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Insect Muscarinic Acetylcholine Receptor: Pharmacological and Toxicological Profiles of Antagonists and Agonists

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The insect muscarinic acetylcholine receptor (mAChR) is evaluated as a potential target for insecticide action. The mammalian M2/M4-selective antagonist radioligand [^{3}H]AF-DX 384 (a pirenzepine analogue) binds to *Drosophila* mAChR at a single high-affinity site identical to that for the nonselective antagonist [^{3}H]quinuclidinyl benzilate (QNB) and with a pharmacological profile distinct from that of all mammalian mAChR subtypes. Three nonselective antagonists (QNB, scopolamine, and atropine) show the highest affinity ($K_i = 0.5-2.4$ nM) at the *Drosophila* target, and AF-DX 384 and M3-selective 4-DAMP (dimethyl-4-(diphenylacetoxy)piperidinium iodide) rank next in potency ($K_i = 5-18$ nM). Eleven muscarinic antagonists generally exhibit higher affinity than eight agonists. On injection into houseflies, the antagonists 4-DAMP and (*S*)-(+)-dimethindene produce suppressed movement, the agonist (methyloxadiazolyl)quinuclidine causes knockdown and tremors, and all of them inhibit [^{3}H]-QNB binding ex vivo, indicating possible mAChR-mediated intoxication. The insect mAChR warrants continuing study in lead generation to discover novel insecticides.

KEYWORDS: antagonists AF-DX 384 and QNB; *Drosophila*; *Musca*; muscarinic acetylcholine receptor

INTRODUCTION

Insect cholinergic neurotransmission is the major target for current insecticides. Organophosphates and methylcarbamates as inhibitors of acetylcholinesterase lead to aberrant accumulation of endogenous acetylcholine (ACh). Nicotine and neonicotinoids act as agonists of the nicotinic ACh receptor (nAChR)/ion channel complex. All of these insecticides induce hyperexcitation by nAChR channel opening and then paralysis by receptor desensitization (*1*). Another insecticide, cartap, blocks this channel (*2*). An alternative target in the cholinergic system is the muscarinic ACh receptor (mAChR) (*3*, *4*). No commercial insecticide modulates insect mAChR function.

mAChR is a metabotropic G-protein-coupled receptor which initiates continuous intracellular signaling events. There are two groups of mAChRs coupled with either stimulatory G-protein G_q or inhibitory G-protein G_i. G_q activates phospholipase C to produce inositol 1,4,5-trisphosphate, and G_i negatively modulates adenylate cyclase to reduce cytosolic cAMP levels. In mammals, five mAChR subtypes with diverse pharmacological profiles are identified and classified into two groups on the basis of coupled effector systems. Subtypes M1, M3, and M5 are associated with G_q, while M2 and M4 are coupled with G_i (*5*). In insects the mAChR pharmacological profile is somewhat similar to that of mammalian M3/M1 receptors (*6*−*8*). In *Drosophila* only a single gene encoding mAChR (*Dm1*) has been cloned, and the structure is most similar to the human M3, sharing 33% overall amino acid identity (50% when the variable intracellular loop i3 is excluded) (*9*, *10*). The pharmacology of this *Drosophila* mAChR expressed in COS-7 cells, *Xenopus* oocytes, or *Drosophila* S2 cells resembles that of mammalian M3/M1 subtypes (*11*, *12*).

The insect mAChR provides a continuing fascination and challenge to discover new chemicals with no target site cross-resistance against known insecticides. One goal of this investigation is to examine the pharmacological properties of the *Drosophila* native mAChR using for the first time the mammalian M2/M4-selective radioligand [^{3}H]AF-DX 384 (*13*, *14*) in comparison with the nonselective [^{3}H]quinuclidinyl benzilate (QNB) (**Figure 1**). The second aim is to determine whether muscarinic antagonists and agonists with diverse chemical structures (**Figure 1**) display intrinsic toxicity to houseflies (*Musca domestica* L.).

MATERIALS AND METHODS

Chemicals. Sources were as follows: [^{3}H]AF-DX 384 (115 Ci/mmol) and [^{3}H]QNB (36.5 Ci/mmol) from Perkin-Elmer (Boston, MA); most of the muscarinic antagonists and agonists from TOCRIS (Ellisville, MO) except for (±)-QNB, scopolamine hydrobromide trihydrate, atropine methyl nitrate, methoctramine tetrahydrochloride, and ACh bromide from Sigma (St. Louis, MO); 3-(3-methyl-1,2,4-oxadiazol-5-yl)quinuclidine (MOQ) described by Saunders et al. (*15*); organophosphates chloropyrifos oxon and paraoxon from ChemService Inc. (West Chester, PA); nicotinic agonist imidacloprid from a previous study in this laboratory (*16*).

Radioligand Binding. *Drosophila* was used as the mAChR source because it is the best known insect relative to genomics and neurobi-

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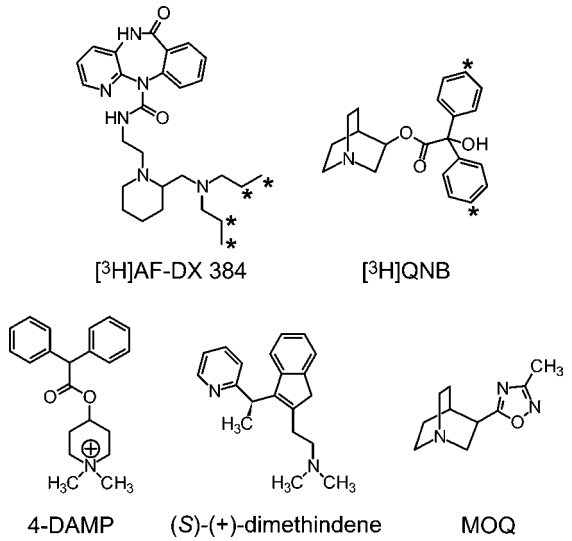

Figure 1. Structures of muscarinic antagonists and an agonist. Two radiolabeled antagonists, [3H]AF-DX 384 and [3H]QNB, are shown with positions of tritium indicated by asterisks. Antagonists 4-DAMP and (*S*)-(+)-dimethindene and agonist MOQ are representative of the chemicals studied.

ology. Head membranes were prepared according to Abdallah et al. (*7*). Radioligand binding assays were performed using a published methodology (*16*) with minor modifications. In brief, the receptor preparation (150−200 μg of membrane protein/assay) was incubated in 50 mM sodium phosphate buffer (pH 7.4) (250 μL final volume) for 60 min at 25 °C with one or two (for a simultaneous dual-probe binding experiment described later) radioligands alone or plus unlabeled displacer. Concentrations of radioligand were 2 or 0.5 nM [3H]AF-DX 384 or [3H]QNB, respectively, for determination of inhibitory potencies of test compounds. The binding reaction was terminated by rapid filtration on a GF/B filter presoaked in 0.1% polyethylenimine. The filter was rinsed three times with ice-cold saline and was transferred into a vial for scintillation counting. Nonspecific binding was defined as the difference between the absence and the presence of 2 μM atropine (10 μM was employed for saturation experiments). Binding parameters [dissociation constant (K_D), maximum binding capacity (B_{max}), and Hill coefficient (n_H)] were calculated from Scatchard and Hill plots. IC_{50} values, molar concentrations of test compounds necessary for 50% displacement of specific radioligand binding, were determined by iterative nonlinear least-squares regression using the Sigmaplot program (SPSS Inc., Chicago, IL). IC_{50} values were converted to inhibition constant (K_i) values using the equation $K_i = IC_{50}/(1 + [L]/K_D)$ (*17*). All experiments were repeated three or more times to give the mean and SD values reported.

Toxicity to Houseflies. *Musca* was employed for toxicity tests because of the ease of intrathoracic treatments. A standard insecticide-susceptible strain was obtained as pupae from Benzon Research (Carlisle, PA). Adult female houseflies (about 25 mg of body mass per fly) were treated with *O*-propyl *O*-(2-propynyl) phenylphosphonate (PPP) at 2.5 μg/fly applied topically in 0.5 μL of acetone to the ventrum of the abdomen. Although not directly tested here with muscarinic agents, PPP was used as a potential P450 and esterase inhibitor because of its remarkable synergistic effect for many insecticides (*18*) and particularly injected nicotinic agents with the same treatment protocol (*19*). After 90 min at room temperature, the test chemical (as the active ingredient) was administered in 0.22 μL of water or 25−50% Me_2SO/water solution by intrathoracic injection. Knockdown effects (including flies showing aberrant behavior) and mortalities were observed at 0.5, 1.0, 1.5, 2.5, and 24 h after administration; flies treated with the vehicle only were completely recovered within 0.5 h.

Ex Vivo Experiment. Flies (PPP-pretreated) were injected with the test compounds and the heads removed 0.5 or 24 h thereafter to determine mAChR ex vivo inhibition measured as [3H]QNB binding. This procedure involves translocation of the chemical from the site of administration (thorax) into the brain (*20*). At the predetermined times, the treated flies in a plastic tube were quickly frozen using liquid nitrogen. The capped tube was shaken vigorously to break the frozen flies into their body regions, which were placed on a dry ice block, and the heads only were collected with forceps. A batch of 50 heads in 2 mL of ice-cold 50 mM sodium phosphate buffer (pH 7.4) was homogenized for three 10 s periods with 60 s intervals between using a Polytron. The homogenate was taken up in a 5 mL plastic syringe and filtered by passing through four layers of attached cheesecloth. A 200 μL aliquot of the filtrate (~800 μg of total protein) was immediately used for incubation with 0.5 nM [3H]QNB in a total volume of 500 μL of phosphate buffer for 60 min at 25 °C prior to filtration. Nonspecific binding was determined in the presence of 2 μM atropine.

RESULTS

Comparative Binding Kinetics of [3H]AF-DX 384 and [3H]-QNB in *Drosophila* mAChR (Figure 2). Specific [3H]AF-DX 384 binding was saturable and showed a single high-affinity site with $K_D = 4.7$ nM, $B_{max} = 155$ fmol/mg of protein, and $n_H = 1.02$. [3H]QNB also gave a single high-affinity binding site with $K_D = 0.21$ nM, $B_{max} = 206$ fmol/mg of protein, and $n_H = 0.99$. Interestingly, similar B_{max} values were obtained for each radioligand.

Simultaneous Dual Radioligand Binding in *Drosophila* mAChR (Table 1). The simultaneous binding or direct competition of two radioligands was determined with dual probes in the same receptor preparation; this approach provides direct evidence that two radioligands bind either to the same domain or to distinct sites (*16*, *21*, *22*). With single individual radioligands, [3H]AF-DX 384 and [3H]QNB bindings were 65% and 35%, respectively, of that for the theoretical total of 100%. The two radioligands together gave only 54% of the expected summation value; i.e., on this basis the binding site for [3H]-AF-DX 384 clearly overlaps with that for [3H]QNB, and they compete with each other for the same binding domain.

Pharmacological Profiles of Muscarinic Antagonists and Agonists in Competing for [3H]AF-DX 384 and [3H]QNB Binding Sites in *Drosophila* mAChR. The pharmacological profiles were compared for 11 muscarinic antagonists, 8 muscarinic agonists, 2 anticholinesterases, and 2 nicotinic agonists (**Table 2**). Three nonselective muscarinic antagonists (QNB, scopolamine, and atropine) were extremely potent at binding sites for the two radioligands (K_i = 0.5−2.4 nM). Among the antagonists with subtype selectivity in mammalian mAChRs, the M2/M4-selective AF-DX 384 had the highest affinity (4.8−6.1 nM). The M3-selective 4-DAMP was next in activity (15−18 nM), whereas DAU 5844 (also M3-selective) was moderate in potency (K_i = 570−640 nM). Pirenzepine and dimethindene with M1 and M2 selectivities, respectively, also had moderate affinity (390−660 nM). The M2- and M4-selective AF-DX 116 and PD 102807, respectively, showed similar but low affinity (1030−1430 nM). Methoctramine (an M2 ligand) had the lowest affinity (2850−3860 nM). All of the test muscarinic agonists generally had lower affinities compared with the antagonists. Among the agonists, MOQ and arecaidine but-2-ynyl ester possessing M1 and M2 selectivities, respectively, showed the highest affinity (1320−1560 nM), and arecaidine propargyl ester was several-fold less potent (4490−5200 nM) than the but-2-ynyl ester analogue. The natural product pilocarpine had moderate affinity (2800−2980 nM), and all other agonists showed very low potencies (7000−130000 nM). Mammalian M2-selective chlorpyrifos oxon and paraoxon and insect-selective nicotinic agonist imidacloprid gave little or no inhibition at 100000 nM.

An important relationship is evident on comparing the K_i values for muscarinic antagonists and agonists at the [3H]AF-

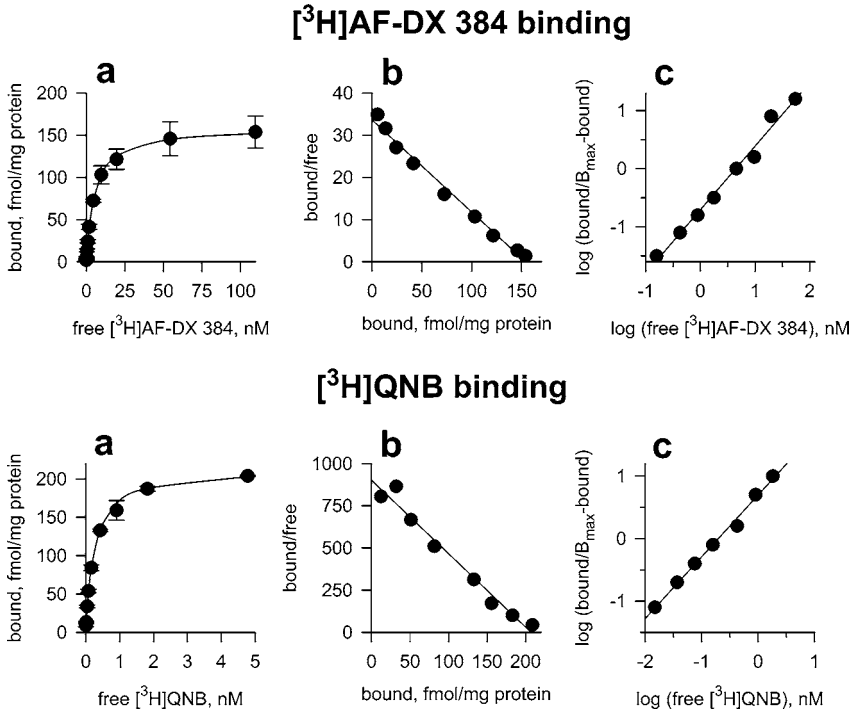

Figure 2. Comparative binding kinetics of [^{3}H]AF-DX 384 and [^{3}H]QNB in *Drosophila* mAChR showing saturation isotherms (**a**), Scatchard plots (**b**), and Hill plots (**c**) for specific binding. The Scatchard and Hill plots are representative data from three individual experiments. The binding parameters (±SD, $n = 3$) are as follows: for [^{3}H]AF-DX 384, $K_D = 4.7 \pm 0.6$ nM, $B_{max} = 155 \pm 15$ fmol/mg of protein, and $n_H = 1.02 \pm 0.08$; for [^{3}H]QNB, $K_D = 0.21 \pm 0.02$ nM, $B_{max} = 206 \pm 4$ fmol/mg of protein, and $n_H = 0.99 \pm 0.02$. Nonspecific binding for [^{3}H]AF-DX 384 was 10–20% at 0.08–4.5 nM (no concentration dependency) and 25–70% at 9.6–110 nM (with concentration dependency); that is, the high level of nonspecific binding at higher concentrations interferes to some extent in obtaining an accurate B_{max} value. In the [^{3}H]QNB assay, the nonspecific binding percentages were 4–10% through the entire concentration range.

Table 1. Simultaneous Dual Radioligand Binding in *Drosophila* mAChR

radioligand(s)	assay level[a] (nM)	specific binding[b] (dpm/mg of protein)	dual binding[c] (% of expected value)
[^{3}H]AF-DX 384	20	28500 ± 3160	65
[^{3}H]QNB	2	15500 ± 1200	35
[^{3}H]AF-DX 384 + [^{3}H]QNB	20 + 2	23900 ± 1050	54

[a] Radioligand concentrations were at or near saturation levels on the basis of their saturation isotherms (**Figure 2**). [b] Means ± SD ($n = 4$). [c] The expected value is the theoretical total of 100% defined as the sum of the dpm/mg of protein for each individual radioligand.

DX 384 and [^{3}H]QNB binding sites (**Figure 3**). The K_i values for the 19 muscarinic antagonists and agonists at the [^{3}H]AF-DX 384 binding site of *Drosophila* mAChR perfectly correlated to those at the [^{3}H]QNB site ($r^2 = 0.996$). This correlation was also evident considering separately either the antagonists or agonists.

Toxicity of Muscarinic Antagonists and Agonists to *Musca* (Table 3). The organismal effects of the muscarinic agents were evaluated as knockdown or lethality 0.5−24 h after administration and were consistently time- and dose-dependent. The classic antagonists scopolamine and atropine were slightly toxic at the early times. AF-DX 384 and 116 (tested only at a low dose due to solubility limitations) also had a little effect. 4-DAMP, DAU 5884, and (*S*)-(+)-dimethindene displayed distinct knockdown effects and mortalities, while pirenzepine and PD 102807 were weak toxicants. Interestingly, methoctramine showed an immediate lethality at the higher dose. Among seven agonists, MOQ, pilocarpine, arecaidine propargyl ester, and aceclidine were toxic at 75 μg/fly for 67−100% of the test organisms, whereas the other three were less effective, or solubility limited the test dose to 25 μg/fly. The active antagonists generally induced suppressed movement, particularly (*S*)-(+)-dimethindene with a toxic effect similar to that of ether or carbon dioxide (but persisting much longer). The agonist MOQ showed knockdown with slight leg tremors, and the two arecaidine esters caused abnormal behavior including aberrant motions of the wings and legs. The insecticidal activities of the muscarinic agents with *Musca* were not directly correlated with their in vitro mAChR target site potency with either *Drosophila* or *Musca* (**Table 2**).

Ex Vivo Inhibition of [^{3}H]QNB Binding Sites in *Musca* Brain. This study was designed to test whether the toxic effects of muscarinic agents were mediated by interaction with the *Musca* brain mAChR. The three most toxic but nonlethal compounds at 25 μg/fly were selected for investigation, i.e., 4-DAMP, (*S*)-(+)-dimethindene, and MOQ (**Table 4**). The observed inhibition establishes that each compound moves from the thorax to the brain, and much remains bound during the [^{3}H]QNB reporter assay. Time and dose dependency were observed for the three compounds. Consistent with the toxic effects, the percentage mAChR inhibition was higher at 0.5 h compared to 24 h in each case ($P < 0.01$). Direct ex vivo potency comparisons among the test compounds are not appropriate because of the large differences in in vitro potency and hydrophobicity and probable variations in distribution, e.g., translocation into the brain and dissociation from the binding site.

DISCUSSION

Single Class of Native *Drosophila* mAChR with a Distinctive Pharmacological Profile. This study establishes that the

Table 2. Pharmacological Profiles of Muscarinic Antagonists and Agonists in Competing for [^{3}H]AF-DX 384 and [^{3}H]QNB Binding Sites in *Drosophila* mAChR

ligand	selectivity[b]	K_i[a] (nM) ± SD (n = 3) [^{3}H]AF-DX 384	[^{3}H]QNB
Muscarinic Antagonists[c]			
(±)-QNB	none	0.5 ± 0.2	0.5 ± 0.3
scopolamine	none	0.7 ± 0.2	1.1 ± 0.6
atropine	none	1.8 ± 0.5	2.4 ± 0.8
AF-DX 384	M2/M4	6.1 ± 2.9	4.8 ± 2.5
4-DAMP	M3	18 ± 6.2	15 ± 6.6
pirenzepine	M1	390 ± 130	430 ± 150
DAU 5844	M3	570 ± 320	640 ± 370
(*S*)-(+)-dimethindene	M2	660 ± 210	510 ± 82
AF-DX 116	M2	1380 ± 410	1220 ± 120
PD 102807	M4	1430 ± 460	1030 ± 24
methoctramine	M2	3860 ± 1330	2850 ± 250
Muscarinic Agonists[c]			
MOQ	M1	1330 ± 280	1320 ± 380
arecaidine but-2-ynyl ester	M2	1560 ± 670	1500 ± 230
pilocarpine	none	2800 ± 630	2980 ± 89
arecaidine propargyl ester	M2	5200 ± 1120	4490 ± 1270
ACh (+50 μM paraoxon)	none	8000 ± 5200	7000 ± 2040
5-methylfurmethiodide	none	12300 ± 2500	14900 ± 1500
oxotremorine	none	21500 ± 6200	19400 ± 4750
aceclidine	none	114000 ± 52000	130000 ± 76000
Anticholinesterases			
chlorpyrifos oxon	M2	>100000 (29%)[d]	>100000 (17%)[d]
paraoxon	M2	>100000 (13%)[d]	>100000 (0%)[d]
Nicotinic Agonist			
imidacloprid		>100000 (3%)[d]	>100000 (1%)[d]

[a] The following K_D values (see **Figure 2**) and concentrations of radioligands, [L], were used in converting IC_{50} to K_i values: K_D = 4.6 and [L] = 2.0 nM for [^{3}H]AF-DX 384, and K_D = 0.21 and [L] = 0.5 nM for [^{3}H]QNB. [b] Selectivity based on potencies among the mammalian mAChR subtypes (*5*, *15*, *23–28*). [c] n_H values of the 19 muscarinic antagonists and agonists were 0.88–1.29 (average 1.03) in the [^{3}H]AF-DX 384 assay and 0.82–1.13 (average 1.02) in the [^{3}H]QNB assay, except for PD 102807 with 1.77 and 1.91 and methoctramine with 1.64 and 1.79 in the [^{3}H]AF-DX 384 and [^{3}H]QNB assays, respectively. Potencies (K_i, nM) of QNB, atropine, 4-DAMP, pirenzepine, AF-DX 116, and MOQ as inhibitors of [^{3}H]QNB binding to the *Musca* receptor are 0.17, 1.0, 19, 484, 1560, and 620, respectively (*3*, *7*), i.e., essentially the same as those tabulated for *Drosophila*. [d] Inhibitory percent at 100000 nM.

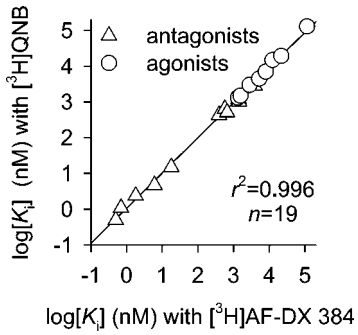

Figure 3. Correlation plot of K_i values for mAChR antagonists and agonists at the *Drosophila* mAChR [^{3}H]AF-DX 384 and [^{3}H]QNB binding sites. Data are from **Table 2**. r^2 = 0.995 (n = 11) for the antagonists only, and r^2 = 0.993 (n = 8) for the agonists only.

mammalian M2/M4-selective radioligand [^{3}H]AF-DX 384 binds to the *Drosophila* brain mAChR with a single high-affinity site pharmacologically characterized here using various muscarinic antagonists and agonists with differential subtype selectivity among mammalian mAChRs. The three nonselective antagonists QNB, scopolamine, and atropine show the highest affinity, and

Table 3. Toxicity of Muscarinic Antagonists and Agonists to *Musca*

compound	dose (μg/fly)	toxicity[a] (%) at indicated time after administration[b] (h) 0.5	1.0	1.5	2.5	24
Antagonists						
scopolamine	25	10	3	3	3	3
	75	20	3	0	0	0
atropine	25	17	7	7	7	13
	75	23	23	20	20	7
AF-DX 384[c]	6.25	30	3	3	3	3
4-DAMP	7.5	10	10	7	3	7
	25	60	47	40	20	10
	75	100	67	57	60	33
pirenzepine	25	20	7	7	7	7
	75	27	27	20	13	10
DAU 5884	25	23	17	10	0	0
	75	70	43	23	17	7
(*S*)-(+)-dimethindene	7.5	10	0	0	0	0
	25	100	70	40	17	3
	75	100	100	100	97	23
AF-DX 116[c]	6.25	17	10	10	10	3
PD 102807	25	20	13	13	13	13
methoctramine	7.5	17	10	0	0	3
	25	100	100	100	100	100
Agonists						
MOQ	25	47	20	13	3	3
	75	100	83	60	33	13
arecaidine but-2-ynyl ester	25	3	3	3	3	3
	75	10	9	9	8	4
pilocarpine	25	3	0	0	0	0
	75	67	23	10	7	7
arecaidine propargyl ester	25	17	0	0	0	0
	75	100	100	100	100	87
5-methylfurmethiodine[c]	25	13	3	3	3	3
oxotremorine[c]	25	3	3	3	3	3
aceclidine	25	33	3	3	3	0
	75	100	30	27	10	3

[a] Average SD values (%) at various ranges of toxicity (knockdown or lethal): 6% for 3–19%, 14% for 20–39%, 9% for 40–59%, 11% for 60–79%, and 9% for 80–99%. [b] Pretreated with the synergist PPP. [c] Treatment dose limited by solubility in the injection vehicle. Compounds not tested including QNB were not soluble in the vehicle even at the minimum test dose of 6 μg/fly.

Table 4. Ex Vivo Inhibition of [^{3}H]QNB Binding Sites in *Musca* Brain

compound	dose (μg/fly)	inhibition[a] (% ± SD) at indicated time after administration (h) 0.5	24
4-DAMP	7.5	79 ± 5	49 ± 7[b]
	25	92 ± 7	59 ± 7[b]
(*S*)-(+)-dimethindene	25	41 ± 7	0 ± 0[b]
	75	81 ± 11	28 ± 8[b]
MOQ	25	49 ± 11	9 ± 15[b]
	75	80 ± 11	16 ± 26[b]

[a] Compound-injected flies compared with vehicle-treated controls based on two independent experiments involving a total of 6–8 measurements. [b] Significant difference between the two time points (P < 0.01).

the M2/M4-selective AF-DX 384 and M3-selective 4-DAMP rank next in potency, while other ligands with selectivity to M1, M2, M3, or M4 are rather moderate to relatively low in their affinity. This specificity profile does not follow those for any of the five mammalian mAChR subtypes. Importantly, the *Drosophila* binding site for [^{3}H]AF-DX 384 is identical to that for [^{3}H]QNB on the basis of three substantial types of evidence: (1) perfect correlation in their pharmacological

profiles; (2) similar B_{max} values of the two radioligands; (3) shared binding domain based on the simultaneous dual-binding experiment. The antagonist profile of recombinant *Drosophila* mAChR (*12*) is also consistent with the present finding. Therefore, only a single class of native mAChR appears to be expressed in *Drosophila* brain, and the pharmacological profile is distinctive from all of the mammalian mAChR subtypes.

Antagonist/Agonist Comparison. All of the test muscarinic agonists show generally lower affinity compared to those of antagonists to *Drosophila* mAChR assayed by the two antagonist radioligands. In mammalian mAChRs, the agonists have high potency (antagonists also show high affinity) in agonist radioligand binding but show diminished potency in antagonist radioligand assays (*15*, *29*) as with our results in *Drosophila*. There is no suitable agonist radioligand available for insect mAChR (although not detailed here, we also found that [^{3}H]-oxotremorine-M has no specific binding in the *Drosophila* preparation). The potency difference in mammalian mAChR observed in [^{3}H]agonist/[^{3}H]antagonist assays is interpreted in terms of separate binding subsites for an agonist and an antagonist, with hydrogen-bonding interactions characterizing agonist behavior and lipophilic features determining antagonist interaction (*15*). Muscarinic agonists tend to be small molecules with very little tolerance for steric bulk, and introduction or extension of one carbon unit to an agonist may lead to an antagonist or a partial agonist (*29*).

Structure of the *Drosophila* Muscarinic Receptor. There are distinct structural differences between insect and mammalian mAChRs. The *Drosophila* mAChR has an extremely large intracellular loop (i3, between the fifth and sixth putative transmembrane domains). In *Drosophila* it is almost twice as long as in most mammalian mAChR subtypes and contains 17 potential phosphorylation sites (*8*). Loop i3 apparently involves the alternative splicing variants of the *Dm1* gene, because the sequence reported by Shapiro et al. (*10*) has an insert of 17 amino acids in this region that is not present in the sequence by Onai et al. (*9*). Intracellular loops i2 and i3 represent the approximate region that determines receptor−G-protein coupling; thus, the difference in structure of i3 with and without the 17 amino acid extension might reflect an associated effector system for differential roles in neurotransmission. Interestingly, the presynaptic mAChR mediates inhibition of ACh release coupled to reduction of cAMP levels (mammalian M2-like), while the postsynaptic receptor plays a stimulatory role associating an increase in cAMP levels and phosphatidiylinositol turnover, supporting the differential effector coupling and/or suggesting that possibly more than one receptor subtype exists in cockroach and locust (*8*, *30*, *31*). The other curious structural aspect is that the extracellular N-terminus of the *Drosophila* mAChR is much longer than that in the mammalian receptor; however, the functional or pharmacological significance is unknown (*8*).

Toxicological Features of Muscarinic Antagonists and Agonists to *Musca*. Some of the antagonists and agonists elicit distinct toxicological effects in insects. Although direct correlation is not observed between in vitro mAChR target site potency and insecticidal actions of muscarinic agents, the ex vivo inhibitory effect on radioligand binding provides support for mAChR-mediated intoxication. The structure−activity relationships of muscarinic agonists in terms of receptor or agonist potency and insecticidal activity have been explored with quinuclidine, azabicycloheptane, and azabicyclooctane analogues in studies based on therapeutic agents modulating mammalian mAChR functions (*3*, *4*). We find that some muscarinic antagonists also display toxicity to *Musca* at levels comparable to those of the agonists.

Concluding Remarks. The insect mAChR is a potential but poorly developed target for insecticide action. This investigation and two others (*3*, *4*) establish the high potency of muscarinic agents at *Drosophila* or *Musca* mAChRs and that some antagonists and agonists have moderate insecticidal activity. The receptor studies were made with the antagonist radioligands [^{3}H]-AF-DX 384 and [^{3}H]QNB, but for now there is no useful agonist radioligand for insects. The mAChR target warrants continuing study in lead generation programs to discover novel insecticides.

ABBREVIATIONS USED

ACh, acetylcholine; AF-DX 384 or [^{3}H]AF-DX 384, an antagonist pirenzepine analogue or its tritiated radioligand; B_{max}, maximum binding capacity; K_D, dissociation constant; K_i, inhibition constant; mAChR, muscarinic ACh receptor; MOQ, 3-(3-methyl-1,2,4-oxadiazol-5-yl)quinuclidine (an mAChR agonist); n_H, Hill coefficient; PPP, *O*-propyl *O*-(2-propynyl) phenylphosphonate; QNB or [^{3}H]QNB, quinuclidinyl benzilate or its tritiated form; SD, standard deviation.

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