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Effects of Eserine and Neostigmine on the Interaction of α-Bungarotoxin with *Aplysia* Acetylcholine Receptors

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SUMMARY

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Binding of [^{125}I]α-bungarotoxin to acetylcholine receptors of ganglionic homogenate of the marine mollusc *Aplysia* is blocked by the anticholinesterases eserine (I_{50} = 4 μM) and neostigmine (I_{50} = 0.2 mM). The classical acetylcholine antagonist *d*-tubocurarine blocks with an I_{50} of 2 μM. Eserine (I_{50} = 3.2 μM) and neostigmine (I_{50} > 1 mM) also block toxin binding to a solubilized receptor preparation. In contrast to their relative potency in blocking toxin binding, neostigmine is a more potent inhibitor of *Aplysia* acetylcholinesterase (I_{50} = 14 nM) than is eserine (I_{50} = 250 nM). α-Bungarotoxin does not affect esterase activity or interfere with the ability of eserine to block the esterase. The response to acetylcholine recorded through intracellular microelectrodes is blocked by α-bungarotoxin. Neither eserine nor neostigmine blocks the acetylcholine response; rather, they prolong and increase it, as expected from their effects on the esterase. Eserine (0.1 mM) blocks the α-bungarotoxin inhibition of the physiological acetylcholine response. These results indicate that eserine and neostigmine block the binding of α-bungarotoxin by interacting with a site which is different from both the esterase and the cholinergic sites of the acetylcholine receptor.

INTRODUCTION

Acetylcholinesterase, the catabolic enzyme for acetylcholine, has structural similarities to and is found in high concentration near the acetylcholine receptor. At one time it was questioned whether the receptor and the esterase might be the same molecule (1, 2). However, techniques that have been developed for solubilization of the acetylcholine receptor have demonstrated a clear separation of esterase and receptor (3-5).

In this report we examine the action of drugs which affect the esterase and acetylcholine receptor in the nervous system of the marine mollusc *Aplysia*. Many *Aplysia* neurons are large and can be identified from one preparation to the next. All these large identified neurons are sensitive to acetylcholine (6) and all contain acetylcholinesterase (7). Furthermore, receptors for acetylcholine which cause both a voltage shift and a conductance increase are found over most of the neuronal surface, includ-

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ing the cell body. Although functional synapses are located only in the neuropile, the pharmacological characteristics of the acetylcholine receptors in nonsynaptic regions appear to be identical with those at synapses (8, 9).

α-Bungarotoxin blocks nicotinic acetylcholine responses in many preparations and binds specifically to the acetylcholine receptor (3, 5). In the *Aplysia* nervous system acetylcholine can elicit on different neurons three different responses (due to conductance increases to Na^+, Cl^-, and K^+, respectively) (9), and all are blocked by α-bungarotoxin (10). [^{125}I]α-Bungarotoxin binds to crude ganglionic homogenates with a saturation concentration of 24 pmoles of toxin per milligram of protein. The toxin binding in *Aplysia*, unlike many mammalian systems, is reversible and has a dissociation constant of 0.8 nM. More than 90% of the labeled toxin binding is blocked by *d*-tubocurarine. Other classical agonists and antagonists of acetylcholine also are effective in preventing toxin binding (10).

One surprising observation of our previous study (10) was that the anticholinesterases eserine (physostigmine) and neostigmine also blocked α-bungarotoxin binding to ganglionic homogenates. These drugs do not usually affect the responses to acetylcholine except through inhibition of the esterase. However, the ability of eserine and neostigmine to block binding of α-bungarotoxin in this preparation could be the result of binding of these drugs to any of three different sites. (a) Eserine and neostigmine bound to acetylcholinesterase could block α-bungarotoxin binding if the toxin also bound to the esterase or a nearby site. If this were the case, α-bungarotoxin might affect esterase activity. (b) Eserine and neostigmine in *Aplysia* might interact with the binding site for acetylcholine on the acetylcholine receptor complex. If so, they should block the physiological response to acetylcholine as well as inhibit toxin binding. (c) Eserine and neostigmine might bind to a site in addition to the esterase which is near or on the acetylcholine-receptor complex but distinct from the acetylcholine binding site. In this case the relative affinities of eserine and neostigmine for esterase inhibition and blockade of toxin binding would likely be different. These drugs might not block the physiological responses to acetylcholine, depending on the relationships of this second binding site to the acetylcholine binding site.

In this study we have attempted to determine how eserine and neostigmine interact with both acetylcholinesterase and the acetylcholine receptor by measuring their relative effectiveness as inhibitors of acetylcholinesterase and in blocking the binding of [^{125}I]α-bungarotoxin to a ganglionic homogenate and a Triton X-100 extract of this homogenate. In addition, we report on the effects of these drugs on the acetylcholine responses recorded by standard electrophysiological techniques.

MATERIALS AND METHODS

All experiments were performed on *Aplysia dactylomela*. Animals for electrophysiological experiments were obtained from Marine Specimens, Unlimited, Marathon, Fla., and were maintained in artificial seawater at 18° until used. Recording and iontophoresis procedures were those previously described (10).

Studies on acetylcholinesterases and binding of [^{125}I]α-bungarotoxin were performed on homogenates of the nervous tissue of animals collected at the Bermuda Biological Station. Crude ganglionic homogenates were prepared as previously described (10). A solubilized ganglionic preparation was made by extraction of the crude homogenate with 1% Triton X-100, centrifugation, and resuspension in 10 mM phosphate buffer as described by Schmidt and Raftery (11). Binding of [^{125}I]α-bungarotoxin to the crude ganglionic preparation was studied during the initial, linear portion of toxin binding. The homogenate was incubated first for 15 min in the presence of drug and then for 1 min with toxin in Millipore-filtered seawater. The techniques for collecting membrane-bound toxin on Millipore filters have also been described (10). Binding of labeled toxin to the solubilized ganglionic preparation was studied by collecting the receptor-toxin

complexes on DEAE-cellulose paper discs after the method of Schmidt and Raftery (11). The inhibition experiments were performed as described above for the ganglionic homogenate.

Acetylcholinesterase activity was assayed after the method of Ellman *et al.* (12), using acetylthiocholine as substrate. The absorbance of the reaction product of thiocholine and 5,5'-dithiobis(2-nitrobenzoic acid) was measured at 412 nm as a function of time on a Bausch & Lomb model Spec 20 spectrophotometer. The reaction was carried out at room temperature (22–24°) in a 3-ml reaction mixture containing homogenate, 5,5'-dithiobis(2-nitrobenzoic acid), acetylthiocholine, and inhibitors as noted, all in Tris$^+$-buffered, Millipore-filtered seawater, pH 8.0.

RESULTS

α-Bungarotoxin binding. Figure 1 illustrates the effects of various concentrations of *d*-tubocurarine, eserine, and neostigmine on the binding of [^{125}I]α-bungarotoxin to samples of ganglionic homogenate. All three drugs depressed α-bungarotoxin binding, even though eserine and neostigmine would not be expected to do so if they acted exclusively on the acetylcholinesterase. Eserine was very potent, blocking 50% of the binding at 5 μM and more than 90% at 1 mM. This is similar to the blocking ability of *d*-tubocararine (I_{50} = 2 μM), a classical acetylcholine receptor antagonist. Neostigmine was much less potent in blocking toxin binding, showing 50% inhibition at 1 mM.

Figure 2 shows the effects of eserine and neostigmine on [^{125}I]α-bungarotoxin binding to a solubilized ganglionic preparation. Both eserine and neostigmine were still effective in blocking toxin binding and showed the same relative potencies as in Fig. 1. Although 3.2 μM eserine inhibited 50% of toxin binding, only 80% of the total toxin binding was inhibited at 1 mM. Neostigmine showed lower affinity, blocking only 28% at 1 mM.

Acetylcholinesterase activity. In order to determine whether α-bungarotoxin binds to the acetylcholine receptor or to the esterase, experiments were performed to determine the interaction of toxin and eserine and/or neostigmine on acetylcholinesterase activity. Figure 3 shows inhibition of esterase activity in the presence of various concentrations of eserine and neostigmine. Unlike their relative efficacy in blocking toxin binding, neostigmine was more potent (I_{50} = 14 nM) than eserine (I_{50} = 250 nM) in blocking esterase activity. These findings support the hypothesis that the anticholinesterases affect binding of α-

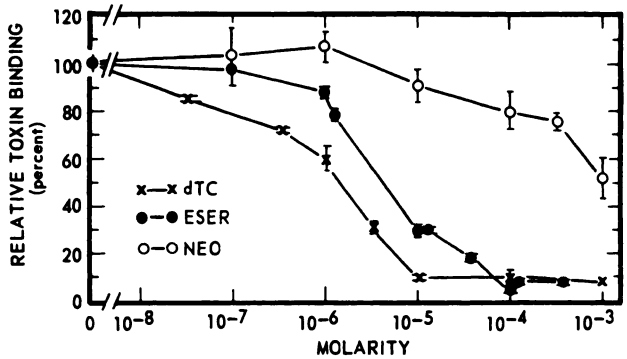

FIG. 1. *Inhibition of [^{125}I]α-bungarotoxin binding to ganglionic homogenate at various concentrations of neostigmine (NEO, o——o), eserine (ESER, ●——●), and d-tubocurarine (dTC, ×——×)*

The amount of binding in the absence of added drug was 4.1 pmoles/mg of protein. All drugs were incubated with the ganglionic homogenate for 15 min before addition of the toxin, and toxin binding was assayed after an additional 5 min of incubation. The final concentration of [^{125}I]α-bungarotoxin per assay was 3.4 nM. An aliquot of ganglionic homogenates containing 2.8 μg of protein was used in each assay. The concentrations of drugs necessary to inhibit 50% of [^{125}I]α-bungarotoxin binding were 1 mM neostigmine, 5.0 μM eserine, and 2.0 μM *d*-tubocararine. The data are plotted as means ± standard errors (n = 5).

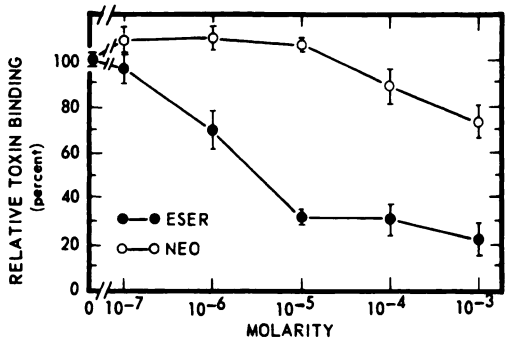

FIG. 2. *Inhibition of [^{125}I]α-bungarotoxin binding to solubilized ganglionic preparation by eserine (ESER, ●——●) and neostigmine (NEO, ○——○)*

The amount of binding in the absence of added drug was 57 pmoles/mg of protein. All drugs were incubated with the ganglionic homogenate for 15 min before addition of the toxin, and toxin binding was assayed after 1 min of additional incubation. The final concentration of [^{125}I]α-bungarotoxin per assay was 37 nM. An aliquot of solubilized preparation containing 16.5 μg of protein was used in each assay. The concentration of eserine necessary to inhibit 50% of [^{125}I]α-bungarotoxin binding was 3.1 μM; 1 mM neostigmine blocked only 28% of toxin binding. The data are plotted as means ± standard errors (n = 6).

bungarotoxin by binding to a site other than the acetylcholinesterase.

Table 1 shows the effects of eserine and/or α-bungarotoxin on acetylcholinesterase activity in the ganglionic homogenate. α-Bungarotoxin alone (0.7 μM) did not affect esterase activity. Moreover, concentrations of eserine sufficient to block either all or about 60% of the esterase activity, when tested alone, were similarly effective in the presence of the toxin. These results indicate that α-bungarotoxin does not affect the interaction of acetylcholine or eserine with the acetylcholinesterase, and consequently suggest that the toxin may not bind to esterase.

Electrophysiological studies. The physiological counterpart of this binding experiment is shown in Fig. 4. Iontophoretic application of acetylcholine to an unidentified neuron of the abdominal ganglion caused a hyperpolarizing response (A-1). The response was due to a conductance increase in Cl^-, since when Cl^- in the perfusing seawater was replaced with the impermeant anion acetate, the hyperpolarizing response was abolished and became slightly depolarizing consequent to the movement of the equilibrium potential for Cl^- in a depolarizing direction (A-4). Application of 3.6 μM α-bungarotoxin for 10 min greatly reduced the response to acetylcholine (A-2). Upon perfusion with high-Mg^{++} seawater for 30 min, the response returned to control levels (A-3).

The effects of eserine and its blockade of the action of α-bungarotoxin on the acetylcholine response are shown in Fig. 4B. The control response was considerably increased and prolonged after exposure to 0.1 mM eserine for 15 min (B-2). Similar effects of eserine were observed in experi-

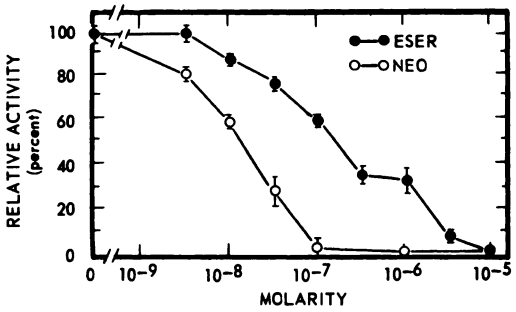

FIG. 3. *Inhibition of acetylcholinesterase activity of ganglionic homogenate by eserine (ESER, ●——●) and neostigmine (NEO, ○——○)*

Esterase activity in the absence of inhibitors was 0.148 absorbance unit/mg of protein per minute. An aliquot of ganglionic homogenate containing 35.8 μg of protein was used in each assay. The concentrations of drugs necessary to inhibit 50% of the esterase activity were 14 nM neostigmine and 250 nM eserine. Data points represent means ± standard errors (n = 4).

TABLE 1

Effects of eserine and α-bungarotoxin on acetylcholinesterase activity

Each assay contained 35.8 μg of protein. All drugs were incubated with the ganglionic homogenate for 15 min before the addition of acetylthiocholine. Values are means ± standard errors (n = 5).

Eserine	α-Bungarotoxin	Activity
μM	*μM*	*% control*
0	0	100 ± 3.6
0	0.7	102 ± 3.4
150	0	3 ± 1.2
150	0.8	0 ± 0.0
0.93	0	41 ± 2.5
0.93	0.7	41 ± 4.6

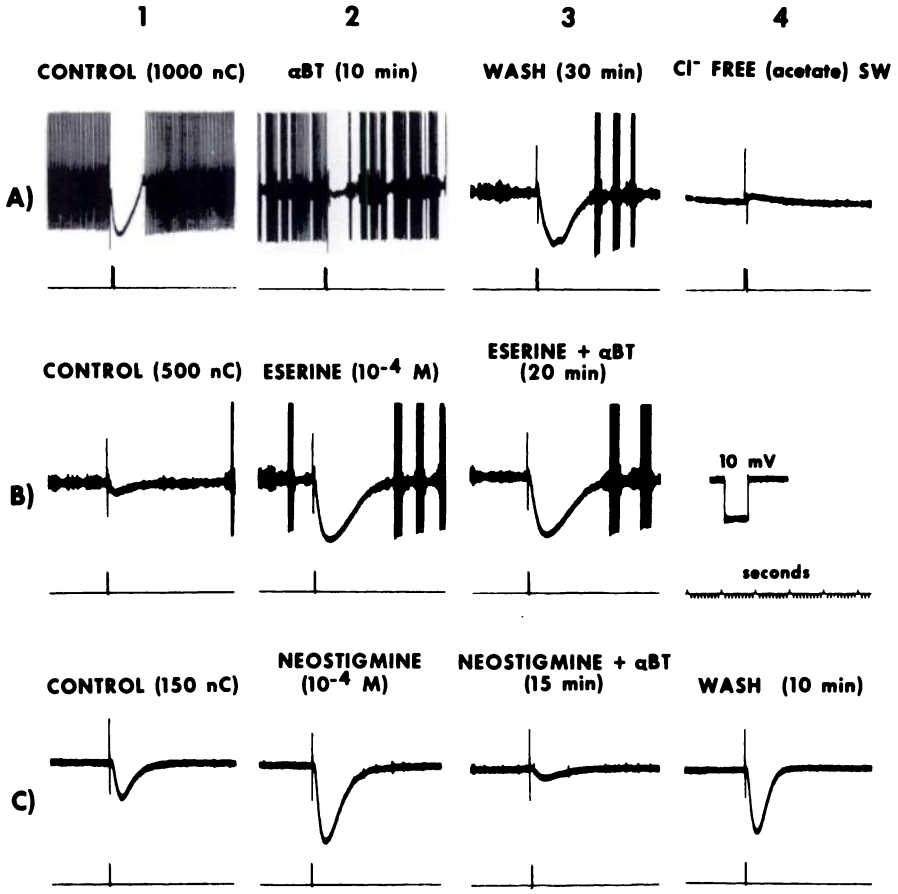

FIG. 4. *Interactions of α-bungarotoxin (αBT) with eserine and neostigmine on response to iontophoretic acetylcholine*

Responses were recorded from an unidentified neuron in the lower left quadrant of the abdominal ganglion of *Aplysia*. The intracellular recording of electrical activity is shown in trace A. The neuron was initially spontaneously active but became silent with time. Trace B indicates the duration of the iontophoretic pulse. The total charges passed were different for records A, B, and C, as indicated in column 1. The preparation was perfused with artificial seawater containing 100 mM added $MgSO_4$ to block spontaneous synaptic transmission, and drugs were added as indicated. All records were taken at 22°. A-2 shows the reduction of the response to 1000 nanocoulombs of acetylcholine observed after 10 min of perfusion with high-Mg^{++} seawater containing 2 mg/ml of bovine serum albumin and 3.6 μM α-bungarotoxin. A-3 shows the effect of the same amount of acetylcholine given after 30 min of washing with high-Mg^{++} seawater. A-4 shows the response 10 min after beginning perfusion with seawater (SW) in which all Cl^- was replaced by acetate. B-1 is a smaller control response before addition of eserine, while B-2 shows the effect of the same amount of acetylcholine 15 min after addition of 0.1 mM eserine. α-Bungarotoxin (3.6 μM) did not affect this response, even after 20 min. After the eserine effect had been at least partially reversed by washing, another control was taken (C-1) and neostigmine (0.1 mM) was added (C-2). The facilitated response in neostigmine was depressed by 3.6 μM α-bungarotoxin (C-3). After washing (C-4), the blockade was reversible.

ments on other neurons, which had acetylcholine responses due to conductance increases to either Na^+ or K^+. In no experiment did eserine depress any of the responses to iontophoretic acetylcholine. When α-bungarotoxin was added in the presence of eserine the toxin displayed no significant blocking effect, even after 20 min (B-3). Neostigmine (0.1 mM for 15 min) also increased the acetylcholine response (C-2), as expected by virtue of its known effects in blocking the acetylcholin-

esterase. However, when α-bungarotoxin was added in the presence of neostigmine, considerable inhibition of the electrophysiological response was observed (C-3), and this inhibition was reversed on washing with high-Mg^{++} seawater. Thus, at a concentration of 0.1 mM, eserine appears to be effective in preventing the inhibitory effects of α-bungarotoxin on the acetylcholine response, while neostigmine is considerably less effective. These results are consistent with the relative potencies of the two drugs in blocking binding of [^{125}I]α-bungarotoxin to both homogenized and solubilized ganglionic preparations.

DISCUSSION

These experiments show that in *Aplysia* nervous tissue there are at least two binding sites for the classical antiacetylcholinesterase agents, eserine and neostigmine. For both drugs the most sensitive of these sites is on the esterase, where neostigmine (I_{50} = 14 nM) is more potent than eserine (I_{50} = 250 nM). This value for eserine is to be compared with a K_i value of 190 nM for eserine action on erythrocyte acetylcholinesterase (13). However, both drugs also block [^{125}I]α-bungarotoxin binding, with eserine (I_{50} = 4 μM) more potent than neostigmine (I_{50} = 200 μM). The esterase site is distinct and remote from the acetylcholine receptor, as indicated by their separation in Triton X-100-solubilized preparations from eel and *Torpedo* electroplax (3–5) and the observation that in *Aplysia* α-bungarotoxin does not affect the esterase or block the inhibition of esterase by eserine. These results are in agreement with the studies of Chang and Su (14) on rat diaphragm, but do not accord with the conclusion of Štalc and Župančič (15), who reported binding of α-bungarotoxin to and activation of acetylcholinesterase of rat diaphragm.

The other eserine binding site appears to be intimately associated with but distinct from the acetylcholine binding site at the receptor. The close association is indicated by the efficacy of eserine in blocking [^{125}I]α-bungarotoxin binding. Since more than 90% of toxin binding to the preparation is inhibited by classical agonists and antagonists of the acetylcholine receptor (10), it appears very unlikely that any sizable fraction of toxin binding occurs to other than functional acetylcholine receptors. Furthermore, the interaction between this eserine site and toxin binding is not changed by receptor solubilization with Triton X-100, a procedure which in other preparations has been shown to separate the acetylcholine receptor and esterase (3–5).

Since eserine does not block responses to acetylcholine, this second eserine binding site must be distinct from both the acetylcholine binding site and the associated ionophore. In the electrophysiological studies the only effect on responses to acetylcholine of a concentration of eserine which blocks more than 90% of [^{125}I]α-bungarotoxin binding was an increase in both the amplitude and duration of the response, as expected from inhibition of the esterase. However, eserine protected against toxin blockade under these circumstances, while neostigmine was very much less effective, in contrast to their relative potencies against the esterase. Thus, while binding of eserine to this second site does not interfere with the binding of or response to acetylcholine, it can block binding of the relatively large α-bungarotoxin molecule.

Several recent developments in our understanding of the organization of receptors for acetylcholine and other putative neurotransmitters are relevant to these effects of eserine. Invertebrate neurons show at least three different ionic responses to a number of putative neurotransmitters, including serotonin (16), dopamine (17), glutamic acid (18), and γ-aminobutyric acid (19), as well as acetylcholine (8, 9).

Swann and Carpenter (17) have compared the different ionic responses to acetylcholine and dopamine and concluded that many properties of responses caused by permeability changes to the same ionic species were independent of which neurotransmitter receptor elicited the response. They also observed the same relative efficacy of compounds structurally similar to dopamine in eliciting Na^+, Cl^-, or K^+ conductance increase responses, and suggested that a single transmitter binding site could be coupled to any of at least three ionophores. They proposed that the

functional receptor unit, which they called the receptor complex, consists of at least two interchangeable moieties, the receptor binding site and ionophore. The present experiments suggest that the receptor complex may contain, in addition, another class of sites to which drugs may bind without necessarily affecting the normal transmitter action.

The three kinds of acetylcholine responses on *Aplysia* neurons are pharmacologically distinguishable in that only the Na^+ response is blocked by hexamethonium, only the Na^+ and Cl^- responses by curare, and only the K^+ response by tetraethylammonium (8, 9). However, α-bungarotoxin, under the conditions of our experiments, blocks all three responses; furthermore, the above three drugs each will block most of the toxin binding of the ganglionic preparation (10). The action of the toxin against all three responses is consistent with there being a single acetylcholine receptor. We have also observed linear competitive inhibition between α-bungarotoxin and the stable cholinergic agonist carbamylcholine, implying mutually exclusive binding, but hyperbolic competitive inhibition of hexamethonium, *d*-tubocurarine, tetraethylammonium, and eserine with α-bungarotoxin, implying that the binding sites of these substances are near but not identical with that for α-bungarotoxin (20).[3]

In another series of experiments (21)[3] we have determined the ability of hexamethonium to block [^{125}I]α-bungarotoxin binding to single, identified neurons which exhibit only one or two of the ionic responses to acetylcholine. Hexamethonium, which might be expected to block binding on only Na^+ neurons, was equally effective on all neurons, including those which show only Cl^- or K^+ responses. Thus, on these neurons, hexamethonium has an apparently anomalous binding site similar to that for eserine, in that it does not affect the response to acetylcholine and is detected only when binding of a large molecule like α-bungarotoxin is measured.

Kehoe, Sealock, and Bon (22) have recently reported a failure to confirm our observation that α-bungarotoxin blocks all three responses to acetylcholine; they found only the Cl^- response blocked, and only at very high toxin concentrations. This apparent discrepancy appears to result from a species difference (*Aplysia californica* binds α-bungarotoxin less well than *A. dactylomela*) and seasonal variability, in which toxin binding is great in the summer but very much smaller at other times.[4]

There have been reports of a depression of acetylcholine responses in other tissues by high concentrations of eserine. Quilliam and Strong (23) demonstrated in both rabbit heart and frog muscle that addition of eserine following treatment with the anticholinesterase diisopropyl fluorophosphate resulted in a decreased sensitivity to acetylcholine, while if eserine was given first, the addition of diisopropyl fluorophosphate caused no change. They suggested that both drugs block the esterase but that, in addition, eserine acts to block some acetylcholine receptors. Similar conclusions were reached by Fatt (24), who found that 0.1 mM eserine greatly decreased the depolarizing effect of acetylcholine at the frog neuromuscular junction. Levitan and Tauc (25) reported that in the mollusc *Navanax* eserine selectively blocks the depolarizing but not the hyperpolarizing responses to acetylcholine. Our results show that this is not the case for iontophoretic application of acetylcholine on *Aplysia* neurons. Tauc and Gerschenfeld (26), however, have reported depression of orthodromic synaptic potentials in *Aplysia* by eserine. This observation, if confirmed, is important, since it would be the only evidence that receptors for acetylcholine at natural synapses are different from those distributed over the neuronal soma.

O'Brien and Gilmour (27) have shown that 0.1 mM eserine will inhibit binding of labeled muscarone to acetylcholine receptors of electroplax. It seems likely that this is due to an interaction similar to the one we have studied between eserine and α-bungarotoxin. Raftery *et al.* (5) have found that eserine blocks some α-bungarotoxin

[3] W. Shain, L. A. Greene, and D. O. Carpenter, unpublished observations.

[4] W. Shain, P. Kebabian, and D. O. Carpenter, unpublished observations.

binding to electroplax acetylcholine receptors, but only at much higher concentrations than used in these experiments.

The simplest explanation of the present observations and these reports is that there exists in several preparations a binding site for eserine on the acetylcholine-receptor complex. In some, such as the frog neuromuscular junction and the cells depolarized by acetylcholine in *Navanax*, this site is sufficiently close to the acetylcholine binding site and/or its associated ionophore to block the acetylcholine response. In other preparations, such as *Aplysia*, this site does not block the binding and subsequent response to relatively small molecules like acetylcholine, but may very effectively block the binding of a relatively large molecule, such as α-bungarotoxin, to the acetylcholine receptor.

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