kinetic assays to measure ligand competition with the initial rate of [^{125}I]-α-toxin binding or with the initial rate of carbamylcholine-mediated $^{22}Na^+$ influx were performed as described by Brown and Taylor (20). The following depolarizing buffer solution was used for both toxin binding and sodium flux assays: 5.4 mM NaCl, 140 mM KCl, 1.8 mM $CaCl_2$, 1.7 mM $MgSO_4$, 1.0 mM Na_2HPO_4, 5.5 mM glucose, 25 mM HEPES, acid and 0.06 mg/ml bovine serum albumin, adjusted to pH 7.4 with 1 N NaOH. All experiments were performed at 20–22°. Nonspecific binding of [^{125}I]-α-Bgt was determined in the presence of 1 μM unlabeled purified *Naja naja siamensis* toxin. The nonspecific $^{22}Na^+$ influx in the presence of maximally effective concentrations of carbamylcholine (0.6–1 mM) and 500 μM dTC was 15 ± 3% of the total influx obtained in the absence of dTC. The fractional bimolecular rate constants for binding of [^{125}I]-α-Bgt (k_T) and the relative permeability factor (k_G) were calculated according to the method of Sine and Taylor (14).

Concentration dependence for the drug inhibition of carbamylcholine-induced $^{22}Na^+$ influx. Influx was measured under the same conditions described by Brown and Taylor (20) except that the assay solution contained fixed concentrations of carbamylcholine (60 μM) and $^{22}Na^+$ (1 μCi/ml), together with the specified concentrations of the test drug. The preincubation solution contained only specified concentrations of the drug.

Analysis of carbamylcholine binding to desensitized AChR and carbamylcholine-mediated response of desensitized AChR. The binding of carbamylcholine to desensitized AChR was measured indirectly by monitoring its effect on the initial rate binding of [^{125}I]-α-Bgt. Cells were initially rinsed free of growth medium with 1 ml of depolarizing buffer and incubated with a solution containing specified concentrations of carbamylcholine with or without the drug under study. In order to measure toxin binding, the initial incubation solution was replaced by an identical solution supplemented with [^{125}I]-α-Bgt (12–15 nM), and the initial rate of binding was measured over a 30- to 60-sec interval. Initial rates of carbamylcholine-stimulated sodium influx were monitored under the same conditions employed for estimation of ligand occupation except that the assay solution contained 0.3 mM carbamylcholine and tracer $^{22}Na^+$ (1 μCi/ml), and the initial rate of Na^+ uptake was monitored for a 15-sec period.

Competitive inhibition of [^{3}H]PCP equilibrium binding to *Torpedo* receptor. The equilibrium binding of [^{3}H]PCP was measured as described by Heidmann *et al.* (21) with the following modifi-

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cations. AChR-enriched membranes were suspended in 100 mM NaCl, 10 mM sodium phosphate, pH 7.4, at a final concentration of 1 μM α-toxin sites. Binding of [^{3}H]PCP was determined under the following conditions: 1) membranes were not treated with cholinergic ligand before [^{3}H]PCP addition, 2) a 10-fold molar excess of α-toxin was incubated with AChR for 1 hr before addition of [^{3}H]PCP, and 3) 200 μM carbamylcholine was incubated with AChR for at least 10 min before the addition of [^{3}H]PCP. Nonspecific binding was determined from bound [^{3}H]PCP in the presence of 1 mM PCP. A 20 μM [^{3}H]PCP stock solution was prepared so that the ratio of the concentrations of radiolabeled to unlabeled PCP was 0.05. The final concentration of PCP in the samples was 1.0 μM. After addition of the noncompetitive inhibitor, [^{3}H]PCP was added and samples were incubated for at least 1 hr at 20° in Beckman polyallomer Airfuge tubes. Bound ligand was separated from free ligand by ultracentrifugation in a Beckman Airfuge for 5 min at 30 psi (160,000 × g). Duplicate 10-μl aliquots of the supernatant were removed or, alternatively, aliquots were withdrawn prior to centrifugation to determine total counts. The supernatant was then aspirated, and a 3-mm end of the tube containing the pellet was excised. The membrane pellets and supernatant aliquots were solubilized and counted in 5 ml of Biofluor (New England Nuclear) using LKB 1211 Reckbeta.

Data analysis. The fits to the experimental data obtained from BC3H-1 cells were carried out using Marquardt's nonlinear least squares analysis (20). Radioligand competition binding experiments with *Torpedo* AChR were analyzed by a weighted nonlinear regression computer program, LIGAND, for a single class of binding sites (22). In our analysis, we used the following K_D values for PCP: 0.4 μM in the presence of carbamylcholine and 2.0 μM in the presence of α-toxin or no cholinergic ligand. Data points are the average of duplicate samples. Figs. 2–5 (see Results) show the results of individual experiments, each of which was performed at least three times with either different membrane preparations or different platings of cells. Figs. 6 and 7 (see Results) represent average data obtained from three different experiments.

Results

Inhibition of concentration-dependent agonist activation of receptor permeability response. Fig. 2, A and B, shows that aprophen and benactyzine inhibit the carbamylcholine activation of the receptor-mediated permeability response in intact BC3H-1 cells. In these experiments, the cells were first exposed to a fixed concentration of inhibitor, followed by measurement of the carbamylcholine-elicited $^{22}Na^+$ permeability over a 15-sec period in the presence of the inhibitor. The fit to the experimental data presented in Fig. 2 was analyzed using Eq. 1:

$$k_G = k_G(\max)[L + K_{act})]^2 \quad (1)$$

where k_G is the observed rate of carbamylcholine-stimulated $^{22}Na^+$ influx, k_G(max) is the maximal influx rate observed at saturating carbamylcholine concentrations, L is the carbamylcholine concentration, and K_{act} is the carbamylcholine concentration producing half-maximal $^{22}Na^+$ influx. The best fit values for k_G(max) and K_{act} are summarized in Table 1. If aprophen and benactyzine were competitive inhibitors of the receptor, then a shift toward larger K_{act} values without any effect on the maximal permeability response would have been expected (20). However, Fig. 2A shows that 3 and 6 μM aprophen decrease the maximal response by 70.4% and 77.2%, respectively, whereas benactyzine diminishes k_G(max) by 30.8% and 40.5% at 10 and 20 μM, respectively (Fig. 2B; the effect of 10 μM benactyzine is not shown). Furthermore, K_{act} does not shift to larger values but shows a slight shift toward smaller K_{act} values in the presence of either aprophen or benactyzine (Table 1). The combined effects of the diminished maximal response [k_G(max)] and the absence of a shift to larger K_{act} values in the permeability response curve are consistent with a noncompetitive mechanism of inhibition by aprophen and benactyzine.

Comparison of aprophen and benactyzine inhibition of agonist-stimulated $^{22}Na^+$ permeability and the initial rates of [^{125}I]-α-toxin binding. Fig. 3, A and B, describes the concentration dependence of the effect of aprophen and benactyzine on initial rates of [^{125}I]-α-Bgt binding and carbamylcholine-stimulated $^{22}Na^+$ influx in BC3H-1 cells. The various parameters derived from these curves, together with data obtained for adiphenine and atropine, are summarized in Table 2. Whereas 83 μM aprophen is required to decrease the initial rate of toxin binding by 50% (K_p), only 3 μM aprophen is required to reduce the maximal agonist-induced permeability increase by 50% (K_{ant}) (Fig. 3A, Table 2). In five experiments, benactyzine displayed a biphasic pattern for its concentration-dependent inhibition of the agonist-induced permeability response (Fig. 3B). Two apparent K_{ant} values for benactyzine were derived: 0.6 and 50 μM (Table 2). Atropine inhibits the agonist-induced permeability response only at significantly higher concentrations (K_{ant} = 150 μM) than for aprophen and

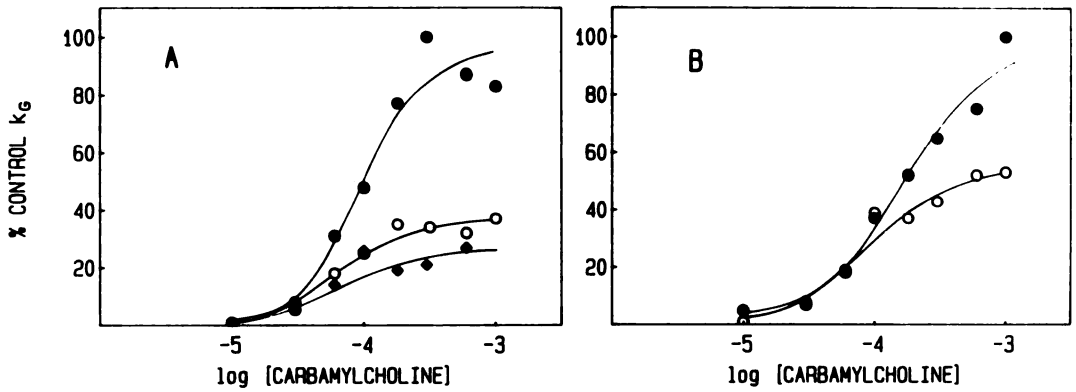

Fig. 2. Inhibition of carbamylcholine activation of AChR permeability response. Sets of cultures were equilibrated with a fixed concentration of noncompetitive inhibitors, following which the $^{22}Na^+$ permeability responses to the specified concentration of carbamylcholine in the presence of the conditioning concentrations of the inhibitors were measured over a 15-sec interval. Results are expressed as percentage of maximal rate in the absence of ligand. A. Aprophen: ●, no added aprophen; ○, 3 μM aprophen; ◆, 6 μM aprophen. B. Benactyzine: ●, no added benactyzine; ○, 20 μM benactyzine.

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TABLE 1

Effects of aprophen and benactyzine on activation parameters of carbamylcholine-elicited $^{22}Na^+$ permeability increase

Ligand	$k_G(max)$*	K_{act}*
	sec^{-1}	μM
Aprophen		
Control	0.0899 ± 0.0068	42.7 ± 12.6
3 μM	0.0266 ± 0.0019	30.0 ± 7.2
6 μM	0.0205 ± 0.0020	28.7 ± 10.0
Benactyzine		
Control	0.0656 ± 0.0043	81.7 ± 12.2
10 μM	0.0454 ± 0.0032	45.1 ± 9.9
20 μM	0.0390 ± 0.0008	44.9 ± 2.9

* Data from Fig. 2 were fit according to Eq. 1 ($k_G = k_G(max)[1/(L + K_{act})]^2$) by nonlinear regression analysis. The values are the mean ± standard error.

benactyzine, and the initial rate of α-toxin binding was inhibited by 50% at mM concentrations of atropine (Table 2).

Influence of aprophen and benactyzine on agonist occupation in the desensitized state. Prolonged exposure to AChR to agonist converts the receptor to a state which possesses a higher affinity for agonists together with a decreased responsiveness. This desensitization process is enhanced by certain noncompetitive inhibitors which convert the receptor to a state with higher agonist affinity. Many of the noncompetitive inhibitors that possess this capacity are synthetic and naturally derived amines such as local anesthetics (23), histrionicotoxin (24, 25), PCP (26), dimethisoquin (23), and certain antibiotics (20). In previous studies, agonist occupation in the desensitized state was correlated quantitatively with the state functions for receptor desensitization in the presence of noncompetitive inhibitors (27).

Binding of carbamylcholine to the receptor in BC3H-1 cells (determined by its competition with [^{125}I]-α-toxin) was measured in the absence and presence of either aprophen or benactyzine. Fig. 4 depicts the influence of these ligands on equilibrium receptor occupation by carbamylcholine. The control curve for carbamylcholine occupation of the desensitized receptor is similar to those obtained in the presence of either 3 μM aprophen or 8 μM benactyzine (Fig. 4). In contrast, PCP (8 μM) shifts the curve to lower carbamylcholine concentrations, as expected for this noncompetitive inhibitor (26).

Effect of aprophen and benactyzine on [^{3}H]PCP equilibrium binding to *Torpedo* AChR. [^{3}H]PCP was selected as a marker of the allosterically coupled noncompetitive inhibitor site because its stoichiometry of binding has been determined as 1/receptor monomer (28). Fig. 5 describes the concentration dependence for inhibition by aprophen (Fig. 5A) and benactyzine (Fig. 5B) of [^{3}H]PCP equilibrium binding to AChR-enriched membranes from *Torpedo*. The displacement of specifically bound [^{3}H]PCP from the noncompetitive site was measured in the absence or presence of either 200 μM carbamylcholine or 10 μM α-toxin. The K_D values for the competitive binding by aprophen and benactyzine with [^{3}H]PCP are presented in Table 3. The K_D values obtained for aprophen are 0.65 μM in the presence of carbamylcholine, 22.0 μM in the presence of α-toxin, and 16.4 μM in the control. The K_D values for benactyzine are 28.0 μM in the presence of carbamylcholine, 824 μM in the presence of α-toxin, and 384 μM for control.

Influence of aprophen and benactyzine on desensitization of nAChR. Fig. 6, A and B, depicts, respectively, the effects of aprophen and benactyzine on the state-function for desensitization in BC3H-1 cells. Aprophen at 3 μM and 6 μM decreases by 35% and 57%, respectively, the maximal permeability response, without lowering the effective concentration of carbamylcholine which elicits 50% of the maximal response (K_{des}). Benactyzine acts similarly but at slightly higher (8 μM) concentrations (Fig. 6B).

We have further characterized the interaction of aprophen with the desensitized receptor by measuring its effect in the presence of another noncompetitive inhibitor which is known to enhance desensitization. The rationale for this experiment is based on the assumption that two noncompetitive ligands, both having "local anesthetic-like" activity, will have an additive effect on desensitization, irrespective of their exact binding site on the receptor oligomer. For example, a typical additive effect is obtained with dimethisoquin and PCP as shown in Fig. 7A. Addition of the noncompetitive inhibitors dimethisoquin (3 μM) and PCP (3 μM) decreased the K_{des} value for carbamylcholine from 39.0 μM to 25.0 μM and 19.7 μM, respectively. Simultaneous addition of both ligands effected a further shift in K_{des} to 9.8 μM (Fig. 7A).

A similar experiment was conducted in which dimethisoquin was replaced by aprophen. Fig. 7B demonstrates that aprophen alone decreased the maximal response by 60% without decreas-

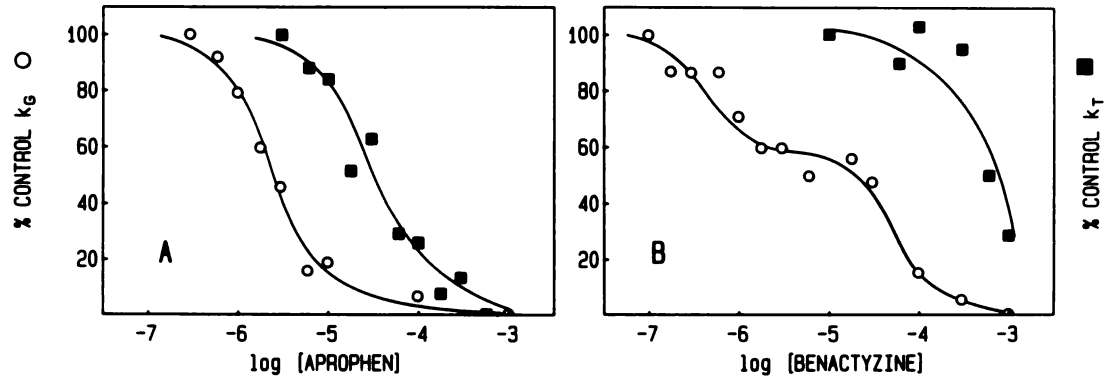

Fig. 3. Concentration-dependent inhibition of initial rates of [^{125}I]-α-Bgt binding- and carbamylcholine-stimulated $^{22}Na^+$ influx of BC3H-1 cells. Monolayer cultures of BC3H-1 cells were equilibrated with specified concentrations of aprophen (A) or benactyzine (B) for 30 min. Following this interval, the conditioning solution was replaced by solution containing the same concentration of inhibitor plus a fixed concentration of either [^{125}I]-α-toxin (12–15 nM) or carbamylcholine plus $^{22}Na^+$ (100 μM carbamylcholine and $^{22}Na^+$, 0.5–1.0 μCi/ml). The initial rates of toxin binding- and carbamylcholine-stimulated $^{22}Na^+$ influx were measured over 20- and 60-sec intervals, respectively. Results are expressed as percentage of control rate in the absence of inhibitor. ○, Inhibition of $^{22}Na^+$ influx; ●, inhibition of initial rate [^{125}I]-α-toxin binding.

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TABLE 2

Parameters for inhibitor competition with ^{125}I-α-toxin binding and inhibition of the $^{22}Na^+$ permeability increase by carbamylcholine in the active and desensitized states of AChR in BC3H-1 cells

Inhibitor	Active state		Desensitized state	
	K_{ant}[a]	K_p[b]	K_{des}[c]	[L][c]
	μM		μM	
Aprophen	2.8 ± 0.6	83 ± 20	18 ± 6	0
			25 ± 4	3
			22 ± 5	6
Benactyzine	0.6 ± 0.1	800 ± 100	15 ± 3	0
	50 ± 10		14 ± 2	4
			12 ± 4	8
Atropine	150 ± 20	~1000		
Adiphenine	4.0 ± 0.5	100 ± 20		

[a] K_{ant} is the antagonist concentration which diminishes 50% of maximal $^{22}Na^+$ influx obtained in the presence of 60 μM carbamylcholine in 15 sec.

[b] K_p is the antagonist concentration decreasing the initial rate of ^{125}I-α-Bgt binding by 50%.

[c] K_{des} is the equilibrium concentration of carbamylcholine which diminishes 50% of the maximal $^{22}Na^+$ influx (15 sec) elicited by 0.3 mM carbamylcholine with partially desensitized AChR in the absence or presence of inhibitor [L].

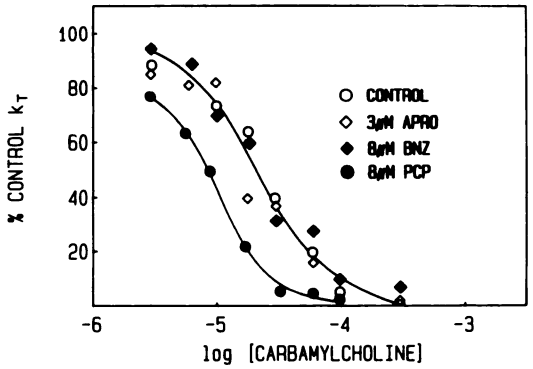

Fig. 4. Effects of noncompetitive inhibitors aprophen (*APRO*), benactyzine (*BNZ*), and PCP on the concentration dependence of agonist occupation of the receptor. Sets of BC3H-1 cultures were equilibrated with a fixed concentration of either aprophen (3 μM), benactyzine (8 μM), or PCP (8 μM) plus the specified concentrations of carbamylcholine for 30 min. After this interval, the conditioning solution was replaced by a fresh solution containing the same concentration of ligand and carbamylcholine, plus a fixed concentration of [^{125}I]-α-toxin. The binding of toxin to AChR was measured over a 60-sec interval. The concentration dependence of carbamylcholine inhibition of toxin binding rate reflects the affinity of the agonist for AChR. Results were expressed as percentage of toxin binding rate in the absence of carbamylcholine or ligands.

ing the K_{des} value, whereas PCP decreased the maximal response by 40% and also caused a leftward shift in the K_{des} value from 24 μM to 10 μM. In the presence of both PCP and aprophen there was an additional 15% decrease in the maximal response. No further decrease was observed, however, in the value of K_{des}.

Variability in absolute values of K_{des} obtained from two different sets of experiments (presented in Fig. 7, A and B) may arise from alterations in receptor sensitivity in different cell line passages. Due to this intrinsic variability, each experiment included an internal control which presents the carbamylcholine-induced desensitization obtained in the absence of ligand. The ratios of control K_{des} values and K_{des} in the presence of 3 μM PCP, obtained from two different sets of experiments, are: 39:20 = 1.95 (Fig. 7A) and 24:10 = 2.4 (Fig. 7B). The estimated error for the determination of K_{des} is ~20% (see Table 2). Therefore, the leftward shift in control K_{des} values elicited by 3 μM PCP in both sets of experiments is practically of the same magnitude.

Discussion

In the results presented above, it was demonstrated that the diethylaminoethanol esters, aprophen and benactyzine, noncompetitively block the carbamylcholine-elicited $^{22}Na^+$ permeability increase mediated by the AChR in the intact muscle cell. Furthermore, these muscarinic antagonists compete with [^{3}H]PCP, a noncompetitive inhibitor of the AChR in receptor-enriched membranes from *Torpedo*. Since ligand occupation and the functional response can be monitored simultaneously in the intact cell, the BC3H-1 cell line offers the opportunity to assess specific mechanisms of postjunctional activity of inhibitors in the absence of presynaptic constituents. In addition, the absence of cholinesterase activity in these cells[1] permits examination of the effect of drugs exclusively on receptor occupation and response.

K_{ant} for aprophen, determined by the inhibition of the functional response, is 30-fold smaller than the K_p value (Table 2). In addition, aprophen (3 μM) does not shift the K_{act} value but diminishes the maximal response by 70% (Table 1, Fig. 2A). These results are consistent with a noncompetitive mechanism for the antagonistic effect of aprophen on nAChR. The biphasic pattern obtained for the concentration dependence of benactyzine inhibition of $^{22}Na^+$ influx (Fig. 3B) suggests that benactyzine is identifying two putative intermediate states of receptor desensitization (21). The existence of two populations of receptor channels differing in their sensitivity to benactyzine is less likely since it was recently shown by Sine and Steinbach (29), using the patch clamp technique, that AChR in BC3H-1 cells is coupled to a homogeneous population of ion channels.

In the neuromuscular junction, in which rapid destruction of ACh by acetylcholinesterase prevails, the significance of desensitization in physiological response may be questioned. However, under conditions where, due to the presence of acetylcholinesterase inhibitors (e.g., OPs or carbamates) ACh is likely to reside in the synaptic cleft for significantly longer periods, substantial desensitization has been shown to occur following repetitive nerve stimulation (30). It is, therefore, of pharmacological importance to study the effect of noncompetitive antagonists on the occupation and state-function of desensitized AChR.

At least three distinct receptor states have been defined by both electrophysiological and ligand binding studies. These states are presented in the following scheme (31):

$$\begin{array}{ccccccc} L + RR & \overset{K_R/2}{\rightleftharpoons} & LRR & \overset{2K_R}{\rightleftharpoons} & LRRL & \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} & LR^*R^*L \\ M \uparrow\downarrow & & \uparrow\downarrow & & \uparrow\downarrow & & \\ L + R'R' & \overset{K_{R'}/2}{\rightleftharpoons} & LR'R' & \overset{2K_{R'}}{\rightleftharpoons} & LR'R'L & & \end{array}$$

where L represents the agonist, R the activatible or resting receptor state, R^* the active or open channel state, and R' the desensitized state or state with a refractory channel. RR designates the two agonist-binding sites on the receptor which give rise to potentially cooperative interactions. M, the allosteric constant, equals $R'R'/RR$ and defines the ratio of receptor in the desensitized to activatible states in the absence of agonist. K_R and $K_{R'}$ are the dissociation constants for the activatible and desensitized states. α and β are the closing and opening rates for the channel.

Noncompetitive antagonists may interact essentially by two

[1] G. Amitai and X. Futerman, unpublished observations.

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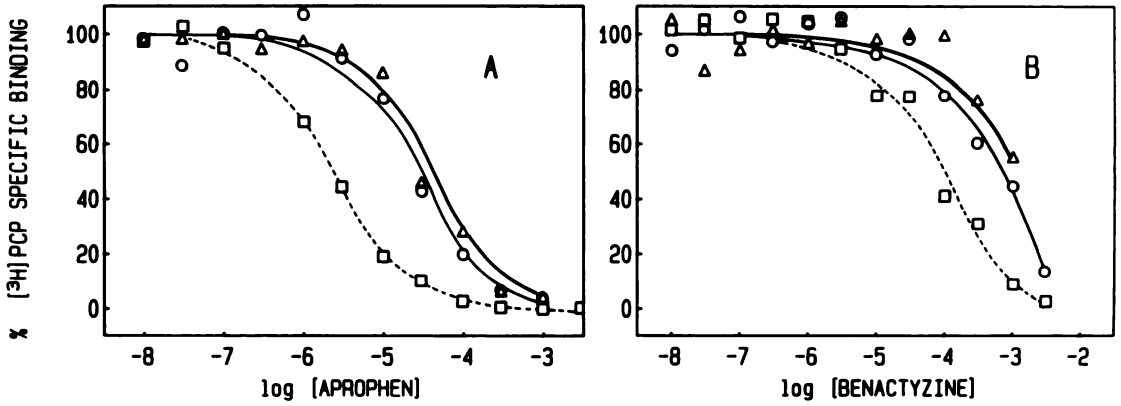

Fig. 5. Concentration dependence for aprophen or benactyzine inhibition of [^{3}H]PCP equilibrium binding to nAChR-enriched membranes from *Torpedo*. Binding of [^{3}H]PCP was determined under the following conditions: 1) membranes were not treated with any cholinergic ligand prior to [^{3}H]PCP addition; 2) a 10-fold molar excess of α-toxin was incubated with nAChR for 1 hr before addition of [^{3}H]PCP; or 3) AChR was incubated with 200 μM carbamylcholine for at least 10 min before addition of [^{3}H]PCP. Nonspecific binding was determined from bound [^{3}H]PCP in the presence of 1 mM unlabeled PCP. The final concentration of [^{3}H]PCP in the samples was 1.0 μM. Samples were incubated with the noncompetitive inhibitor for 1 hr at 20°. A. Binding in the presence of aprophen with the following pretreatment: □- - -□, 200 μM carbamylcholine; ○—○, control; △—△, 10 μM α-toxin. B. Binding in the presence of benactyzine with the following pretreatment: □- - -□, 200 μM carbamylcholine; ○—○, control; △—△, 10 μM α-toxin.

TABLE 3

Dissociation constants for the noncompetitive inhibitor site of the *Torpedo* AChR

Dissociation constants were determined under the following conditions: 1) membranes were not treated with cholinergic ligand before [^{3}H]PCP addition (K_D); 2) 200 μM carbamylcholine was incubated with AChR for at least 10 min before the addition of [^{3}H]PCP (K_{carb}); or 3) a 10-fold molar excess of α-toxin (10 μM) was incubated with AChR for 1 hr before the addition of [^{3}H]PCP (K_{tox}). Nonspecific binding was determined from bound [^{3}H]PCP in the presence of 1 mM PCP. The final concentration of [^{3}H]PCP in the samples was 1 μM. After addition of noncompetitive inhibitor, samples were incubated for at least 1 hr at 20°. The values of the dissociation constants are presented as mean ± standard error (n = 3).

Ligand	K_D	K_{carb}	K_{tox}	K_D/K_{carb}	K_{tox}/K_{carb}
		μM			
Aprophen	16.4 ± 2.5	0.7 ± 0.1	22.0 ± 9.7	23	31
Benactyzine	384 ± 59	28.0 ± 0.5	824 ± 186	14	29
Adiphenine	4[a]	5[a]		0.8	
Proadifen		0.1[b]			
Meproadifen	25[a]	0.3[a]		83	

[a] Data from Cohen *et al.* (31).
[b] One determination only.

mechanisms. First, a ligand may bind to a site allosteric to the agonist-antagonist site, reduce the time intervals between channel openings (32), and further stabilize the receptor conformation in a high affinity refractory state ($R'R'$). Such an inhibitor binds preferentially to the desensitized receptor state ($R'R'$) and increases the population of desensitized receptors, thus increasing effectively the allosteric constant M (31). Typical ligands which enhance desensitization are most local anesthetics, histrionicotoxin, dimethisoquin (23, 24), PCP (26), and certain antibiotics (20). Second, a ligand may interfere with the coupling of the ACh-binding site and the ion channel without stabilizing the high affinity state. The efficacy of certain noncompetitive ligands in their action on the receptor may vary according to the receptor sources. For example, tetracaine does not enhance conversion of *Torpedo* membrane AChR to the high affinity state (33), but stabilizes the high affinity state of the receptor in the intact BC3H-1 muscle cell (31).

Our data demonstrate that, in the intact mammalian muscle cell, aprophen and benactyzine do not enhance agonist affinity to the AChR, nor do they promote desensitization of the receptor response (cf. Figs. 4 and 6). However, in the *Torpedo* receptor preparation the mode of action of these ligands resem-

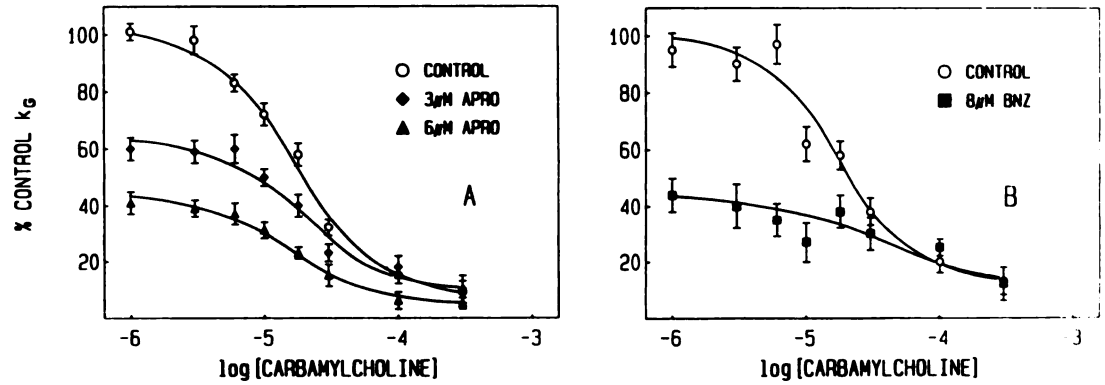

Fig. 6. Effects of aprophen or benactyzine on the concentration dependence of agonist-induced desensitization of receptor responsiveness. BC3H-1 cells were equilibrated with fixed concentrations of either aprophen or benactyzine plus the specified concentrations of carbamylcholine. Following the conditioning interval, the initial rate of $^{22}Na^+$ influx was measured in the presence of the conditioning concentration of aprophen or benactyzine plus a maximally stimulating concentration of carbamylcholine (0.3 mM). Results are expressed as percentage of maximal carbamylcholine-stimulated $^{22}Na^+$ influx in the absence of conditioning carbamylcholine or drug. Each *point* represents average data with the standard error obtained from three different experiments. A, aprophen (*APRO*); B, benactyzine (*BNZ*).

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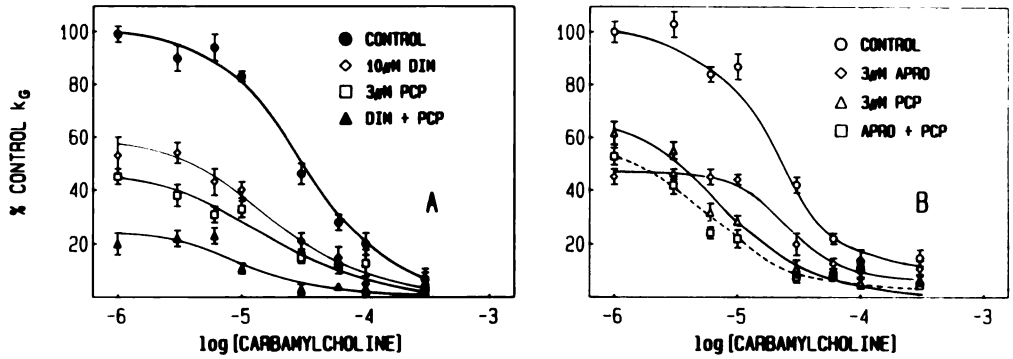

Fig. 7. Additive effect of various inhibitors on the concentration dependence of agonist-induced desensitization of receptor responsiveness. Experimental details are the same as those specified in Fig. 6. Each *point* represents average data with the standard error obtained from three different experiments. A. Additive effect of dimethisoquin (*DIM*) and PCP. ▲, 10 μM dimethisoquin + 3 μM PCP. B. Effect of PCP and aprophen (*APRO*). □, 3 μM aprophen plus 3 μM PCP.

bles that of local anesthetics, inasmuch as their affinity for their noncompetitive site is enhanced in the presence of 200 μM carbamylcholine (Fig. 5). This difference between the receptor systems may result from: 1) species differences intrinsic to differences in primary structure of the various receptor subunits (34), or 2) the receptor within the membrane environment of the BC3H-1 cell which, likely, is different from the receptor in purified *Torpedo* membranes.

Boyd and co-workers (10, 35) studied the effect of several diethylaminoethanol esters on the high affinity receptor state for agonists in *Torpedo*. They have noted that these ligands may either antagonize or enhance desensitization produced by local anesthetics or H_{12} histrionicotoxin. For instance, adiphenine (in the presence of H_{12} histrionicotoxin) does not convert the receptor to its high affinity state and antagonizes the conformational perturbation induced by dibucaine. In contrast, proadifen stabilizes a high affinity state identical to that stabilized by agonists at equilibrium (10). In the present report we have shown that aprophen does not decrease the K_{des} value for carbamylcholine beyond that induced by PCP in the intact muscle cell. This demonstrates that aprophen possesses equivalent affinity for both the resting (*RR*) and desensitized (*R'R'*) states.

During the acute phase of OP intoxication, ACh resides within the synaptic cleft of the neuromuscular junction for prolonged intervals which causes continuous depolarization of the postsynaptic membrane. Aprophen and benactyzine may diminish depolarization by blocking the receptor at a site distinct from the agonist-antagonist site. The use of these compounds also possesses the advantage of not having to compete directly with ~0.3 mM ACh (36) (K_D of ~1 μM) at the agonist sites (37). Thus, in terms of the framework presented in Scheme I, the number of available ACh-binding sites (*RR*) remains unchanged in the presence of aprophen or benactyzine, although fewer activatible receptors (*LRRL*) are being converted to their functional open channel state (*LR*R*L*). In contrast to a "local anesthetic-like" action, the above mode of action may favor the use of aprophen in a pretreatment schedule for the therapy of OP poisoning (11), since further enhancement of desensitization prior to intoxication (increase in *R'R'*) would diminish the receptor response.

It is noteworthy that the K_{ant} value obtained for inhibition by aprophen of the mammalian AChR *in vitro* corresponds very well with the plasma concentration (8–20 μM) (38) required to produce a therapeutic response against OP poisoning. Thus, the blocking effect of aprophen on the AChR may indeed contribute, together with its antimuscarinic activity (8), to its significant antidotal efficacy in especially severe cases of OP poisoning (11).

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