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Ligand Binding Properties of Muscarinic Acetylcholine Receptors in *Caenorhabditis elegans*

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Abstract: Ligand binding properties of muscarinic acetylcholine receptors (mAChRs) in the nematode *Caenorhabditis elegans* (*C. elegans*) were characterized by using filtration binding assays. Scatchard analysis using [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) showed that the dissociation constant (K_d) and the maximum binding value (B_{max}) were $3.3\pm 0.8\times10^{-10}$ M and 9.0 ± 1.1 fmol/mg protein, respectively. Binding competition experiments indicated that the affinities of *C. elegans* mAChRs to atropine, scopolamine, and oxotremorine were similar to those of mammalian mAChRs. Pirenzepine binding experiments revealed that the binding pattern of mAChRs in *C. elegans* closely resembled that of mAChRs in rat brain, suggesting that the receptors consist primarily of M1 subtype. The affinity of mAChRs for oxotremorine was significantly affected by guanylylimidodiphosphate (Gpp(NH)p), a nonhydrolyzable GTP analog, suggesting that mAChRs in *C. elegans* might be coupled to G proteins. The data presented here indicate the possibility that *C. elegans* provides a living animal model to study the action mode of the muscarinic cholinergic system.
Key words: *Caenorhabditis elegans*, G protein, ligand binding, muscarinic acetylcholine receptor, pirenzepine.

Muscarinic acetylcholine receptors (mAChRs) are members of a superfamily of G protein-coupled receptors that have a predicted structure of seven transmembrane domains (Dohlman *et al.*, 1991). mAChRs regulate functions of the organs of the autonomic nervous system (*e.g.*, beating of heart, contraction of smooth muscle, and secretion from exocrine glands). In addition, mAChRs are thought to be the major type of cholinergic receptors in the central nervous system and play important roles in learning, memory, and arousal (Nathanson, 1987).

Genomic and cDNA clones encoding mAChRs have been isolated from porcine (Kubo *et al.*, 1986), rat (Bonner *et al.*, 1987), human (Bonner *et al.*, 1987), mouse (Shapiro *et al.*, 1988), *Drosophila* (Shapiro *et al.*, 1989), and chicken (Tietje *et al.*, 1990). Five subtypes of mAChRs (m1−m5) have been genetically classified and characterized. The m1, m3, and m5 subtypes activate phospholipase C (Peralta *et al.*, 1988) and phospholipase A_2 (Conklin *et al.*, 1988), and stimulate mitogenesis (Gutkind *et al.*, 1991). The m2 and m4 subtypes inhibit adenylyl cyclase (Peralta *et al.*, 1988), weakly stimulate phosphatidylinositol turnover (Stein *et al.*, 1988), and activate inwardly rectifying K^+ channels (Yatani *et al.*, 1987).

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mAChRs have been pharmacologically classified (M1 to M3 subtypes) on the basis of their differences in the affinity for muscarinic ligands (Doods *et al.*, 1987). Pirenzepine, an M1-specific ligand, showed a high affinity binding to the muscarinic binding sites in rat brain and a low affinity binding to those in rat heart (Hammer *et al.*, 1980). Pirenzepine was used to distinguish M1 (high affinity), M2 (low affinity), and M3 (moderate affinity) subtypes (Doods *et al.*, 1987).

Previous efforts to investigate the muscarinic cholinergic system in higher vertebrates have produced a good amount of information on the biochemical aspect of the system, but the knowledge on the behavioral aspect of the system is still limited mainly due to the enormous complexity of the nervous system in the higher vertebrates. We chose the nematode *Caenorhabditis elegans* (*C. elegans*) as a model system to study the action mechanism of the muscarinic cholinergic system because *C. elegans* possesses a relatively simple nervous system (302 neurons in hermaphrodites and 381 neurons in males). Its small size (adults 1 mm), short life cycle (3 days), small number of linkage groups (a pair of X chromosomes and five pairs of autosomes), small genome size (8×10^7 base pairs), and ability of self-fertilization greatly facilitate genetic analysis (Kenyon, 1988). As the cell lineage from embryo to adult is completely established and individual cells can be observed with Nomarski optics, the developmental fate

of each neuron can be conveniently monitored. Furthermore, germline transformation technique (Mello *et al.*, 1991) and the use of green fluorescent protein (Chalfie *et al.*, 1994) provide a unique opportunity to examine the expression of neuron-specific genes at the cellular level in intact, living animals. These attractive features make *C. elegans* a popular experimental animal to investigate the molecular mechanisms underlying signal transmission in the nervous system. In this report, ligand binding properties of mAChRs in *C. elegans* were investigated and compared with those of mammalian mAChRs.

Materials and Methods

Materials

[^{3}H]*N*-methylscopolamine ([^{3}H]NMS) (specific activity, 79.5 Ci/mmol) was purchased from New England Nuclear (Boston, USA). Guanylylimidodiphosphate (Gpp(NH)p) was a product of Boehringer Mannheim (Indianapolis, USA). Atropine, scopolamine, pirenzepine, carbachol, oxotremorine, and other chemicals were obtained from Sigma (St. Louis, USA).

Preparation of crude homogenate of *C. elegans*

C. elegans (N2 strain) was grown in a shaking incubator in S medium (100 mM NaCl, 50 mM KPO_4, 13 μM cholesterol, 10 mM potassium citrate, 3 mM $CaCl_2$, 3 mM $MgSO_4$, 50 μM EDTA, 25 μM $FeSO_4$, 10 μM $MnCl_2$, 10 μM $ZnSO_4$, 1 μM $CuSO_4$) at 25℃ for 5~10 days. *E. coli* OP50 was added as a food every 2 days. The medium was removed using an aspirator, and *C. elegans* was collected by centrifugation at 2,000 rpm for 2 min using a tabletop centrifuge (Vision VS-5000). The pellet was washed with M9 buffer (90 mM Na_2HPO_4, 20 mM KH_2PO_4, 8.6 mM NaCl, 18 mM NH_4Cl) and centrifuged at 2,000 rpm for 2 min. Remaining OP50 was separated by centrifugation at 2,000 rpm for 2 min on a 30% sucrose solution. *C. elegans* was homogenized by freeze-powdering in liquid nitrogen and resuspended in 20 mM sodium phosphate buffer (pH 7.4). The sample was stored at −70℃ until use. Membrane samples of rat brain and heart were prepared as described by Min *et al.* (1994).

Receptor binding assays

For saturation studies, crude homogenates (0.5 mg to 1 mg protein/assay tube) were incubated with increasing concentrations (0.02~2 nM) of [^{3}H]NMS at 20℃ for 40 min. For competitive inhibition studies, crude homogenates were incubated with 0.2 nM [^{3}H]NMS in the presence of unlabeled muscarinic ligands. To investigate the effect of Gpp(NH)p on ligand binding affinity, crude homogenates were pretreated with Gpp(NH)p for 5 min before the addition of [^{3}H]NMS and muscarinic ligands. The binding reactions were terminated by rapid filtration under vacuum through GF/C glass fiber filters (Whatman Inc.). Filters were washed twice with 5 ml of ice-cold 20 mM sodium phosphate buffer (pH 7.4) and dried at room temperature overnight. Each filter was transferred to a vial containing 4.5 ml of scintillation cocktail (toluene 1 L, 2,5-diphenyloxazole (PPO) 4 g, 1,4-bis[2-(5-phenyloxazoly)] benzene (POPOP) 0.1 g). Radioactivity was determined in a Beckman LS-5000TA liquid scintillation counter. Protein content was determined according to the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Nonspecific binding that was determined by adding 10 μM atropine was typically less than 10% of the total binding.

Data analyses

Data from saturation studies were fitted by using Scatchard analysis to obtain the dissociation constant (K_d) and the total number of binding sites (B_{max}). To get IC_{50} value that is the concentration of drug causing 50% inhibition of the specific radioligand-receptor complex formation, data from competitive inhibition studies were fitted to the equation $Y=100/(1+(X/IC_{50})^{nH})$ for a one-site model, where X is the concentration of a competing ligand, nH is the Hill coefficient, and Y is the fractional occupancy of all the binding sites. The dissociation constant for the inhibitor (K_i) was determined by the equation $K_i=IC_{50}/(1+L/K_d)$ according to the method of Cheng and Prusoff (1973), where L is the concentration of the radioligand. Scatchard plot and competitive inhibition curves were analyzed by iterative linear and nonlinear least-squares regression, respectively, using the SIGMA PLOT® on an IBM-PC. In this study, a one-site model is better fitted than a two-site model in the analysis of agonist binding data. Paired Student's *t*-test was performed using the MINITAB® on an IBM-PC.

Results and Discussion

Scatchard analysis

To investigate ligand binding properties of mAChRs from crude homogenates of *C. elegans*, we carried out filter binding assays using [^{3}H]NMS as a ligand. Specific binding saturated at 1 nM [^{3}H]NMS (Fig. 1). Scatchard analysis indicated that the dissociation constant (K_d) for [^{3}H]NMS binding to mAChRs and the total binding sites (B_{max}) were 0.33± 0.08 nM and 9.0± 1.1 fmol/mg protein (mean± SE, n=5), respectively (Fig. 1, *inset*). These results are consistent with the data reported

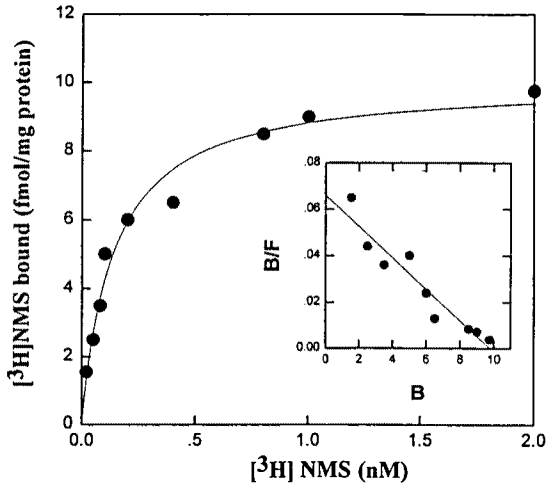

Fig. 1. Saturation binding curve of [^{3}H]NMS to crude homogenates of *C. elegans*. A crude homogenate of *C. elegans* was mixed with various concentrations of [^{3}H]NMS as indicated. The reaction mixtures were incubated at 20℃ for 40 min in 20 mM sodium phosphate buffer (pH 7.4). The reaction mixtures were filtered through GF/C filters and the filters were dried and counted with a liquid scintillation counter. Total binding and nonspecific binding (not shown) were determined in the absence and presence of 10^{-5} M atropine, respectively. Specific binding (●) was calculated by subtracting the nonspecific binding from the total binding. *Inset*: Scatchard analysis of the binding data.

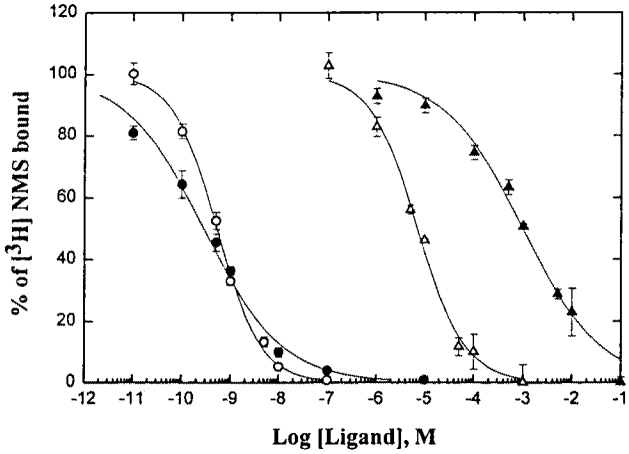

Fig. 2. Competitive binding between [^{3}H]NMS and muscarinic ligands to crude homogenates of *C. elegans*. A crude homogenate of *C. elegans* was incubated at 20℃ for 40 min with 0.2 nM [^{3}H]NMS and increasing amounts of competing ligands (●, scopolamine; ○, atropine; △, oxotremorine; ▲, carbachol). The reaction mixtures were filtered and counted as described in Fig. 1. Specific [^{3}H]NMS binding activity in the absence of the competing ligand was set to be 100%. Filters were prepared in triplicate and the error bars indicate the standard error (SE). Three to seven separate experiments were carried out with similar results.

by Culotti and Klein (1983). The dissociation constant value is similar to that obtained from rat brain (0.17± 0.02 nM) (Min *et al.*, 1994), rat heart (0.4± 0.1 nM) (Min *et al.*, 1994), or *Drosophila* (0.52± 0.12 nM) (Blake *et al.*, 1993), suggesting that the binding property of mAChRs to [^{3}H]NMS has been conserved from nematodes to mammals. The receptor density is about 10 to 100 fold lower compared to rat heart (148± 23 fmol/mg protein) and rat brain (1030± 10 fmol/mg protein) (Min *et al.*, 1994). It should be noted, however, that this experiment was performed with the whole body of *C. elegans*. Presumably mAChRs are locally concentrated in *C. elegans*, but it is hard to determine the local concentration of the receptors because of the small size of the animal.

Competitive inhibition binding analysis

The affinity of mAChRs for various muscarinic ligands was examined by observing the displacement of [^{3}H]NMS binding by the ligands. The dissociation constants (K_i) for atropine, scopolamine, oxotremorine, and carbachol were estimated to be $4.2\pm0.9\times10^{-10}$ M (n=3), $4.9\pm1.7\times10^{-10}$ M (n=3), $3.4\pm0.5\times10^{-6}$ M (n=7), and $6.7\pm0.9\times10^{-4}$ M (n=3), respectively (Fig. 2 and Table 1). The receptors exhibited a very high affinity for antagonists (atropine and scopolamine) and a much lower affinity for agonists (oxotremorine and carbachol), like vertebrate receptors. As shown in Table 1, the K_i values were close to those of rat tissues, except for carbachol binding that was at least 100-fold weaker. These results imply that the conformation of the ligand binding site is similar, but not identical, to that of the mammalian mAChRs. Previous studies with vertebrate mAChRs reported that antagonists bind to a single population of mAChRs while agonists bind to

Table 1. Comparison of ligand binding to mAChRs between *C. elegans* and rat

	Binding affinities of muscarinic ligands (K_i values, M)			
	Atropine	Scopolamine	Oxotremorine	Carbachol
C. elegans	$4.2\pm0.9\times10^{-10}$	$4.9\pm1.7\times10^{-10}$	$3.4\pm0.5\times10^{-6}$	$6.7\pm0.9\times10^{-4}$
Rat brain	$4.3\pm1.1\times10^{-10}$	$2.7\pm0.6\times10^{-10}$	$1.1\pm0.2\times10^{-6}$	$2.2\pm1.6\times10^{-7}$
Rat heart	$2.3\pm0.4\times10^{-9}$	$6.5\pm5.5\times10^{-9}$	$0.6\pm0.3\times10^{-6}$	$1.2\pm0.6\times10^{-6}$

All the data were analysed by nonlinear least-squares regression and presented as the mean± SE (standard error) from three to seven separate experiments. Ligand binding data for rat brain and heart were from Min *et al.* (1994).

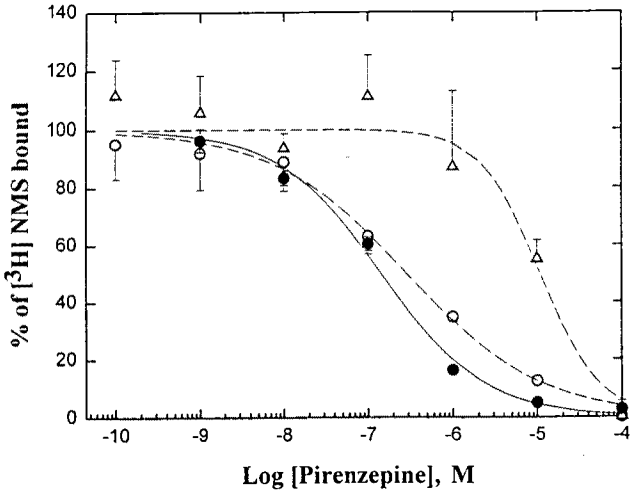

Fig. 3. Pirenzepine competition of [^{3}H]NMS binding to *C. elegans* mAChRs. A crude homogenate of *C. elegans* (●) was incubated at 20℃ with 0.2 nM [^{3}H]NMS for 40 min in the presence of various concentrations of pirenzepine as indicated. For comparison, membrane preparations of rat brain (○) and heart(△) were incubated at 37℃. The reaction mixtures were filtered and counted as described in Fig. 1. Specific [^{3}H]NMS binding activity in the absence of the competing ligand was set to be 100%. Filters were prepared in triplicate and the error bars indicate the standard error (SE). Similar results were obtained from four separate experiments.

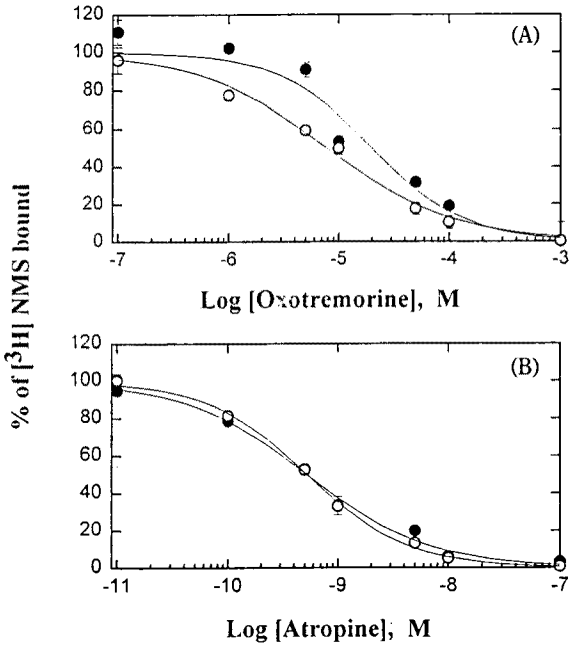

Fig. 4. Effect of guanylylimidodiphosphate (Gpp(NH)p) on the binding of muscarinic ligands. A crude homogenate of *C. elegans* was incubated in the absence (○) and presence (●) of 100 μM Gpp(NH)p at 20℃ for 5 min and further incubated for 40 min with 0.2 nM [^{3}H]NMS and various concentrations of oxotremorine (A) or atropine (B). The reaction mixtures were filtered and counted as described in Fig. 1. Specific [^{3}H]NMS binding activity in the absence of the competing ligand was set to be 100%. Filters were prepared in triplicate and the error bars indicate the standard error (SE). The affinity for oxotremorine decreased about 2-fold by Gpp(NH)p (n=7), whereas the affinity for atropine did not alter significantly (n=3).

multiple populations (usually two populations, high- and low-affinity) of mAChRs. In our study, however, computer analysis of the binding data indicated that both antagonists and agonists bound to a single population of mAChRs in *C. elegans*. We cannot exclude the possibility, however, that the high-affinity population of the receptors in *C. elegans* occupies a very small fraction (less than 10%) of the total population.

C. elegans mAChRs may belong to M1 subtype

Pharmacological subtypes of mAChRs are designated M1−M3. Pirenzepine has been used to discriminate these three subtypes (Hammer *et al.*, 1980; Doods *et al.*, 1987). The dissociation constant (K_i) of mAChRs in *C. elegans* for pirenzepine was calculated to be $2.4 \pm 1.0 \times 10^{-7}$ M (n=4) (Fig. 3). This value was very close to that of mAChRs in rat brain ($1.7 \pm 0.4 \times 10^{-7}$ M), in which M1 subtype is predominant. This result suggests that mAChRs in *C. elegans* are primarily M1 subtype. Did M1 subtype come first in evolution? We are currently trying to isolate the gene(s) encoding mAChRs in *C. elegans* and we expect that information on the gene(s) will provide some insight into the question.

Coupling of *C. elegans* mAChRs to G proteins

Vertebrate mAChRs are believed to exert their biochemical functions through the activation of G proteins. We asked whether mAChRs in *C. elegans* are coupled to G proteins by analyzing the effect of Gpp(NH)p, a nonhydrolyzable GTP analog, on the agonist oxotremorine binding. The affinity for oxotremorine decreased significantly by Gpp(NH)p (n=7, paired Student's *t* test, p=0.014) (Fig. 4A). The K_i values were $7.2 \pm 1.4 \times 10^{-6}$ M and $3.4 \pm 0.5 \times 10^{-6}$ M in the presence and absence of Gpp(NH)p, respectively. In contrast, the affinity for atropine was not appreciably affected by Gpp(NH)p (n=3, p=0.2) (Fig. 4B). These results suggest that mAChRs in *C. elegans* interact with G proteins in the receptor-mediated signal transduction pathway.

Interactions between neurotransmitter receptors and G proteins are believed to play important roles in modulating neuronal activities in *C. elegans*. An example of this type of interaction has been reported by Kaplan's group. A couple of mutants which failed to respond to serotonin turned out to lack a functional G protein, and these mutants displayed abnormal behaviors that are ordinarily regulated by serotonin (Segalat *et al.*, 1995), implying that serotonin receptors transmit serotonin signals to the cell interior via the G protein. It will be of considerable interest to determine how mAChRs interact with G proteins in transmitting acetylcholine

signals.

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