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Drosophila Nervous System Muscarinic Acetylcholine Receptor: Transient Functional Expression and Localization by Immunocytochemistry

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SUMMARY

The pharmacological properties of a cloned *Drosophila* muscarinic acetylcholine receptor (mAChR) were investigated using two independent transient expression systems. The binding characteristics of the expressed receptor were determined using transfected COS-7 cells, whereas the mAChR functional properties were analyzed using nuclearly injected *Xenopus* oocytes. Competition displacement studies with transfected COS-7 cell membranes showed that *N*-[^{3}H]methylscopolamine binding was displaced most effectively by atropine, followed by 4-diphenylacetoxy-*N*-methylpiperidine methiodide, pirenzepine, and AFDX-116. This same order of effectiveness (4-diphenylacetoxy-*N*-methylpiperidine methiodide > pirenzepine > AFDX-116) was observed in oocytes expressing Dm1 when carbamylcholine-induced currents were inhibited by the same antagonists. Thus, the expressed *Drosophila* mAChR (Dm1) exhibits a pharmacology that broadly resembles that of the vertebrate M_1 and M_3 mAChR subtypes. To determine the anatomical localization of the *Drosophila* mAChR, polyclonal antiserum was raised against a peptide corresponding to the predicted carboxyl-terminal domain of the receptor. Immunocytochemistry on fly sections demonstrated that the mAChR gene product was found in the nervous system and was not seen in skeletal muscle. The most intense staining was localized to the glomeruli of the antennal lobes, an area of the insect brain where first-order synaptic processing of olfactory information occurs.

mAChRs belong to a superfamily of predicted seven-transmembrane region proteins that transduce hormonal and neurotransmitter responses by coupling to heterotrimeric G proteins. Pharmacological studies on mAChRs in a variety of vertebrate and invertebrate tissues have indicated the presence of multiple tissue-specific subtypes (1, 2). All tissue characterization work has relied on the ability of selective antagonists to discriminate between the M_1, M_2, and M_3 subtypes of the receptor; however, similarities in the pharmacology of members of this receptor family make convincing detection of the individual receptors difficult (1–3).

Molecular cloning studies indicate that the number of possible mAChR subtypes is greater than that shown by the use of currently available subtype-selective antagonists (3). To date, five separate but homologous genes (m1–m5) have been identified in mammalian tissues. Although each of the expressed genes possesses distinct pharmacological and functional properties, all have been placed into one of two general classes depending upon the type of intracellular signaling pathway to which the receptor is coupled (3). Heterologous expression studies in either *Xenopus laevis* oocytes or mammalian cell lines indicate that the m1, m3, and m5 gene products activate phospholipase C and possibly regulate intracellular cAMP levels in some cell lines. In contrast, the m2 and m4 subtypes of the receptor inhibit adenylate cyclase activity while providing weak or negligible stimulation of the phosphatidylinositol response (3, 4). The division of the expressed m1–m5 genes into these classes is not absolute, with second messenger responses varying with the cell line, the levels of receptor expression, and the concentration of agonist used (3, 4).

Immunocytochemical studies, using antisera raised against gene-specific fusion proteins, have shown that some vertebrate neuronal tissues that respond to mAChR agonists contain two or more subtypes (5). This immunological study is in agreement

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ABBREVIATIONS: mAChR, muscarinic acetylcholine receptor; NMS, *N*-methylscopolamine chloride; QNB, quinuclidinyl benzilate; AFDX-116, 11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepine-6-one; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methiodide; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SOS, standard oocyte saline; EC_{50}, effective concentration for 50% stimulation; BCG, Bacillus-Calmette-Guerin.

TABLE 1

Antagonist binding properties of a *Drosophila* mAChR transiently expressed in COS-7 cells

Affinity constants (K_d) for [^{3}H]QNB and [^{3}H]NMS were determined as described in Experimental Procedures. B_{max} values indicate the maximum number of binding sites/mg of membrane protein. Data shown are the means ± standard errors for three independent transfections.

	Membranes		Monolayers	
	K_d	B_{max}	K_d	B_{max}
	pM	pmol/mg	pM	pmol/mg
[^{3}H]QNB	90 ± 9	2.0 ± 0.6	123 ± 32	ND[a]
[^{3}H]NMS	520 ± 120	2.5 ± 0.7	540 ± 90	0.48 ± 0.03

[a] ND, not determined.

with data from *in situ* hybridization experiments (6, 7) and suggests that the binding studies with subtype-selective antagonists may not have accurately portrayed the complexity of mAChR tissue distribution.

The characterization and identification of mAChRs in invertebrates have proceeded slowly, relative to the vertebrate work. mAChR binding studies in insect tissues show that the highest concentration of antagonist binding sites appears to be in the nervous system (8–14). Additional characterization of these binding sites has also been performed using the vertebrate subtype-selective mAChR antagonists pirenzepine (M_1) and AFDX-116 (M_2) (12, 13). Taken together, these biochemical results indicate that the invertebrate nervous system may contain several mAChR subtypes, based upon analogy with the vertebrate classification system (1). Only recently has a mAChR gene been isolated from *Drosophila* (15, 16). Two separate laboratories reported that the cloned mAChR from the fly nervous system has a predicted amino acid sequence that is highly homologous to the genes of the vertebrate m1, m3, and m5 subtypes. In addition, one study described (16) the pharmacology and intracellular signaling mechanism of the stably expressed receptor. This work demonstrated that the expressed receptor bound the mAChR antagonist pirenzepine and stimulated phosphatidylinositol turnover in response to carbamylcholine.

In the present study we characterize this cloned *Drosophila* mAChR, using transiently receptor-expressing COS-7 cells and *Xenopus* oocytes, to examine the receptor antagonist pharmacology. Data from displacement binding studies with mAChR antagonists are in accord with the observed inhibition of agonist responses, suggesting that the pharmacology of this mAChR shares properties with that of the vertebrate M_1 or M_3 subtypes. Immunocytochemical localization, using antiserum produced against a peptide from the predicted carboxyl-terminal amino acid sequence of the receptor, demonstrates that this gene product is expressed in the antennal lobes of the *Drosophila* nervous system, a region for which there is evidence of primary afferent cholinergic input (17).

Experimental Procedures

Materials. The COS-7 cell line was a gift from Sandra Gould, Merck Research Laboratories (Rahway, NJ). [^{3}H]NMS (83 Ci/mmol), [^{3}H]QNB (43 Ci/mmol), and *myo*-[^{3}H]inositol (23 Ci/mmol) were purchased from DuPont, Ltd. (Stevenage, Hertfordshire, UK). Freon, perchloric acid, tri-*n*-octylamine, formic acid, and ammonium formate were obtained from BDH (Poole, Dorset, U.K.). The anion exchange resin AG-1X8 was purchased from Bio-Rad (Richmond, CA). AFDX-116 was obtained from Boehringer Ingelheim UK, Ltd. (Lewes, Sussex, U.K.); atropine and 4-DAMP were purchased from Research Biochemicals Inc. (Natick, MA). Tissue culture reagents were obtained from GIBCO-BRL and immunocytochemical reagents were from either Calbiochem or Vector Laboratories. All other reagents were from Sigma Chemical Co. (Poole, Dorset, UK) unless otherwise indicated.

Expression in mammalian cells. The nucleotide sequence of the *Drosophila* mAChR (Dm1) cDNA has been published (16). For transient expression studies this cDNA was subcloned into the eukaryotic expression vector pHEI. This plasmid contains a human cytomegalovirus promoter/enhancer for the expression of the gene of interest, in addition to simian virus 40 polyadenylation and splice signals. The receptor construct was introduced into COS-7 cells grown to 75% confluence in 10-cm dishes, using the DEAE-dextran method of transfection (18). Cells were passaged and routinely maintained in a 5% humidified atmosphere at 37°, in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

Membrane preparation and receptor binding assays. Cells

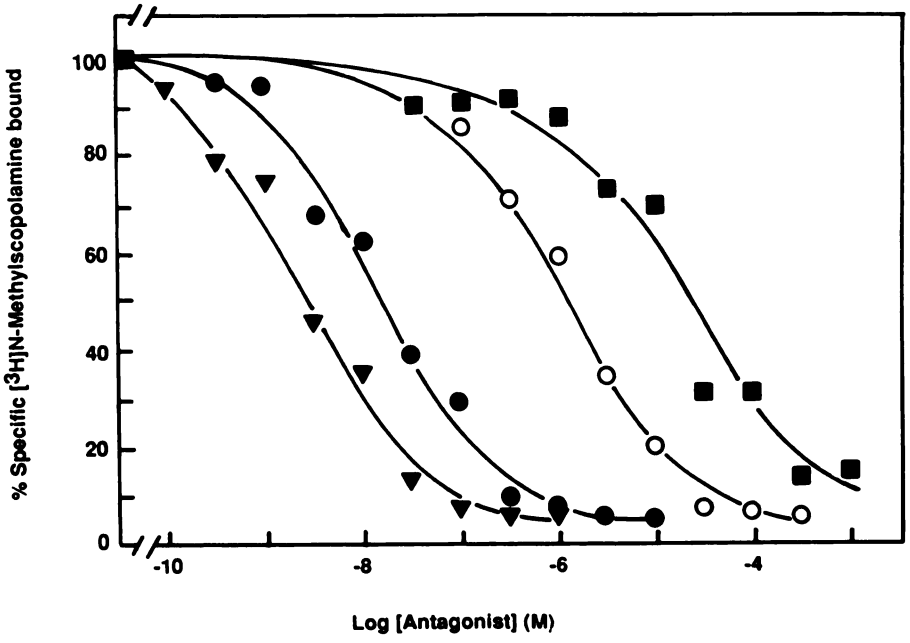

Fig. 1. Competitive displacement of [^{3}H]NMS by subtype-selective mAChR antagonists. A membrane fraction from receptor-expressing COS-7 cells was prepared for binding as described in Experimental Procedures. Duplicate binding assays were incubated for 90 min at room temperature before being filtered through presoaked Whatman GF/C filters using a Brandel cell harvester. Each data point represents the mean of three independent cell transfections. ▼, Atropine; ●, 4-DAMP; ○, pirenzepine; ■, AFDX-116. The [^{3}H]NMS concentration was 300 pM.

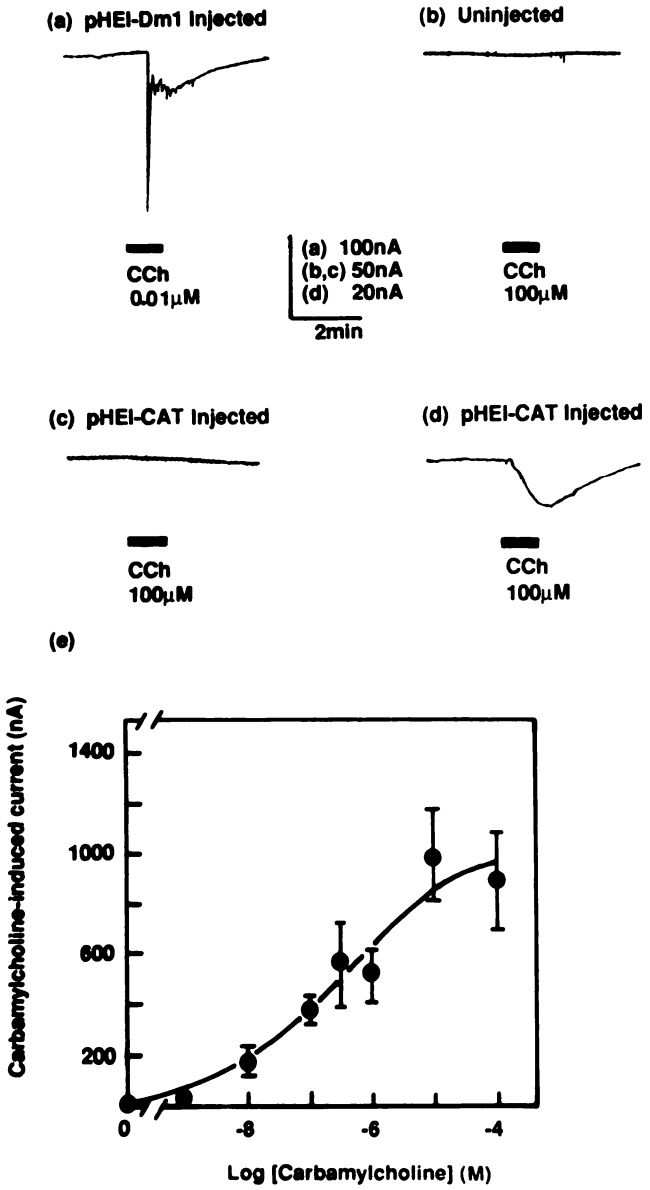

Fig. 2. a-d, Electrophysiological recordings of Dm1-injected *Xenopus* oocytes and controls. Nuclear injection of defolliculated oocytes was performed using 20 ng of the Dm1-containing vector pHEI (a), water (b), or 20 ng of the pHEI vector containing the bacterial reporter gene chloramphenicol acetyltransferase (*CAT*) in place of the Dm1 cDNA (c and d). The responses are to 0.10 μM and 100 μM carbamylcholine (*CCh*). d, Endogenous mAChR response detected in one batch of oocytes. *Vertical scale bar*, 100 nA (a), 50 nA (b and c), or 20 nA (d); *horizontal bar*, 2 min. e, Dose dependence of the carbamylcholine-stimulated response. The EC_{50} for carbamylcholine was approximately 0.5 μM. These responses are the mean ± standard error for seven to 30 independent observations.

were harvested 48 hr after transfection. The monolayers were rinsed with ice-cold PBS, pH 7.2, and scraped on ice into the same buffer containing 5 mM EDTA. Centrifugation at 500 × *g* (4°) resulted in a cell pellet, which was then disrupted by homogenization in PBS plus 5 mM EDTA. Cell membranes were collected by centrifugation in a microfuge at 13,000 × *g* at 4°. The membrane fraction was resuspended in assay binding buffer and washed twice at 4° before assay. Receptor binding assays were carried out at room temperature for 90 min in 10 mM HEPES, pH 7.2, containing 5 mM $MgCl_2$. Approximately, 100 μg/ml total membrane protein was used in each assay. In [^{3}H]NMS and [^{3}H]QNB saturation binding experiments, eight concentrations of radioligand were used (25–2500 pM and 10–500 pM, respectively), in a final volume of 0.25 ml. Nonspecific binding at each concentration was determined in the presence of 1 μM atropine. For [^{3}H]NMS competition experiments, eight concentrations of unlabeled ligand were used with approximately 40 μg/ml total membrane protein. Duplicate binding assays were terminated by dilution with ice-cold 50 mM Tris·HCl, pH 7.4, and filtered over Whatman GF/C filters with a Brandel cell harvester. Filters were then washed three times with 10 volumes of ice-cold buffer and transferred to scintillation vials that contained 5 ml of scintillation fluid. The radioactivity retained on the filter was determined using a Beckman scintillation counter. Receptor binding studies were analyzed using the nonlinear regression computer program LIGAND (19). IC_{50} values were converted to K_i values by the method of Cheng and Prusoff (20). Protein concentration was determined as described by Bradford (21), with a Bio-Rad assay kit.

Measurement of cell monolayer binding. Cells that had been transfected in 10-cm dishes were washed with PBS before being scraped, pelleted, and resuspended for transfer to 24-well plates. Radioligand binding was carried out 40 hr later in PBS with 5 mM $MgCl_2$. The radioligand concentrations were the same as for the membrane binding described above. Incubations were stopped after 90 min by placing the plates on ice and rapidly rinsing the monolayers with ice-cold PBS. Bound radioactivity was determined by incubating the monolayers at room temperature for 30 min in 50 mM Tris, pH 7.4, containing 0.5% sodium dodecyl sulfate. Duplicate wells were rinsed repeatedly and the samples were added to 5 ml of scintillation fluid before being counted in a Beckman scintillation counter.

Measurement of phosphoinositol accumulation. Before transfection, cells were grown for 24 hr in inositol-free Dulbecco's modified Eagle's medium containing 10% dialyzed fetal 0calf serum. After transfection, the cells were rinsed with PBS, scraped from the 10-cm dishes, pelleted by centrifugation, resuspended in inositol-free medium, and aliquoted into 24-well plates. Eight hours later, 2 μCi/ml *myo*-[^{3}H] inositol was added and the incubation was continued for 40 hr. The cell monolayers were rinsed and incubated for 10 min at room temperature with PBS containing 10 mM LiCl. The appropriate concentration of agonist was then added to each well for 15 min at room temperature. To terminate the reactions, individual wells received 0.5 ml of 5% ice-cold perchloric acid containing 1 mM inositol and the plates were transferred to ice. Cell supernatants were collected into 1.5-ml microfuge tubes and prepared for inositol phosphate accumulation as described (22). The levels of radiolabeled inositol phosphates were determined by anion exchange chromatography (23) and were quantified using a Beckman scintillation counter.

Oocyte preparation and nuclear injection. Follicular oocytes were obtained from anesthetized mature *Xenopus laevis* females. Ovarian lobes were surgically removed, placed in SOS (100 mM NaCl, 2.0 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.0 mM HEPES, pH 7.6), and teased into small clumps. Stage 5 or 6 oocytes were selected and manually defolliculated by removal of the outer ovarian epithelium and theca, followed by collagenase treatment (Sigma type 1A, 1 mg/ml in Ca^{2+}-free SOS) for 10 min at room temperature. Defolliculated oocytes were placed in supplemented SOS containing 10 μg/ml gentamicin and 2.5 mM sodium pyruvate. Oocytes were allowed to recover for 1–3 hr at 17–19° before nuclear injection.

Nuclear microinjection of oocytes was carried out with a digital microdispenser (Drummond Series 500). After immobilization of the oocytes, 20 ng of the Dm1-containing plasmid pHEI were injected into the nucleus and the oocytes were transferred to fresh SOS. Controls were performed with oocytes that were either uninjected or injected with the pHEI vector containing the bacterial reporter gene chloramphenicol acetyltransferase instead of the Dm1 cDNA. Oocytes were incubated for 24 hr at 18° before electrophysiological analysis.

Electrophysiology. Individual oocytes were voltage-clamped at room temperature using a conventional two-microelectrode amplifier (Warner oocyte clamp OC-725A). The microelectrodes used to impale the oocytes were filled with 2.0 M KCl and had resistances between 2

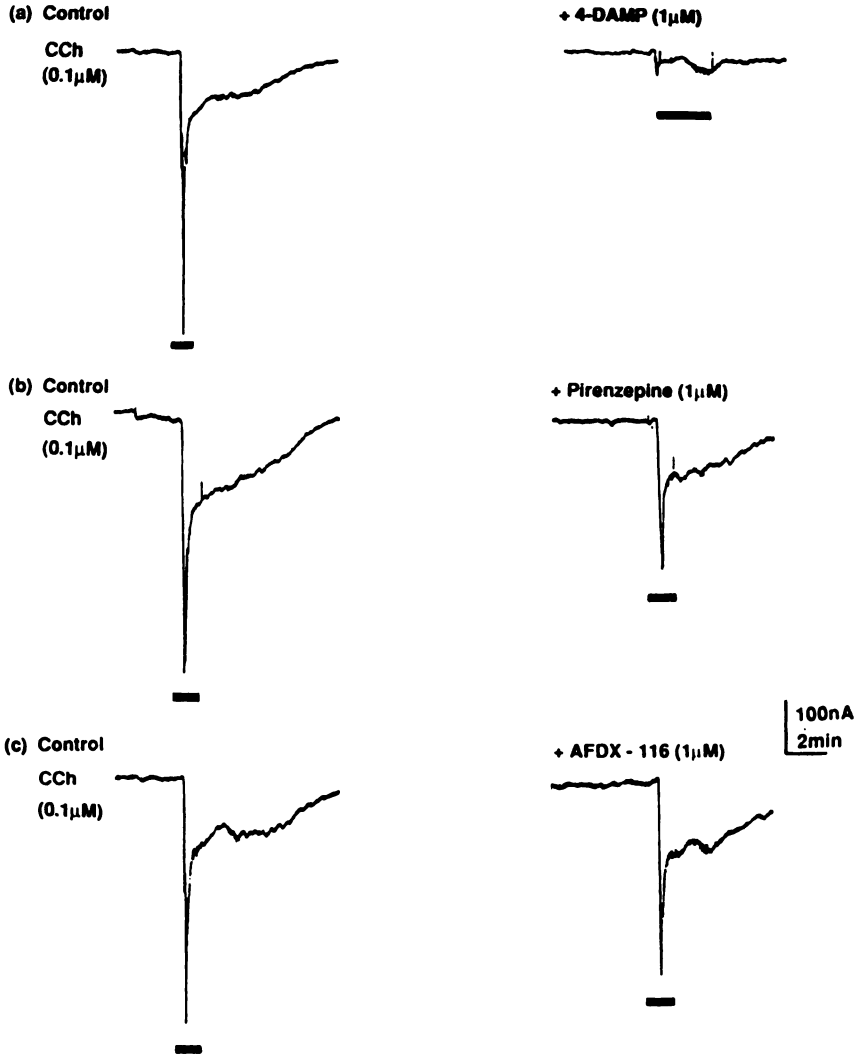

Fig. 3. Inhibition by muscarinic antagonists of carbamylcholine (*CCh*)-induced currents in nuclearly injected *Xenopus* oocytes. Nuclearly injected oocytes were exposed to 0.1 μM carbamylcholine before being treated for 5 min with mAChR antagonists. a, Control carbamylcholine response and antagonist blockade by 1 μM 4-DAMP. b, Inhibition of carbamylcholine-induced responses by 1 μM pirenzepine. c, Reduction in agonist response by 1 μM AFDX-116. Solid bars below each electrophysiological trace denote CCh application. The results are representative of three or four independent experiments.

and 5 MΩ. In all experiments the holding potential was set at −60 mV. The holding potential and transmembrane currents were monitored on separate channels of an oscilloscope and displayed simultaneously on a dual-channel Gould (BS-272) chart recorder.

Drugs were applied to individual oocytes that had been immobilized in an experimental chamber (0.5-ml volume) and perfused continuously with SOS at 2.0 ml/min. All agonists were bath applied in SOS for 1–2 min and antagonists were applied for 5 min.

Generation of an mAChR antipeptide antibody. A carboxyl-terminal peptide corresponding to amino acids 772–778 of the predicted protein sequence of the Dm1 clone (16) was synthesized on a Biosearch 9500 synthesizer using *N-tert*-butoxycarbonyl chemistry. For each synthesis, 0.5 g of *t*-butoxycarbonyl-alanine-*O*-resin was used. The reaction was started at −196°, allowed to warm to 0° over 80 min, and then maintained at 0° for an additional 40 min to complete the reaction. Cleavage of the peptide from the resin was achieved using 10 ml of hydrogen fluoride and 1 ml of anisole.

The peptide was washed from the resin with glacial acetic acid and water, collected, and freeze dried. To purify the peptide, it was dissolved in 20 ml of 67% acetic acid and eluted at 4° through a Biogel P2 column. The eluted fractions were pooled and lyophilized for storage.

For immunization, the peptide was conjugated to a purified protein derivative of tuberculin (24), using sulfosuccinimidyl 4-(*N*-maleoimidomethyl)cyclohexane-1-carboxylate (Pierce Chemical Co.). Conjugation was performed overnight under nitrogen at pH 7.0. The conjugate was diluted in distilled water and stored at −70° until used.

Dutch rabbits were subcutaneously primed with BCG vaccine before immunization with the peptide-purified protein derivative conjugate. Initial injections were subcutaneous and intramuscular, followed by three subcutaneous boosts at 3-week intervals. Each rabbit was bled 10 days after injection. Preimmune serum was collected from the rabbits before the BCG priming.

The antibody titer of the rabbit antiserum to the peptide was determined by enzyme-linked immunoabsorbent assay. Immulon plates (96-well) were coated with 50 μg of the antigen diluted in 0.1 M $NaHCO_3$, pH 9.2, and were incubated overnight at room temperature. Excess antigen was removed and nonspecific antibody binding was blocked with 2% dried skim milk in Tris-buffered saline for 1 hr at room temperature. Excess blocking agent was removed and the antiserum was applied to the wells at dilutions between 1/100 and 1/51,200, in PBS, for 1 hr at room temperature. The plates were then washed four times with Tris-buffered saline containing 0.05% Tween 20 and four times with distilled water. A horseradish peroxidase-conjugated second antibody was added at a dilution of 1/1000 in PBS, and the plates were incubated for 1 hr at room temperature. The plates were again rinsed with Tris-buffered saline, and *o*-phenylenediamine dihydrochloride (0.4 mg/ml in citrate buffer with 0.01% H_2O_2) was added for 10 min at 37°. The reaction was stopped with 2.5 M H_2SO_4 and the results were determined at 492 nm with a spectrophotometer.

Immunocytochemistry. Adult male *Drosophila melanogaster* (Canton-S) were immobilized at 4° and then dissected under Bouin's fixative at room temperature. The proboscis, mouthparts, and legs were removed to enhance access. Tissues were fixed for 18 hr, dehydrated through a graded series of ethanols to 70%, and immersed in Superced-

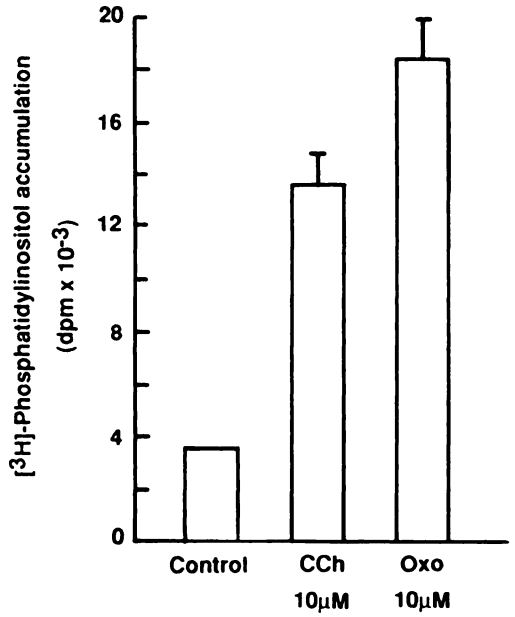

Fig. 4. Effect of carbamylcholine (*CCh*) and oxotremorine (*Oxo*) on inositol phosphate levels in transfected COS-7 cells. Inositol phosphate levels were measured in 24-well dishes as described in Experimental Procedures. The results presented are the mean ± standard error of three independent experiments.

rol (BDH) at 50°, with several changes, before an overnight incubation. The flies were blocked in paraffin wax, and 8–10-μm sections were cut in the horizontal plane and dried onto slides coated with 0.8 mg/ml poly-L-lysine. Before immunocytochemistry, the wax was removed with xylene and the tissue sections were rehydrated through a graded series of alcohols (5 min each) before being placed in PBS.

An immunoperoxidase procedure based on avidin-biotin was used. After brief rinsing of the sections in distilled water, endogenous peroxidase was quenched with 0.3% H_2O_2 in methanol for 30 min, followed by a wash in PBS. To increase the permeability of the membranes, the tissues were treated for 5 min with 3 mg/ml sodium deoxycholate in PBS, transferred to PBS containing 0.4% Triton X-100, and then transferred to incubation medium without antibody for 2 hr. At the end of this time excess solution was withdrawn from the sections by capillary action through a filter paper. The sections were then covered with primary antibody (1/100 final dilution) or a peptide conjugate-preabsorbed antibody control incubation medium, placed under coverslips, and incubated at 4° overnight. Coverslips were floated off the sections with PBS and, after an additional rinse in PBS, the sections were incubated in diluted biotinylated secondary antibody for 2 hr at room temperature. After rinsing with PBS, the ABC reagent (Vector Laboratories) was added for 1 hr at room temperature, followed by another wash in PBS. The sections were exposed to a diaminobenzamidine solution for times between 2 and 10 min, depending upon the observed intensity of the reaction. A positive reaction resulted in a brown-black coloration within the tissue section. The sections were then rinsed in PBS followed by tap water, dehydrated through an ascending series of alcohols to xylene, and mounted. The slides were analyzed on a Leitz Diaplan microscope.

The specificity of the antipeptide antibody for the Dm1 gene product was confirmed by indirect immunofluorescence on transiently receptor-expressing COS-7 cells (data not shown). However, this does not rule out the possibility that this antibody may cross-react with additional, uncharacterized, mAChRs in the *Drosophila* nervous system.

Results

To determine the pharmacological properties of the *Drosophila* mAChR, Dm1, this gene was subcloned into a eukaryotic expression plasmid and transiently expressed in either COS-7 cells or *X. laevis* oocytes. Using [^{3}H]QNB and [^{3}H]NMS, saturable, high affinity, mAChR binding was detected at the cell surface of transfected COS-7 cells but not in untransfected controls. Displacement of [^{3}H]NMS was carried out using unlabeled mAChR antagonists. For the functional assessment of antagonist inhibition of agonist-induced responses, nuclearly injected *Xenopus* oocytes were used. The application of mAChR agonists to these transiently receptor-expressing cells stimulated a Ca^{2+}-activated Cl^- current. This allowed a comparision of the three mAChR antagonists, 4-DAMP, pirenzepine, and AFDX-116, in the blockade of carbamylcholine-stimulated currents. Additional information on the agonist pharmacology of Dm1 was obtained by monitoring inositol phosphate accumulation in transfected COS-7 cell monolayers, which were stimulated with either carbamylcholine or oxotremorine. Basal levels of inositol phosphate accumulation were determined in the absence of agonist.

The presence of the Dm1 gene product in the nervous system of *Drosophila* was verified and localized by immunocytochemistry with a polyclonal antipeptide antibody raised against the predicted carboxyl-terminal amino acid sequence of the receptor.

[^{3}H]QNB and [^{3}H]NMS binding studies with COS-7 cells. Transiently receptor-expressing cells were examined for their ability to bind the nonselective mAChR antagonists [^{3}H]QNB and [^{3}H]NMS. No specific binding of either radiolabeled antagonist was found in untransfected cells. However, the Dm1-transfected cells demonstrated high affinity and saturable specific binding for [^{3}H]QNB and [^{3}H]NMS, using 1 μM atropine to define nonspecific binding (data not shown). In agreement with a previous study on a mouse adrenal carcinoma cell line that was stably transfected with Dm1 (16), high affinity [^{3}H]QNB binding was found on COS-7 cell membranes (K_d = 90 ± 9 pM) (Table 1). The apparent dissociation constant for [^{3}H]NMS was lower (K_d = 520 ± 120 pM) (Table 1), with both labeled antagonists recognizing an equivalent total population of receptors on the COS-7 cells (B_{max} = 2.0 ± 0.6 and 2.5 ± 0.7 pmol/mg of protein for [^{3}H]NMS and [^{3}H]QNB, respectively). Hill coefficients derived from the binding data were not significantly different from unity (data not shown). Similiar binding results were also obtained with intact cell monolayers (Table 1).

Competition studies with mAChR antagonists on COS-7 membranes. The ability of four mAChR antagonists to compete for [^{3}H]NMS binding was examined. These antagonists have been previously characterized in vertebrates by using both native tissue preparations and stably transfected cell lines expressing each of the five cloned vertebrate mAChRs, m1-m5 (3, 25). The antagonists atropine and 4-DAMP appear to have high affinities for all mAChR subtypes examined, although 4-DAMP has been reported to have the lowest affinity for the expressed m2 mAChR (25). The antagonist pirenzepine has been shown to have the highest affinity for the vertebrate M_1 subtype and has higher affinity for the expressed m1 mAChR than for the other subtypes studied (3, 25, 26). AFDX-116 has been used to discriminate between the M_1, M_2, and M_3 receptor subtypes, although it has been recently reported to bind with equally high affinity to the expressed m2 and m4 mAChRs (26). The relative potencies of these compounds for inhibition of [^{3}H]NMS binding to Dm1-expressing COS-7 cell membranes

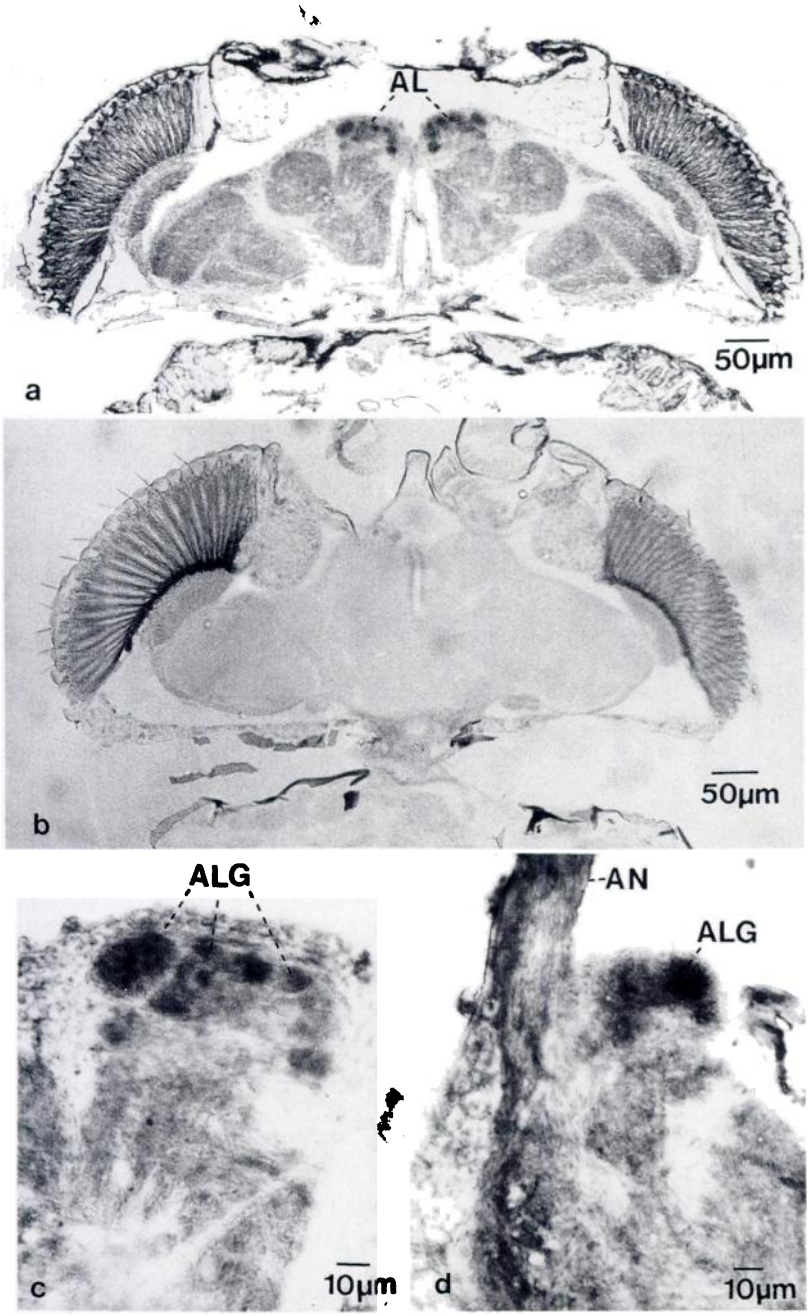

Fig. 5. Immunocytochemical localization of an anti-peptide antibody raised against the predicted carboxyl-terminal domain of a cloned *Drosophila* mAChR. Horizontal *Drosophila* sections were prepared as described in Experimental Procedures. a, Intense immunostaining is observed in the antennal lobes (*AL*); b, control section, using preabsorbed antibody, showing the absence of specific staining; c, detail of antennal lobe region, showing the staining of glomeruli (*ALG*); d, section dorsal to a, showing staining in regions where the antennal nerve fibers enter the antennal lobe (*AN*).

are shown in Fig. 1. In rank order of inhibition, atropine was found to be most effective in displacing [^{3}H]NMS binding, followed by 4-DAMP (K_i = 14 nM), pirenzepine (K_i = 570 nM), and AFDX-116 (K_i = 11 μM) (Fig. 1). Hill coefficients for the antagonist competition binding data were not significantly different from unity (data not shown). These data demonstrate that the Dm1 gene transiently expresses an mAChR when introduced into COS-7 cells, in agreement with the stable cell line expression work (16). The pharmacology of this mAChR generally resembles that of a vertebrate M_3 type, based upon the intermediate affinity for pirenzepine and low affinity for AFDX-116. However, the assignment of a subtype classification is difficult, given the variablity in antagonist affinities that can occur with expression in different cell lines and different assay conditions (26, 27).

Electrophysiological characterization of nuclearly injected oocytes. To further explore the pharmacology of the *Drosophila* mAChR, functional expression in *X. laevis* oocytes was used. Oocytes have been used extensively for the investigation of mammalian mAChR pharmacology and provide a reliable system for monitoring functional receptor responses (4). After introduction of the Dm1 cDNA-containing expression plasmid into the nuclei of oocytes, it was possible to measure large agonist-stimulated Ca^{2+}-activated Cl^- currents. Inward current responses of 175–225 nA were achieved using 0.01 μM carbamylcholine (Fig. 2a). At the holding potential of −60 mV, the current resulting from carbamylcholine applications reversed at approximately −21 mV ± 3 (n = 5), close to the equilibrium potential for chloride ions (28). This reversal potential of the inward current is consistent with the stimulation

of a Ca^{2+}-activated Cl^- current. This has been shown by others to occur in oocytes that have been injected with *in vitro* transcribed RNA derived from the m1 and m3 receptor subtype genes (4). These results indicate that *Xenopus* oocytes are fully capable of producing a functional mAChR response when the Dm1 cDNA is introduced into the nuclei of the cells in a suitable expression vector. No agonist-induced response was obtained in control uninjected oocytes (n = 23; Fig. 2b) or oocytes injected with the expression vector containing the bacterial reporter gene chloramphenicol acetyltransferase (n = 18; Fig. 2c). However, one batch of oocytes from a single animal did show evidence of an endogenous mAChR response, although the response differed from that obtained with Dm1-injected oocytes (Fig. 2d). This endogenous mAChR activity in the control oocytes may correspond to responses that were previously characterized in a small subpopulation of toads (29).

Dose-dependent responses to carbamylcholine were achieved over a range of 0.001–100 μM (Fig. 2e). This concentration range for activation of a mAChR is in agreement with previous work that examined the agonist responses of the vertebrate m1 and m3 subtypes (4). An EC_{50} of approximately 0.5 μM is in agreement with these earlier studies.

Inhibition of agonist-induced currents in oocytes. To examine the functional effects of the subtype-selective mAChR antagonists characterized in the cell line binding studies, Dm1-injected oocytes were stimulated with 0.1 μM carbamylcholine and then individually exposed for 5 min to 1 μM concentrations of each of the antagonists, i.e., 4-DAMP, pirenzepine, or AF-DX 116. As shown in Fig. 3a, agonist-induced currents were abolished by 99 ± 1% with 1 μM 4-DAMP (n = 6), whereas 1 μM pirenzepine reduced the current response by approximately 50% of the carbamylcholine-stimulated value (n = 4; Fig. 3b). AFDX-116 was the least effective in reducing the agonist stimulation, with a 16 ± 4% decrease of the control response (n = 4; Fig. 3c). These results demonstrate that the Dm1-expressing oocytes are producing a functional response that has an antagonist pharmacological profile that is in agreement with the COS-7 cell line binding studies.

Inositol phosphate stimulation in transfected COS-7 cells. An assessment of the ability of two mAChR agonists, carbamylcholine and oxotremorine, to stimulate inositol phosphate accumulation in transiently receptor-expressing COS-7 cells was undertaken. By labeling cell monolayers with *myo*-[^{3}H]inositol, it is possible to monitor changes in inositol phosphate accumulation (23). Carbamylcholine was previously shown to increase inositol phosphate accumulation in mouse adrenal cells that stably express the Dm1 clone (16). It was of interest to determine whether this invertebrate receptor also demonstrated a similar increase in inositol accumulation in response to the mAChR agonist oxotremorine. Fig. 4 shows that transfected COS-7 cells provided an increase in labeled inositol phosphates when stimulated by either carbamylcholine or oxotremorine. Equivalent responses were obtained with the two agonists, with 10 μM carbamylcholine stimulating an approximately 3.5-fold increase in inositol phosphate production, relative to the control. Oxotremorine stimulated inositol phosphate accumulation in an analogous manner, with a 4.5-fold accumulation occurring at 10 μM. This increase in inositol phosphates is comparable to that achieved with carbamylcholine. These results suggest that the Dm1 pharmacology of the transiently receptor-expressing COS-7 cells resembles that of the stably expressed receptor in mouse adrenal cells (16).

Immunocytochemical localization of the mAChR in the *Drosophila* nervous system. To verify the existence of the Dm1 gene product in the *Drosophila* nervous system, a polyclonal antipeptide antibody was used. Antisera generated against the predicted carboxyl-terminal domains of G protein-coupled receptors have proven to be effective in immunological studies of this receptor superfamily (30). Antibody staining of *Drosophila* sections appeared to be confined to the nervous system (Fig. 5). No positive staining was detected on muscle tissue. Within the nervous system, the staining was pronounced in the antennal lobes (Fig. 5a). This region receives chemosensory afferent inputs from the antennae; there is considerable evidence that acetylcholine is the primary afferent neurotransmitter in insects (17, 31). For example, immunocytochemical localization of the enzyme choline acetyltransferase has been detected in the antennal lobes of *Drosophila* (32), where [^{3}H]cholilne uptake has also been detected (33). The specificity of the antennal lobe staining was demonstrated on a corresponding control section exposed to antibody that had been preabsorbed with the peptide conjugate, as described in Experimental Procedures. There was no evidence of specific staining in this horizontal section (Fig. 5b). However, intense staining was present in the neuropiles of the antennal lobe glomeruli (Fig. 5c). An additional area of staining was evident in a more dorsal section, indicating the presence of immunoreactivity near the point of entry for the antennal nerve bundles connecting to the antennal lobe (Fig. 5d). Additional staining occurred in the region of the thoracic ganglion (data not shown). Our results (Fig. 5, a and c) demonstrate for the first time that a mAChR that shares pharmacological characteristics with the vertebrate mAChR subtypes is present in the antennal lobes, suggesting a role for the mAChR in the processing of olfactory information from the antennae.

Discussion

This study presents a pharmacological and functional characterization of a cloned mAChR of *Drosophila* (16). We have used either transfected COS-7 cells or nuclearly injected *X. laevis* oocytes to characterize the binding and functional characteristics of this invertebrate gene. Both systems have been used extensively in studies on vertebrate mAChR subtypes (4, 34). By using two independent transient expression systems, it has been possible to show that the mAChR antagonist atropine is more potent than 4-DAMP, pirenzepine, or AFDX-116 in displacing [^{3}H]NMS binding to COS-7 membranes. The relative affinities of pirenzepine and AFDX-116 have been previously used to define mAChR subtypes in vertebrates (1, 3). In agreement with the binding studies, 4-DAMP was also found to be more potent than pirenzepine when carbamylcholine-activated currents were inhibited on *Xenopus* oocytes. AFDX-116 was found to be the least effective antagonist of the series studied here.

The binding and electrophysiological results presented here are clearly consistent with work that was carried out on neuronal tissue preparations from three invertebrate species (13). In this study the rank order of potency for antagonist displacement of [^{3}H]QNB was found to be 4-DAMP > pirenzepine > AFDX-116. Taken together, our heterologous expression studies and the work on native insect tissues with subtype-selective

antagonists suggest that the invertebrate nervous system contains at least one mAChR that resembles mAChRs of the vertebrate M_1 and M_3 subtypes.

To identify regions of the fly nervous system where mAChR expression occurs, we have used a polyclonal antiserum raised against the predicted carboxyl-terminal region of the *Drosophila* mAChR. This type of approach, using gene-specific antisera, proved to be a valuable tool for mapping regions of mAChR expression in the vertebrate nervous system. That study (5) used subtype-specific antisera as a means of showing that regions of the rat central nervous system can contain several mAChR subtypes. A similar situation may occur in the invertebrate nervous system; however, to date, only a single mAChR gene has been isolated (15, 16). By using immunocytochemistry with an antipeptide antibody, we have been able to demonstrate the presence of this mAChR in neuropiles of the *Drosophila* nervous system. Although no specific staining was apparent in muscle tissue, intense specific staining was detected in the glomeruli of the antennal lobes. Additionally, antibody staining appeared in the antennal lobes around the entry point of the antennal nerve fiber bundle. Both regions were previously shown to be areas of high density [^{3}H]choline uptake, indicating a likely role for cholinergic activity in olfactory processes (33). The localization of this mAChR gene product to the antennal glomeruli, a region that contains terminals of antennal sensory neurons (17), suggests that it may play a role in processing olfactory signals in the fly. It is of interest to note that a recent report has demonstrated the presence of inositol-1,4,5-triphosphate receptor mRNA in the antennae of *Drosophila* (35).

Several studies on mAChRs in insect nervous tissue have indicated the possibility of additional mAChR subtypes. Those reports relied primarily on the ability of pirenzepine to discriminate between M_1- and M_2-type pharmacologies (12, 14, 36–38). Of interest is the demonstration that two putative mAChR subtypes are differentially located in the locust (12, 37) and cockroach (14, 38) nervous systems. Whether the previously designated M_1 receptor subtypes are the homologs of *Drosophila* Dm1 remains to be determined, but preliminary data on cross-hybridization Southern blotting of locust genomic DNA with the Dm1 cDNA indicate the presence of a homologous gene.[1]

The work reported here represents the first characterization of a cloned invertebrate mAChR using a series of mAChR antagonists. The immunocytochemical localization of this mAChR to the antennal lobes of the insect nervous system provides evidence for a putative involvement of this receptor in first-order synaptic processing of antennal sensory information. Although we cannot exclude the possibility that our antiserum cross-reacts with additional mAChR subtypes in the fly, it is apparent from our transient gene expression studies that this cloned mAChR has an antagonist pharmacology that has similiarities to that of vertebrate mAChR subtypes.

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