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Stereoselective L-[^{3}H]Quinuclidinyl Benzilate-binding Sites in Nervous Tissue of *Aplysia californica*: Evidence for Muscarinic Receptors[1]

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Abstract

The muscarinic antagonist L-[^{3}H]quinuclidinyl benzilate (L-[^{3}H]QNB) binds with a high affinity (K_d = 0.77 nM) to a single population of specific sites (B_{max} = 47 fmol/mg of protein) in nervous tissue of the gastropod mollusc, *Aplysia*. The specific L-[^{3}H]QNB binding is displaced stereoselectively by the enantiomers of benzetimide, dexetimide, and levetimide. The pharmacologically active enantiomer, dexetimide, is more potent than levetimide as an inhibitor of L-[^{3}H]QNB binding. Moreover, the muscarinic cholinergic ligands, scopolamine, atropine, oxotremorine, and pilocarpine are effective inhibitors of the specific L-[^{3}H]QNB binding, whereas nicotinic receptor antagonists, decamethonium and *d*-tubocurarine, are considerably less effective. These pharmacological characteristics of the L-[^{3}H]QNB-binding site provide evidence for classical muscarinic receptors in *Aplysia* nervous tissue. The physiological relevance of the dexetimide-displaceable L-[^{3}H]QNB-binding site was supported by the demonstration of the sensitivity of the specific binding to thermal denaturation. Specific binding of L-[^{3}H]QNB was also detected in nervous tissue of another marine gastropod, *Pleurobranchaea californica*. The characteristics of the *Aplysia* L-[^{3}H]QNB-binding site are in accordance with studies of numerous vertebrate and invertebrate tissues indicating that the muscarinic cholinergic receptor site has been highly conserved through evolution.

Cholinergic mechanisms occur ubiquitously in the animal kindgom (Walker et al., 1980). Muscarinic receptors are the major type of acetylcholine (ACh) receptor in mammalian central nervous systems and may have a role in a wide variety of processes including memory storage and learning (Banks and Russell, 1967; Deutsch, 1971; Squire and Davis, 1981) and Alzheimer's and Huntington's diseases (Davies and Maloney, 1976; Yamamura et al., 1979). In fact, ACh can "influence every physiological or behavioral response thus far examined" (Meyers, 1974). Because of such general action, ACh may be necessary in the production of normal behavior but may not itself be responsible for producing any particular behavior (Russell, 1982). Thus, cholinergic mechanisms in higher animals may have such varied expression that their relationship to the principles of neuronal integration underlying behavior may be difficult to assess.

Invertebrates, insofar as they have simpler behaviors and far fewer neurons than vertebrates, offer an opportunity for studies on the relationship between transmitter systems and the integrative properties of the nervous system. For our work, we have used gastropod molluscs, sea slugs, that exhibit simple and higher order behaviors, such as associative learning, which are amenable to analysis at the cellular level (see Kandel and Schwartz, 1982, Mpitsos and Lukowiak, 1985). However, ACh receptors in molluscs and other invertebrates have not fallen easily into Dale's nicotinic-muscarinic classification (Dale, 1914; Kehoe, 1972; Walker et al., 1980). Previous studies of gastropods have examined choline metabolism and the physiologic responses of neurons to ACh agonists and antagonists (e.g., Deutsch, 1971; Kehoe, 1972; Eisenstadt et al., 1973; Kehoe and Marder, 1976; Treistman and Schwartz, 1977; McCaman and Ono, 1982). These studies have demonstrated the importance of cholinergic mechanisms in molluscan nervous systems. However, the relationship of these results to mammalian muscarinic responses has been ambiguous because the physiological measurements often showed mixed nicotinic/muscarinic pharmacology.

Radioligand binding techniques can characterize receptors without some of the complications of physiological assays. From such studies we report specific high affinity binding sites for a potent and specific muscarinic antagonist, L-[^{3}H]quinuclidinyl benzilate (L-[^{3}H]QNB) (Yamamura and Snyder, 1974) in the nervous tissue of the gastropod *Aplysia californica*. The pharmacology of these binding sites resembles that of mammalian muscarinic receptors. The characteristics of such *Aplysia* muscarinic recognition sites are consistent with studies of fruit fly, rat, dog, monkey, and human tissue (Venter et al., 1984) showing that the muscarinic cholinergic receptor site has been highly conserved through evolution.

Materials and Methods

L-[^{3}H]QNB (30.2 Ci/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear (Boston, MA). The enantiomers of benzetimide, levetimide, and dexetimide, were generous gifts of Janssen Pharmaceutica (Belgium). All other drugs and reagents were obtained from commercial sources.

Approximately 650 *Aplysia californica* weighing 150 to 450 gm were obtained from Dr. Rim Fay of Pacific Biomarine (Venice, CA). We maintained them at 18°C and fed them twice weekly. *Aplysia* were anesthetized by injection of isotonic $MgCl_2$ into the body cavity and were opened by longitudinal incision along the entire midline of the dorsal body wall. The cerebral, pleural, pedal, visceral, and buccal ganglia were then dissected, trimmed of excess connective tissue, and cut so as to disrupt the encasing connective tissue capsule in order to facilitate separation of nervous tissue

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from sheath material. We made these dissections at 0°C and then stored the tissue at −80°C for less than 2 weeks prior to use.

Tissue fractionation. Subcellular fractionation and preparation of membranes were performed essentially as described (Dowdall and Whittaker, 1973) for the head ganglion of the squid. *Aplysia* ganglia were homogenized in 6 to 8 vol of ice-cold 0.7 M sucrose using a Potter-Elvehjem-type Telfon-glass homogenizer. This homogenate was filtered through four layers of cheese cloth to remove any remaining connective tissue sheaths. The filtered homogenate was then centrifuged at 1,000 × *g* for 10 min at 4°C. The pellet (P_1) was routinely discarded except in the subcellular distribution studies where it was assayed for L-[^{3}H]QNB binding. The resulting supernatant (S_1) was centrifuged at 17,500 × *g* for 1 hr at 4°C. This procedure resulted in a pellet (fraction P_2H) and, in addition, a pellicle (fraction P_2L) floating on the surface of the supernatant fluid. Due to the limited amount of nervous tissue which may be obtained from individual *Aplysia*, both the P_2L and P_2H fractions were routinely collected to maximize recovery. The supernatant (S_2) was discarded. Suspensions of osmotically disrupted synaptosomal membranes were prepared by the addition of 20 vol of ice-cold 50 mM Na-KPO_4 buffer (pH 7.4) to the combined P_2L and P_2H fractions. This suspension was homogenized using a Brinkman Polytron (20 sec, setting no. 5) and centrifuged at 35,000 × *g* for 30 min at 4°C. The resulting pellet was resuspended in 6 to 8 vol of ice-cold 50 mM Na-KPO_4 buffer and used immediately in the binding assays.

Receptor binding assay. The binding of L-[^{3}H]QNB to *Aplysia* membranes was determined using a previously described (Fields et al., 1978) rapid filtration assay with minor modifications. Aliquots of the membrane preparation (800 μl, 0.2 to 0.5 mg of protein) were incubated in triplicate with 100 μl of L-[^{3}H]QNB and 100 μl of buffer or competing drugs as indicated. The reaction was initiated by the addition of tissue and incubations were routinely carried out for 2 hr at 18°C, which was the ambient temperature at which the animals had been maintained. In preliminary studies it was determined that a 2-hr incubation time was sufficient for the specific binding of L-[^{3}H]QNB to reach equilibrium. The specific binding was found to remain constant from 90 min to 4 hr at 18°C. Incubations were terminated by rapid filtration under vacuum through glass fiber filters (Whatman GF/C; presoaked in 0.1% poly-L-lysine solution). The filters were rapidly washed with 4 × 4 ml of ice-cold Na-KPO_4 buffer. Nonspecific binding was defined as that remaining in the presence of 1 μM dexetimide. Radioactivity present on the filters was measured by liquid scintillation spectrometry at 43% efficiency. Saturation binding data were analyzed by Scatchard analysis (Scatchard, 1949). The protein content of membrane preparations was determined by the method of Lowry et al. (1951) following solubilization of the samples in 0.5 N NaOH.

Results

The specific binding of L-[^{3}H]QNB to *Aplysia* membrane fractions was linear over a membrane protein concentration range of 220 to 510 μm/assay tube. Under the assay conditions employed in these studies, the dexetimide (1 μM)-displaceable binding represented 30 to 50% of the total L-[^{3}H]QNB bound depending on the concentration of L-[^{3}H]QNB employed in the assay. The proportion of the total binding of L-[^{3}H]QNB which was displaceable was independent of the competing muscarinic ligands examined. Thus, similar percentages of the total binding were displaceable by 1 μM dexetimide and 100 μM oxotremorine (Fig. 1). Moreover, when 1 μM dexetimide and 100 μM oxotremorine were co-incubated in the nonspecific binding tubes, a lack of additivity of the resulting blanks was observed (Fig. 1). These results suggest the mutually exclusive binding of these two muscarinic ligands to a common population of binding sites labeled by L-[^{3}H]QNB (Burt, 1978). The possible physiological relevance of the dexetimide-displaceable L-[^{3}H]QNB binding to *Aplysia* nervous tissue was further tested by determining the binding in membranes that had been previously exposed to 60°C for 15 min. This heat-denaturing treatment reduced the specific component of L-[^{3}H]QNB binding by approximately 92% relative to that of control membrane preparations incubated at 18°C prior to the initiation of the binding reaction (Table I). These results are consistent with a proteinaceous binding site as has been shown for numerous other membrane receptors. In addition, parallel assay tubes incubated with aliquots of buffer rather than membranes demonstrated the dependence of the specific binding on the presence of *Aplysia* membranes. Thus, the presoaking of washed GF/C filters virtually eliminated displaceable binding of L-[^{3}H]QNB to filters in the absence of tissue.

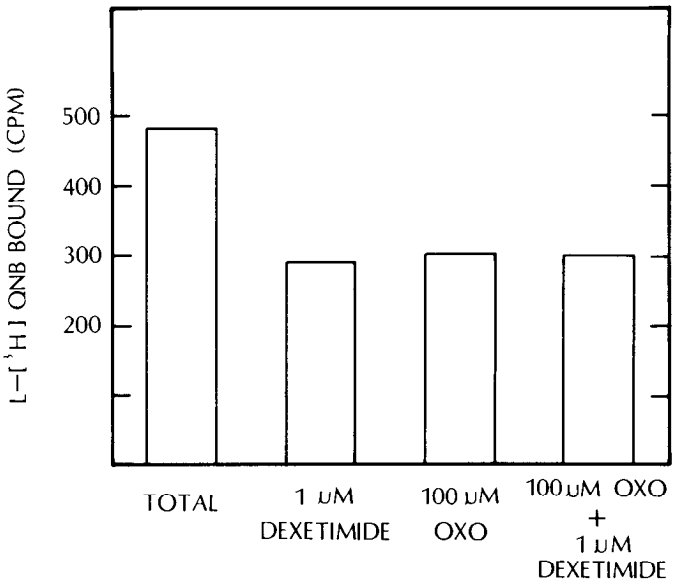

Figure 1. Lack of additivity of L-[^{3}H]QNB binding blanks using 1 μM dexetimide and 100 μM oxotremorine (*OXO*). The binding of 1.9 nM L-[^{3}H]QNB to *Aplysia* ganglia membranes was determined by incubating samples at 18°C for 2 hr prior to termination of the reaction by rapid filtration over glass-fiber filters (Whatman GF/C). The values are the means of three to six determinations which varied by less than 10%.

TABLE I

Binding of L-[^{3}H]QNB to Aplysia ganglia membranes under various assay conditions

Binding of 0.4 nM L-[^{3}H]QNB was determined by incubating the assay mixture as described under "Materials and Methods" at 18°C for 2 hr. Nonspecific binding was that occurring in the presence of 1 μM dexetimide. Values are mean ± SEM counts per minute. The heat-denatured tissue was prepared by heating the *Aplysia* ganglia membrane preparation in a 60°C water bath for 15 min. Samples were then cooled and assayed for L-[^{3}H]QNB binding as described under "Materials and Methods."

Assay Condition	Binding (cpm)		
	Total	Nonspecific	Specific
Control homogenate	239 ± 14	115 ± 6	124 ± 10
Heat-denatured homogenate	277 ± 21	267 ± 24	10 ± 4
Filter alone	77 ± 6	73 ± 8	4 ± 2

A saturation analysis of specific L-[^{3}H]QNB binding to *Aplysia* nervous tissue resembled a rectangular hyperbola, suggesting a single population of saturable high affinity binding sites (Fig. 2). The nonspecific binding measured in the presence of 1 μM dexetimide increased linearly with increasing concentrations of L-[^{3}H]QNB (data not shown). Scatchard analysis of saturation data indicated the presence of a single class of L-[^{3}H]QNB-binding sites with an apparent dissociation constant (K_d) of 0.77 ± 0.08 × 10^{-9} M and a maximum number (B_{max}) of 47.0 ± 2.1 fmol/mg of protein. We have also demonstrated stereoselective L-[^{3}H]QNB-binding sites in nervous tissue of the marine gastropod, *Pleurobranchaea californica*. Using a concentration of L-[^{3}H]QNB of 1.8 nM, nervous tissue of *Pleurobranchaea* bound 62 fmol/mg of protein.

The stereoselectivity and the pharmacological specificity of the L-[^{3}H]QNB-binding site in *Aplysia* nervous tissue were determined by comparing the potencies of various muscarinic and nicotinic drugs as inhibitors of specific L-[^{3}H]QNB binding (Table II). Logit-log plots of the inhibition of L-[^{3}H]QNB specific binding by the various cholinergic ligands employed are depicted in Figure 3. The Hill coefficients

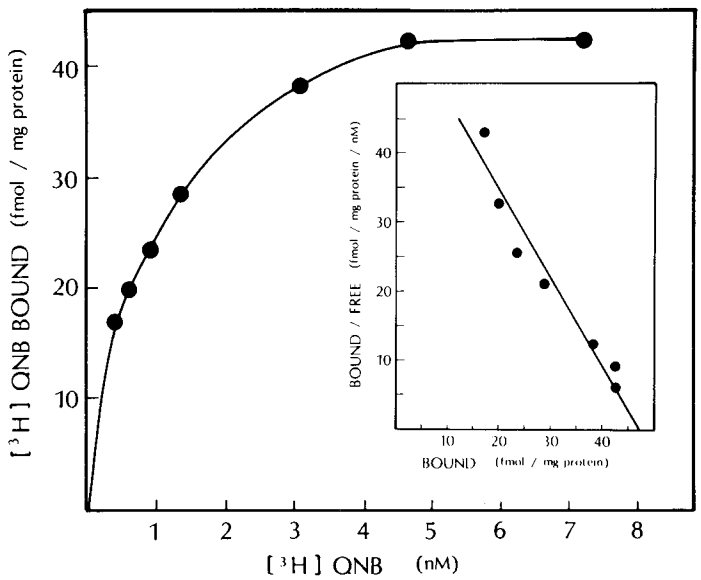

Figure 2. Equilibrium saturation binding of L-[^{3}H]QNB to *Aplysia* nervous tissue. Ganglia, dissected from 150 individual *Aplysia*, were pooled to provide a sufficient amount of tissue for the analysis. Values represent the means of triplicate determinations. Aliquots of membrane preparations (320 μg of protein) were incubated with various concentrations (0.4 to 7.2 nM) of L-[^{3}H]QNB. Specific binding was defined as the total bound minus the amount bound in the absence of 1 μM dexetimide. *Inset*, Scatchard plot of the saturation binding data. Dissociation constant K_d = 0.77 ± 0.08 nM; B_{max} = 47.0 ± 2.1 fmol/mg of protein. The r^2 value for the regression is 0.972.

TABLE II

Relative potencies of drugs in competing for L-[^{3}H]QNB binding to Aplysia ganglia membranes

IC_{50} is the concentration of drug displacing 50% of the specific binding of L-[^{3}H]QNB (1.4 nM) to *Aplysia* membranes. The IC_{50} values (±SE) were determined from the *x*-intercepts of modified Hill plots of competition experiments involving three to five concentrations of each drug. Specific binding was defined as the total binding minus that occurring in the presence of 1 μM dexetimide.

Drug	IC_{50} (μM)
Dexetimide	0.0126 ± 0.0013
Scopolamine	0.059 ± 0.0039
Atropine	0.126 ± 0.012
Levetimide	1.29 ± 0.14
Oxotremorine	2.46 ± 0.22
Pilocarpine	22.4 ± 1.55
Carbachol	323.6 ± 19.4
Decamethonium	>100
d-Tubocurarine	>100

derived from the slopes of these lines did not differ from unity for the antagonists, whereas the corresponding values for the agonists varied from 0.77 for oxotremorine to 0.48 for pilocarpine. This distinction between agonists and antagonists has been reported previously for L-[^{3}H]QNB binding to numerous tissues (e.g., Fields et al., 1978; Dunlap and Brown, 1983). The stereoselectivity of the L-[^{3}H]QNB-binding site in *Aplysia* membranes was apparent for the enantiomers of benzetimide. The pharmacologically active enantiomer of benzetimide, dexetimide, was more than 100 times more potent than the inactive isomer, levetimide, as an inhibitor of specific binding. Dexetimide was the most potent inhibitor of L-[^{3}H]QNB binding tested, with an IC_{50} value of 12.6 nM. The specific muscarinic antagonists scopolamine and atropine were also potent inhibitors, with IC_{50} values of 59 and 126 nM, respectively. Muscarinic agonists such as oxotremorine, pilocarpine, and carbachol were also effective inhibitors of L-[^{3}H]QNB binding, with a rank order potency consistent with an interaction with muscarinic receptors (Yamamura and Snyder, 1974). In contrast, the nicotinic antagonists decamethonium and *d*-tubocurarine produced less than 50% inhibition of specific binding when tested at concentrations as high as 100 μM. Similar pharmacological specificities have been demonstrated for muscarinic receptors in other invertebrates (Dudai and Ben-Barak, 1977; Haim et al., 1979; Jones and Sumikawa, 1981) as well as in numerous vertebrate species (Yamamura and Snyder, 1974; Fields et al., 1978; Jones and Sumikawa, 1981; Dunlap and Brown, 1983).

Discussion

The major findings of our present study are (*1*) that the nervous systems of the opisthobranch molluscs *Aplysia californica* and *Pleurobranchaea californica* contain specific L-[^{3}H]QNB-binding sites, and (*2*) that these binding sites have pharmacologic properties similar to those of muscarinic receptors in other animals including mammals. The observed specific binding of L-[^{3}H]QNB reported herein was characterized by its high affinity, saturability, and sensitivity to thermal denaturation. These characteristics support the physiological relevance of these L-[^{3}H]QNB-binding sites. Moreover, a lack of additivity of blanks was demonstrated using dexetimide and oxotremorine to define specific L-[^{3}H]QNB binding. Additional evidence that the site labeled by L-[^{3}H]QNB represents the pharmacologically relevant muscarinic receptor derives from the demonstration of the stereoselectivity of the observed binding. Pharmacologically, the muscarinic antagonist, dexetimide, has been shown to be much more potent than its stereoisomer, levetimide (Soudijn et al., 1973). In accordance with these results, dexetimide was shown to be more than 100 times more potent than levetimide as an inhibitor of the specific binding of L-[^{3}H]QNB to *Aplysia* nervous tissue.

The density of these specific L-[^{3}H]QNB-binding sites in *Aplysia* nervous tissue is relatively low compared to that of the central nervous system of vertebrates, but similar to that of other invertebrates such as *Drosophila* (Salvaterra and Foders, 1979). Thus, whereas we observed a B_{max} for L-[^{3}H]QNB sites of 47 fmol/mg of protein in the present study, a B_{max} of 65 fmol/mg of protein has been reported for L-[^{3}H]QNB sites in *Drosophila* (Haim et al., 1979). Moreover, the density of L-[^{3}H]QNB recognition sites reported herein is comparable to the densities of other neuroreceptors in molluscan species. The density of [^{3}H]lysergic acid diethylamide-binding sites, including both dopamine and serotonin receptors, in pooled *Aplysia* nervous tissue was reported to be 91 fmol/mg of protein (Drummond et al., 1980a). However, the density of [^{3}H]lysergic acid diethylamide-binding sites in the nervous system of the snail, *Helix pomatia*, is considerably higher (Drummond et al., 1980b). The density of a class of high affinity opiate receptors in nervous tissue of the bivalve mollusc, *Mytilus edulis*, is similar to our values for L-[^{3}H]QNB-binding sites in *Aplysia* (Kream et al., 1980). Other than the present study, we are unaware of any other investigations which have used direct biochemical radioligand binding techniques to quantify molluscan muscarinic receptors.

The pharmacologic profile of L-[^{3}H]QNB-binding sites in sea slugs is similar to that of other animals, and this is consistent with the high degree of conservation of the structure of the muscarinic receptor molecule through evolution (Venter, 1983; Venter et al., 1984). Other receptors, such as the α-adrenergic receptor, do not appear to be as highly conserved (Venter, 1983; Venter et al., 1984). Thus, the muscarinic receptor may be widely important in the functioning of nervous systems. However, it is unknown how the mechanisms which muscarinic receptors control participate in the regulation of activity of neurons and in neural integration. Lower animals, because of their relative simplicity, may be useful in studies inquiring into these principles.

Although our binding studies demonstrate evidence of cholinergic muscarinic receptors, physiologic studies of the responses of single molluscan neurons have not obtained results consistent with the classical nicotinic/muscarinic definition. For example, Kehoe (1972) demonstrated three different membrane responses to iontophoretic application of ACh in *Aplysia* neurons. Two of these, an excitatory

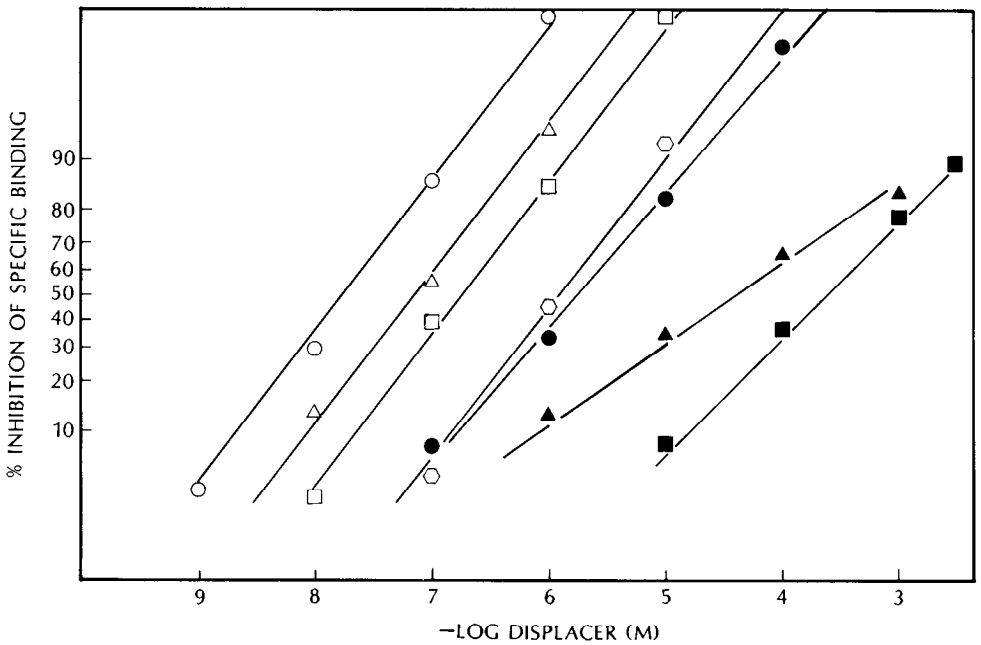

Figure 3. Logit-log plots of the inhibition of specific L-[^{3}H]QNB binding by various cholinergic ligands. Three to five concentrations of each compound were incubated with aliquots of *Aplysia* membranes and L-[^{3}H]QNB (1.4 nM) for 2 hr at 18°C. The percentage inhibition of specific L-[^{3}H]QNB binding was computed for each concentration of competing ligand and plotted as a function of drug concentration on logit-log paper. The cholinergic drugs employed were dexetimide (○), scopolamine (△), atropine (□), levetimide (⬡), oxotremorine (●), pilocarpine (▲), and carbachol (■).

potential and a fast inhibitory one, appeared to be nicotinic because they were sensitive to nicotinic agents, insensitive to muscarine, and were blocked with curare. Nonetheless, hexamethonium, a ganglionic nicotinic antagonist, did not block the fast inhibitory response, and atropine, a classical muscarinic antagonist, blocked the excitatory and, occasionally, the fast inhibitory responses. Thus, these ACh-induced responses exhibited mixed nicotinic and muscarinic properties. The third response to ACh, a slow inhibitory potential, was insensitive to hexamethonium, curare, and atropine and, therefore, exhibited neither nicotinic nor muscarinic properties.

Such problems of classification may arise in part from the nature of the neural tissue and from the experimental methods. Physiologic studies involve the receptor molecule, the ionophore, and other transduction components (Carpenter et al., 1977; McCaman and Ono, 1982). Binding studies examine only the recognition component. Moreover, while physiologic studies of molluscs have required micromolar to millimolar concentrations of cholinergic agents to produce measurable responses, our binding studies were facilitated by the use of potent muscarinic antagonist ligands with nanomolar affinities for the receptor. The reason for the required high concentrations in physiologic experimentation is presently unknown, but may involve the connective tissue surrounding the nervous system, and in marine molluscs, the high salt content of the hemolymph. Nonetheless, with high concentrations the pharmacologic agents may lose their specificity by binding to other receptor populations and by affecting the ionophore (McCaman and Ono, 1982). Thus, although we present evidence of muscarinic receptors in *Aplysia* and *Pleurobranchaea*, we are unable to assign a particular physiologic role to them based on previous physiologic studies. Although there are several known behavioral and physiological cholinergic systems in *Aplysia* (McCaman and Ono, 1982), these also do not easily fit the nicotinic/muscarinic distinction, and it is presently unclear which of these involve the apparent classical muscarinic receptors that we have demonstrated. Toward the goal of establishing a behavioral assay for muscarinic action, we have preliminary evidence showing that muscarinic agents, when injected into *Pleurobranchaea* in concentrations that do not appear to affect normal feeding, affect the ability of animals to learn food-aversion behavior.

The demonstration of muscarinic receptors in sea slugs opens the possibility of avoiding some of the problems encountered in physiologic studies. With highly specific muscarinic agonist and antagonist ligands, it should be possible to intercede into the processes of muscarinic connections without the more promiscuous actions of ACh. Moreover, with such potent and selective pharmacologic tools and with the recent availability of purified muscarinic receptors (Peterson et al., 1984), it may be possible to determine the locus of the muscarinic receptors in the nervous system and thereby guide neurophysiologic studies of the neuronal basis of behavior.

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