Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 9039–9043, November 1989
Pharmacology

Characterization and functional expression in mammalian cells of genomic and cDNA clones encoding a *Drosophila* muscarinic acetylcholine receptor

(guanine nucleotide-binding protein/phospholipase C/Y1 adrenal carcinoma cells/intron)

ROBERT A. SHAPIRO*, BARBARA T. WAKIMOTO†, EMILY M. SUBERS*, AND NEIL M. NATHANSON*‡

*Department of Pharmacology, SJ-30, and †Department of Zoology, NJ-15, University of Washington, Seattle, WA 98195

Communicated by William A. Catterall, August 16, 1989 (received for review July 12, 1989)

ABSTRACT **Genomic and cDNA clones encoding a muscarinic acetylcholine receptor from *Drosophila melanogaster* have been isolated. Sequence analysis demonstrates that this gene encodes a receptor with a high degree of amino acid identity to the mammalian muscarinic acetylcholine receptors and has three introns in the portion of the gene encoding the third putative cytoplasmic loop. A full-length cDNA clone has been placed under the control of the mouse metallothionein promotor and transfected into mouse Y1 adrenal cells. The receptor expressed in these cells exhibits the high-affinity binding for the antagonists quinuclidinyl benzilate and atropine expected of a muscarinic receptor. The *Drosophila* muscarinic receptor, when expressed in Y1 cells, is physiologically active, as measured by agonist-dependent stimulation of phosphatidylinositol metabolism.**

Muscarinic acetylcholine receptors (mAcChoRs) belong to a family of receptors which are involved in the activation of various signal transduction pathways through their interaction with guanine nucleotide-binding proteins (G proteins) (1). These receptors, which include the five subtypes of mammalian mAcChoR (2–9), the adrenergic receptors (10, 11), and the opsin pigments (12), share a predicted seven transmembrane domain structure with highly conserved amino acid sequences, especially within certain transmembrane regions. The region of least identity occurs in a proposed cytoplasmic loop located between the fifth and sixth transmembrane regions, which has been suggested to be involved in the interaction of the receptors with specific G proteins (13, 14). Experiments using point and deletion mutations and chimeric receptors indicate that the membrane-proximal portions of this loop most likely determine the specificity of functional coupling (15–18).

Acetylcholine is believed to be a major excitatory transmitter in the central nervous system of insects (19). Many of the components involved in cholinergic transmission in *Drosophila* have been studied by using genetic and molecular biological approaches. Thus, mutations in both choline acetyltransferase and acetylcholinesterase have behavioral and physiological effects and can cause lethality (20–22). The distribution of both proteins in the developing *Drosophila* nervous system has been determined immunocytochemically (23, 24), and cDNA clones encoding both enzymes as well as nicotinic receptor subunits have been isolated (25–29). In contrast, except for biochemical identification of mAcChoR binding sites (30), little information is available as to the nature or specific subtypes of mAcChoR present in *Drosophila* or their location or function. In this paper we describe the isolation of cDNA and genomic clones encoding a *Drosophila* mAcChoR and demonstrate that the cloned *Drosophila* mAcChoR activates phospholipase C (PLC) when expressed in mouse cells.§ The identification and localization of this gene will greatly facilitate analysis of the role of mAcChoR in insects and the biochemical and genetic dissection of its signal transduction pathway.

MATERIALS AND METHODS

Isolation of Molecular Clones. A genomic library from the wild-type Oregon R strain of *Drosophila melanogaster* (Lambda GEM-11; Promega) was probed with a mixture consisting of a nick-translated full-length cDNA clone encoding the pig m2 mAcChoR (5) and a nick-translated 1.8-kilobase (kb) *Kpn* I–*Bam*HI genomic fragment of the mouse m1 mAcChoR (9). Filters containing the phage were hybridized under moderately stringent conditions (60°C, 6× SSC) and washed at 37°C, 1× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7). DNAs from seven positive clones were isolated and mapped, and a 7.5-kb *Bam*HI fragment found in five of the seven clones was subcloned in the plasmid vector pGEM-3 (Promega).

A *Drosophila* Oregon R head cDNA library constructed in Lambda Zap (Stratagene) was hybridized (65°C, 6× SSC) with a nick-translated 510-base-pair (bp) *Pst* T fragment isolated from the 7.5-kb genomic *Bam*HI/pGEM subclone followed by washing at 55°C in 0.5× SSC. DNA from the 4 out of 12 positive phage that yielded the strongest hybridization signals were analyzed by partial restriction mapping. The cDNAs from isolated plaques were converted into the plasmid form (pBSK) of Lambda Zap according to the supplier's recommendations. Further subcloning and sequencing of both strands were performed in pGEM-3 by the dideoxy chain-termination method using Sequenase (United States Biochemical) according to the supplier's recommendations.

***In Situ* Hybridization to Polytene Chromosomes.** Salivary gland chromosomes from the third instar larvae of the wild-type Canton S *Drosophila* strain were prepared according to the procedure of Todd Laverty (University of California, Berkeley; personal communication). Biotinylated probes were prepared by nick-translating the 7.5-kb genomic *Bam*HI/pGEM subclone using biotinylated-16-dUTP (Enzo Biochemicals). The hybridization conditions (0.6 M NaCl/50 mM $NaPO_4$, pH 6.8/5 mM $MgCl_2$/0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone) and washes (2× SSC, 53°C) and detection with the Detek-1-HRP kit (Enzo Biochemicals) were as described by Langer-Sofer *et al.* (32) with modifications developed by Todd Laverty (University of California, Berkeley; personal communication).

Abbreviations: mAcChoR, muscarinic acetylcholine receptor; QNB, quinuclidinyl benzilate; PtdIns, phosphatidylinositol; PLC, phospholipase C; G protein, guanine nucleotide-binding protein; Ins-*P*s, inositol phosphates.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

‡To whom reprint requests should be addressed.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M27495).

Cell Culture and Expression of mAcChoRs. A 2.5-kb *Eco*RI DNA fragment (FCLR1) containing the entire coding region of the *Drosophila* mAcChoR cDNA plus 172 bp of 5′ noncoding sequences and 165 bp of 3′ noncoding sequences was isolated. The fragment was modified by filling in the ends with the Klenow fragment of DNA polymerase and attaching *Bgl* II linkers and was ligated into the *Bam*HI site of the expression vector ZEM228 (9). This vector allows zinc-inducible expression of the mAcChoR via the metallothionein promoter and also encodes the gene for neomycin phosphotransferase, allowing selection for resistance to the antibiotic G418. Mouse Y1 adrenal carcinoma cells were transfected with FCLR1/ZEM228 and individual G418-resistant subclones were isolated and tested for the ability to bind tritiated quinuclidinyl benzilate ([^{3}H]QNB) as described (9).

Ligand Binding, Inositolphospholipid Hydrolysis, and cAMP Accumulation Assays. The binding of the muscarinic antagonist [^{3}H]QNB to mAcChoR in crude membrane homogenates and antagonist/[^{3}H]QNB and agonist/[^{3}H]QNB competition experiments were carried out as described (9) and analyzed by using the LIGAND program (33). The ability of carbachol to stimulate phosphatidylinositol (PtdIns) breakdown and to alter forskolin-stimulated cAMP accumulation in intact cells was measured as described previously (9).

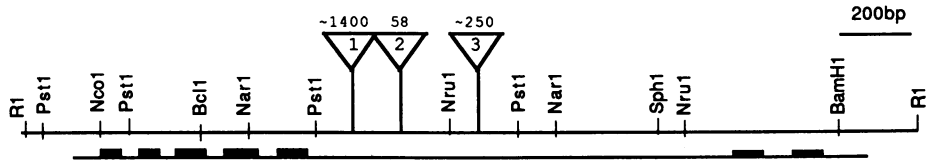
RESULTS

Isolation, Sequence, and Gene Structure of a *Drosophila* mAcChoR Gene. A *Drosophila* mAcChoR gene was isolated from an Oregon R genomic library by using as a hybridization probe a combination of a genomic DNA fragment encoding the mouse m1 mAcChoR (9) and the porcine m2 mAcChoR cDNA (5). A 7.5-kb *Bam*HI fragment was identified that strongly hybridized to the mouse DNA probe under moderate conditions. Partial sequence analysis and subsequent restriction mapping showed that this fragment encoded a protein with considerable amino acid similarity to the sixth and seventh transmembrane regions of the mouse m1 mAcChoR but that the region between the 5th and 6th transmembrane regions was exceptionally long relative to the predicted products of previously isolated mAcChoR genes, suggesting the presence of an intron (or introns) in the third cytoplasmic loop. A *Drosophila* head cDNA library was then screened with a 510-bp genomic *Pst* I fragment encoding transmembrane regions two through five. DNA from four positive clones was isolated and partially sequenced. Two clones were shown, by sequence comparison and restriction analysis, to contain the entire coding region of a *Drosophila* mAcChoR. Southern blot analysis using the 510-bp genomic *Pst* I fragment as probe at moderate stringency (hybridization at 60°C, 6× SSC, followed by washing at 37°C, 1× SSC) shows a single strongly hybridizing band, suggesting that this mAcChoR is encoded by a single-copy gene (data not shown). A few weakly hybridizing bands were observed which may represent either other subtypes of *Drosophila* mAcChoR or other members of the G-protein-coupled receptor gene family.

The complete cDNA sequence of one clone (FCLR1) revealed a single open reading frame encoding a protein of 722

```
        GAATTCCCAACGACGACGTTGGCGGCCGGCGATGACTTCGACGACGTGGACC -120
GGAGACGTGGACGAGTCCTGCAGGGTATTCGCCAGGATACCCGGGCACACTCTACTGACC  -60
GCCCTGTTCGAGAACCTGACGTCCACGGCAGCGAGCGGGCTATACGATCCCTATTCGGGA   -1

ATGTACGGAAACCAGACGAACGGCACCATCGGCTTCGAGACGAAGGGACCTCGGTACTCA   60
MetTyrGlyAsnGlnThrAsnGlyThrIleGlyPheGluThrLysGlyProArgTyrSer   20

CTGGCCTCCATGGTGGTCATGGGCTTCGTGGCGGCGATTTTGAGCACGGTGACGGTGGCG  120
LeuAlaSerMetValValMetGlyPheValAlaAlaIleLeuSerThrValThrValAla   40

GGCAATGTCATGGTGATGATATCCTTTAAGATCGACAAGCAGCTGCAGACGATCAGCAAC  180
GlyAsnValMetValMetIleSerPheLysIleAspLysGlnLeuGlnThrIleSerAsn   60

TACTTCTTGTTCTCGCTGGCCATCGCGGACTTCGCCATTGGCACTATATCGATGCCGCTT  240
TyrPheLeuPheSerLeuAlaIleAlaAspPheAlaIleGlyThrIleSerMetProLeu   80

TTTGCGGTGACCACAATCCTGGGCTACTGGCCCTTGGGTCCCATCGTCTGCGACACCTGG  300
PheAlaValThrThrIleLeuGlyTyrTrpProLeuGlyProIleValCysAspThrTrp  100

CTGGCCCTGGACTACCTGGCATCGAATGCTTCAGTGCTGAACCTGCTGATCATCAATTTC  360
LeuAlaLeuAspTyrLeuAlaSerAsnAlaSerValLeuAsnLeuLeuIleIleAsnPhe  120

GATCGGTACTTCAGTGTCACTCGACCGCTGACGTACCGCGCAAAAGGAACCACCAACCGG  420
AspArgTyrPheSerValThrArgProLeuThrTyrArgAlaLysGlyThrThrAsnArg  140

GCGGCGGTGATGATTGGCGCCTGGGGCATCAGCCTGCTCCTCTGGCCGCCCTGGATCTAC  480
AlaAlaValMetIleGlyAlaTrpGlyIleSerLeuLeuLeuTrpProProTrpIleTyr  160

AGCTGGCCCTACATCGAGGGCAAGCGGACGGTGCCCCAAGGACGAGTGCTACATCCAGTTC  540
SerTrpProTyrIleGluGlyLysArgThrValProLysAspGluCysTyrIleGlnPhe  180

ATCGAGACCAACCAGTACATCACCTTCGGCACGGCACTGGCCGCCTTCTACTTCCCGGTC  600
IleGluThrAsnGlnTyrIleThrPheGlyThrAlaLeuAlaAlaPheTyrPheProVal  200

ACCATCATGTGCTTCCTCTACTGGCGCATCTGGCGCGAGACGAAAAAGCGGCAGAAGGAC  660
ThrIleMetCysPheLeuTyrTrpArgIleTrpArgGluThrLysLysArgGlnLysAsp  220

CTGCCCAACCTGCAGGCGGGCAAGAAAGACTCCAGCAAGAGATCAAACAGCAGTGACGAA  720
LeuProAsnLeuGlnAlaGlyLysLysAspSerSerLysArgSerAsnSerSerAspGlu  240

AACACGGTGGTGAATCACGCCTCTGGGCCCCTGCTGGCCTTCGCCCAGGTGGGCGGAAAC  780
AsnThrValValAsnHisAlaSerGlyProLeuLeuAlaPheAlaGlnValGlyGlyAsn  260

GACCATGACACCTGGCGACGTCCGCGCTCCGAGAGTTCAGCGGACGCGGAGAGTGTCTAC  840
AspHisAspThrTrpArgArgProArgSerGluSerSerAlaAspAlaGluSerValTyr  280

ATGACCAACATGGTCATCGACTCCGGCTACCACGGGATGCACTCGCGCAAGTCAAGCATC  900
MetThrAsnMetValIleAspSerGlyTyrHisGlyMetHisSerArgLysSerSerIle  300

AAAAGCACGAATACAATCAAAAAATCGTACACCTGCTTCGGCAGCATCAAGGAGTGGTGC  960
LysSerThrAsnThrIleLysLysSerTyrThrCysPheGlySerIleLysGluTrpCys  320

ATTGCGTGGTGGCACTCCGGTCGCGAGGACTCCGACGACTTCGCCTACGAGCAGGAGGAG 1020
IleAlaTrpTrpHisSerGlyArgGluAspSerAspAspPheAlaTyrGluGlnGluGlu  340

CCTTCTGATTTAGGGTATGCAACACCAGTAACAATTGAAACTCCTTTACAAAGCTCCGTC 1080
ProSerAspGluGlyTyrAlaThrProValThrIleGluThrProLeuGlnSerSerVal  360

TCCAGATGCACCTCGATGAACGTGATGCGGGACAACTACTCGATGGGCGGCAGCGTGAGC 1140
SerArgCysThrSerMetAsnValMetArgAspAsnTyrSerMetGlyGlySerValSer  380

GGCGTGCGTCCGCCCAGCATTCTCCTGTCGGATGTCTCGCCCACGCCGCTGCCACGCCCA 1200
GlyValArgProProSerIleLeuLeuSerAspValSerProThrProLeuProArgPro  400

CCGCTTGCCTCCATCTCGCAGCTGCAGGAAATGAGTGCGGTTACGGCGAGCACAACGGCG 1260
ProLeuAlaSerIleSerGlnLeuGlnGluMetSerAlaValThrAlaSerThrThrAla  420

AATGTGAATACCAGTGGCAATGGCAATGGCGCCATCAACAACAACAACAACGCCAGCCAC 1320
AsnValAsnThrSerGlyAsnGlyAsnGlyAlaIleAsnAsnAsnAsnAsnAlaSerHis  440

AACGGGAACGGCGCAGTGAACGGGAATGGGACCGGAAACGGAAGTGGAATCGGACTGGGT 1380
AsnGlyAsnGlyAlaValAsnGlyAsnGlyThrGlyAsnGlySerGlyIleGlyLeuGly  460

ACAACCGGAAATGCGACGCACCGCGACTCGCGCACTCTGCCCGTTATCAATCGCATTAAC 1440
ThrThrGlyAsnAlaThrHisArgAspSerArgThrLeuProValIleAsnArgIleAsn  480

TCACGGTCGGTGAGCCAGGACTCCGTGTACACGATACTCATCCGCCTGCCTTCCGACGGC 1500
SerArgSerValSerGlnAspSerValTyrThrIleLeuIleArgLeuProSerAspGly  500

GCATCCAGCAACGCGGCCAACGGAGGAGGGGGTGGCCCTGGTGCCGGGGCAGCTGCCTCC 1560
AlaSerSerAsnAlaAlaAsnGlyGlyGlyGlyGlyProGlyAlaGlyAlaAlaAlaSer  520

GCGTCCTTGAGCATGCAAGGCGACTGTGCGCCCTCCATCAAGATGATACACGAGGACGGG 1620
AlaSerLeuSerMetGlnGlyAspCysAlaProSerIleLysMetIleHisGluAspGly  540

CCCACGACGACGGCAGCGGCAGCTCCTTTGGCAAGCGCCGCAGCCACACGACGACCTTTG 1680
ProThrThrThrAlaAlaAlaAlaProLeuAlaSerAlaAlaAlaThrArgArgProLeu  560

CCCTCGCGAGACTCTGAGTTCAGCCTGCCACTCGGACGGAGGATGTCCCATGCCCAGCAC 1740
ProSerArgAspSerGluPheSerLeuProLeuGlyArgArgMetSerHisAlaGlnHis  580

GATGCCAGGCTACTCAACGCCAAGGTCATACCCAAGCAGCTGGGCAAGGCTGGGGGCGGG 1800
AspAlaArgLeuLeuAsnAlaLysValIleProLysGlnLeuGlyLysAlaGlyGlyGly  600

GCAGCGGGCGGCGGATGCGGAGCCCACGCCCTTATGAACCCCAATGCTGCCAAGAAGAAA 1860
AlaAlaGlyGlyGlyCysGlyAlaHisAlaLeuMetAsnProAsnAlaAlaLysLysLys  620

AAGAAATCGCAAGAGAAGCGGCAGGAGTCGAAGGCGGCCAAGACGCTGTCGGCTATCCTG 1920
LysLysSerGlnGluLysArgGlnGluSerLysAlaAlaLysThrLeuSerAlaIleLeu  640

CTGAGCTTCATCATCACGTGGACTCCGTACAACATCCTGGTGCTGATCAAGCCGCTGACC 1980
LeuSerPheIleIleThrTrpThrProTyrAsnIleLeuValLeuIleLysProLeuThr  660

ACCTGCTCCGACTGCATACCCACGGAGCTGTGGGACTTTTTCTACGCCCTGTGCTACATC 2040
ThrCysSerAspCysIleProThrGluLeuTrpAspPhePheTyrAlaLeuCysTyrIle  680

AACTCGACGATCAACCCCATGTCGTACGCCTTGTGCAACGCCACCTTCCGGAGAACCTAC 2100
AsnSerThrIleAsnProMetSerTyrAlaLeuCysAsnAlaThrPheArgArgThrTyr  700

GTCCGGATCCTCACCTGCAAGTGGCACACCCGGAACCGCGAGGGCATGGTACGCGGCGTC 2160
ValArgIleLeuThrCysLysTrpHisThrArgAsnArgGluGlyMetValArgGlyVal  720

TACAATTAGATAATTAATACTTAATCACCCATCATCTGGCCGGTTAACTACTAGTAGTTG 2220
TyrAsn                                                        722

CAATAAGTGTTGTGTGCTAGAATTTTGGGAGGCAGTAAAACCAAAACTAGGCTTAGGATT 2280
ACCCATTGACAAGCAGTACTGATTTTTTGATGTCTAGCCCCCGACTGGAATTC         2333
```

FIG. 1. Nucleotide sequence and deduced amino acid sequence of *Drosophila* mAcChoR FCLR1 cDNA. Nucleotide residues are numbered from the 5′ to 3′ direction, beginning with the first nucleotide encoding the initiating methionine. The predicted amino acid sequence is shown below the nucleotide sequence. Sequencing of both strands was performed by the dideoxy chain-termination method from double-stranded plasmids. Terminal *Eco*RI sites are artifacts of the cDNA cloning procedure.



Intron 1 TCAAACAGCAGgtgaggc.....tatataactaagcatttgaacaaataatcttattttgtaatgtttgcagTGACGAA

Intron 2 GCAAGTCAAGCgtaggac.....gttcctggttactaattcccactatcctcagATCAAAA

Intron 3 TCCGTCTCCAGgtagtaa.....ttgaaatgctaaaccgatgccanatcctgtcttctctctttcttcacatcgcagATGCACC

FIG. 2. Restriction map of *Drosophila* mAcChoR FCLR1 cDNA clone. Black blocks beneath the restriction map show the location of the seven transmembrane domains predicted from the sequence. Numbered triangles show the location of intron sequences. Intron sizes, in base pairs, are noted above each triangle. The nucleotide sequences of the intron/exon boundaries are shown below. Exon nucleotides are shown as uppercase letters and intron nucleotides as lowercase letters. The introns were located between nucleotides 713–714, 897–898, and 1185–1186 in the sequence of the cDNA clone shown in Fig. 1.

amino acids as shown in Fig. 1. Further restriction mapping and partial sequencing of the genomic DNA confirmed the presence of three introns in the third putative cytoplasmic loop. A restriction map delineating the location of the introns and the nucleotide sequences of the intron–exon borders is shown in Fig. 2. The amino acid sequence is remarkably similar to the sequence of mammalian mAcChoR receptors (Fig. 3). The overall amino acid identities, excluding all of the highly variable third cytoplasmic loop except for 20 amino acids on each side, to the mammalian m1, m2, m3, m4, and m5 receptors are 45–50%. It has been suggested that the membrane-proximal portion of the third cytoplasmic loop closest to the fifth putative transmembrane region plays a key role in determining the functional specificity of the mAcChoR (14). Within these 20 amino acids in the third cytoplasmic loop, the amino acid identity is 25% and 15% for m2 and m4, respectively, and 45%, 50%, and 60% for m1, m3, and m5, respectively.

Localization of *Drosophila* mAcChoR Gene by *in Situ* Hybridization. *Drosophila* salivary gland chromosomes were hybridized to a biotinylated 7.5-kb genomic *Bam*HI fragment containing the *Drosophila* mAcChoR gene. Hybridization was observed at only one location (Fig. 4), on chromosome 2R at band 60C7,8. No hybridization was seen when a 3.1-kb genomic DNA fragment encoding the mouse m1 mAcChoR (9) was used as a control for the specificity of hybridization.

Expression and Ligand Binding in Transfected Cells. We have previously shown that the mouse Y1 adrenal carcinoma cell line does not express detectable levels of endogenous mAcChoR and that clones encoding both the mouse m1 and porcine m2 mAcChoR can be expressed in these cells with functional coupling either to activation of PLC or inhibition of adenylate cyclase, respectively (9). We expressed the *Drosophila* mAcChoR gene under the control of the zinc-inducible metallothionein promotor in Y1 adrenal cells using the expression vector ZEM228 (9). A subclone exhibiting high levels of [^{3}H]QNB binding sites in membrane homogenates was characterized further. The expressed receptor bound the antagonists QNB, atropine, and pirenzipine to a single class of sites. The level of mAcChoR binding sites in cells induced with 120 μM zinc sulfate for 24 hr was 5.0–6.0 pmol/mg of membrane protein. The apparent dissociation constant (K_d) for [^{3}H]QNB was shown by Scatchard analysis to be 86 pM. K_d values for atropine (0.82 nM) and pirenzipine (0.57 μM) were measured in competition binding assays. The values for QNB and atropine are consistent with those obtained from binding studies carried out with *Drosophila* head membranes (30). The binding of carbachol was best fit by the interaction of the agonists with two binding sites (38.5% high-affinity sites, K_{d1} = 0.70 μM; 61.5% low-affinity sites, K_{d2} = 15.0 μM). On the basis of these binding properties, FCLR1 encodes a mAcChoR.

Physiological Responsiveness of the Expressed Receptor in Y1 Cells. The mAcChoR subtypes couple to at least two distinct second messenger pathways: activation of mammalian m2 and m4 results in inhibition of adenylate cyclase activity; activation of m1, m3, and m5 results in increases in membrane phospholipid turnover due to activation of PLC and phospholipase A_2, although the functional specificity can depend on both cell type as well as the levels of receptor

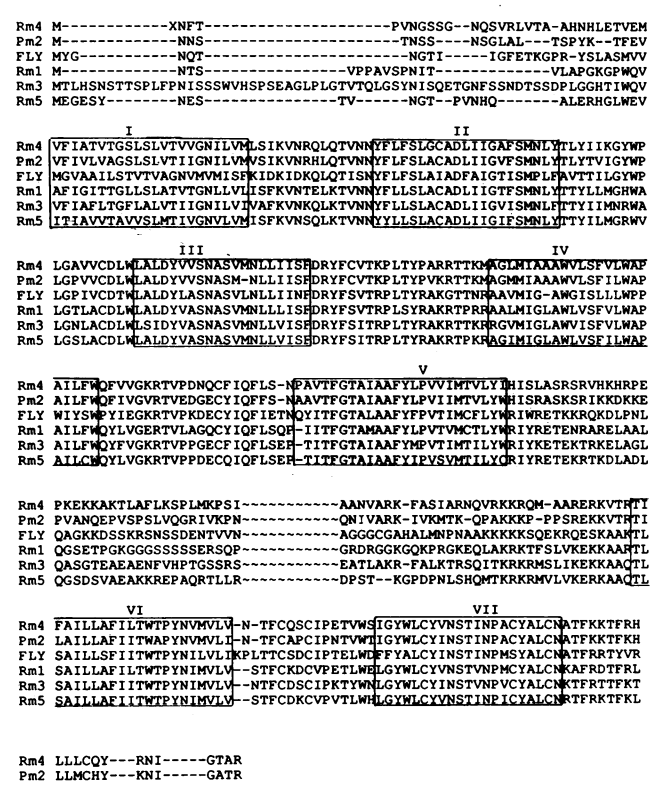


FIG. 3. Amino acid comparison of the *Drosophila* mAcChoR (FLY) and the mammalian mAcChoRs. The amino acid sequences (standard single-letter code) of mammalian mAcChoRs are taken from Bonner *et al.* (7). Boxed regions indicate the seven putative transmembrane regions. Only the membrane-proximal portions of the highly variable third cytoplasmic loop are shown. Wavy lines (~~~~) represent the highly variable portion of the third cytoplasmic loop. Hyphens indicate gaps inserted to maintain the alignment of amino acids.

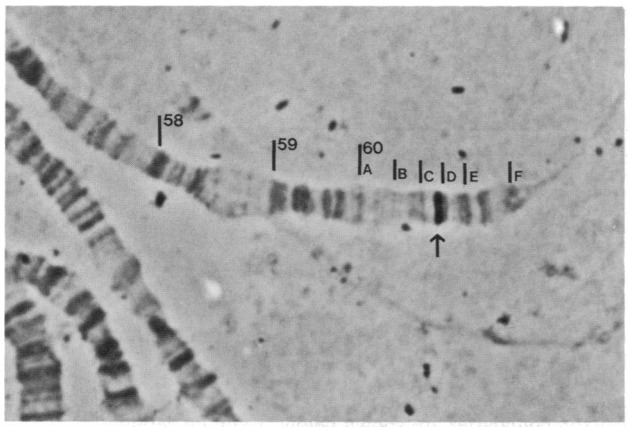


FIG. 4. *In situ* hybridization to *Drosophila* polytene chromosomes. A 7.5-kb genomic DNA fragment in pGEM3 was biotinylated and hybridized to *Drosophila* polytene chromosomes.The region of hybridization (arrow) is located on chromosome 2R at band 60C7,8. (×790.)

expressed (5, 9, 14, 34–39). Due to the sequence similarity between the *Drosophila* receptor and the mammalian m1, m3, and m5 mAcChoRs in the amino terminus of the third cytoplasmic loop, we expected that the *Drosophila* receptor would stimulate PLC activity. The production of total [^{3}H]inositol phosphates ([^{3}H]Ins-*P*s) in response to 1 mM carbachol was measured (9) in cells induced with 120 μM zinc. An 18-fold increase of [^{3}H]Ins-*P*s was observed upon carbachol addition, whereas addition of 1 μM atropine with the carbachol resulted in only a 4-fold increase (control: 152 ± 14 cpm of [^{3}H]Ins-*P* per plate; carbachol: 2824 ± 248 cpm per plate; carbachol plus atropine: 588 ± 21 cpm per plate). The production of total [^{3}H]Ins-*P*s in response to increasing concentrations of carbachol in uninduced and zinc-induced cells with different levels of receptor was then measured (Fig. 5). The maximum response was the same in both induced and uninduced cells; however, basal [^{3}H]Ins-*P*s production was increased in cells with higher receptor levels. The EC_{50} decreased from 10^{-6} M to 10^{-7} M carbachol in cells with the higher level of receptor (Fig. 5).

To determine if the cloned *Drosophila* receptor is able to inhibit adenylate cyclase activity when expressed in Y1 cells, the ability of mAcChoR agonists to inhibit cAMP accumulation in intact cells was determined as described (9). No

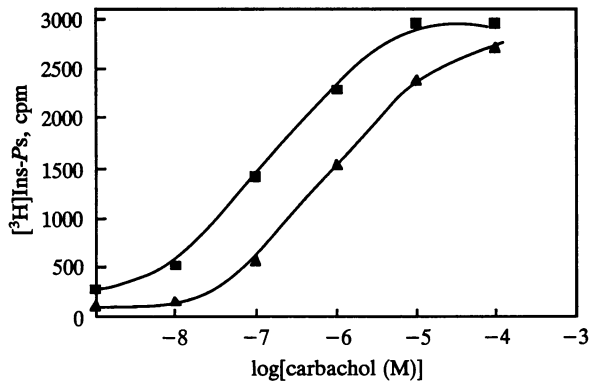


FIG. 5. Stimulation of inositolphospholipid metabolism by *Drosophila* mAcChoR receptor in Y1 cells. Triplicate 35-mm plates of Y1 cells transfected with FCLR1/ZEM228 were treated as described (9) and exposed to carbachol at various concentrations for 15 min at 37°C. The production of [^{3}H]Ins-*P*s was determined as described (9). Concentration of receptor in uninduced (▲) and induced (■) cells was 900 fmol/mg of membrane protein and 7960 fmol/mg, respectively, as measured by [^{3}H]QNB binding.

decrease in forskolin-stimulated cAMP accumulation was observed in response to carbachol; in fact, an increase in cAMP accumulation was observed (control: 250 ± 41 pmol of cAMP per mg of protein; 1 mM carbachol: 471 ± 44 pmol/mg; carbachol plus 1 μM atropine: 248 ± 10 pmol/mg), as has been previously observed in response to the activation of the mammalian m1, m3, and m5 mAcChoR subtypes (7, 9, 34).

Thus, these results suggest that the *Drosophila* mAcChoR gene isolated is structurally and functionally most similar to the mammalian m1, m3, and m5 subtypes. We will refer to it as the *Drosophila* m1 (Dm1) mAcChoR.

DISCUSSION

Genomic and cDNA clones encoding a *Drosophila* mAcChoR were isolated by hybridization at moderate stringency to a mouse genomic clone of the m1 and a porcine cDNA clone of the m2 mAcChoRs. The deduced amino acid sequence is very similar to the sequences of all five mammalian mAcChoRs in the putative transmembrane domains and has a particularly high level of identity to the m1, m3, and m5 subtypes in the membrane-proximal portions of the third cytoplasmic loop (Fig. 3). The *Drosophila* mAcChoR cDNA encodes a polypeptide of 722 amino acids, considerably larger than the mammalian mAcChoRs, with deduced lengths of 460–590 amino acids. Venter *et al.* (40) reported that a number of putative anti-mAcChoR monoclonal antibodies precipitated receptors solubilized from both mammals and *Drosophila*. Because these antibodies were isolated after immunization with crude preparations of receptors and evidence of their specificity for muscarinic or other receptors was not presented, the significance of these results was not clear. The sequence data presented here clearly demonstrate that the *Drosophila* and mammalian mAcChoR are closely related.

Expression of a cDNA encoding the *Drosophila* m1 mAcChoR in mouse Y1 cells results in a functional receptor as measured by ligand binding and the ability to stimulate PtdIns turnover. The *Drosophila* mAcChoR stimulated PtdIns turnover in Y1 cells to the same maximal extent as previously demonstrated for the mouse m1 receptor in Y1 cells (9). Thus, there is a high degree of functional conservation between the mouse and *Drosophila* receptors. No carbachol-induced inhibition of adenylate cyclase activity was observed. These results support the hypothesis that individual receptor subtypes are coupled to specific intracellular second messenger systems.

In contrast to all the mammaliam mAcChoR genes described to date, the *Drosophila* m1 mAcChoR contains introns in the coding region of the gene (Fig. 2). The intron/exon boundaries of the three introns and the internal intron signals are consistent with those reported by Keller and Noon (41), with two exceptions. In the case of intron 1, there are two possible internal splice sequences that are consistent with previously reported intron sequences; in the case of intron 2, the conserved (C/A)AG normally found at the 3′ end of the exon is displaced one nucleotide from the splice site.

Elucidation of the role of mAcChoRs in the development of the nervous system and in behavior would be greatly aided by the use of a system with well-characterized genetics, developmental biology, and neurobiology. The study of genetic neurobiology has been greatly facilitated by the identification and isolation of genes which affect behavior. In *Drosophila*, mutations such as dunce (42) and Shaker (31) have been extremely valuable in identifying the macromolecules involved in neurotransmission and sensory transduction. The identification of the *norpA* gene product as a PLC (43) clearly demonstrates the importance of the PtdIns pathway in signal transduction in insects.

The identification of the *Drosophila* mAcChoR gene and its precise cytogenetic localization should permit a genetic dissection of the physiological role of this mAcChoR. Null mutations, in combination with the reintroduction of altered mAcChoR genes into the *Drosophila* germ line or into *Drosophila* tissue culture cells, should allow functional domains of the *Drosophila* mAcChoR to be defined *in vivo*. The availability of the cloned *Drosophila* mAcChoR gene will also allow both determining the precise tissue-specific expression during development and testing the effects of altered receptor expression on the behavior of the whole organism.

We thank Rick Garber and Bruce Tempel for the genomic and cDNA libraries. This work was supported by a Grant-in-Aid from the American Heart Association and by National Institutes of Health Grants HL30693, HL07312, and NS26920. N.M.N. is an Established Investigator of the American Heart Association.

1. Nathanson, N. M. (1987) *Annu. Rev. Neurosci.* **10,** 195–236.
2. Kubo, T., Fukuda, K., Mikami, A., Maeda, K., Takahashi, H., Mishina, T., Haga, T., Haga, K., Ichiyama, A., Kangawa, K., Kojima, M., Matsuo, H., Hirose, T. & Numa, S. (1986) *Nature (London)* **323,** 411–416.
3. Kubo, T., Maeda, K., Sugimoto, K., Akiba, I., Mikami, A., Takahashi, H., Haga, T., Haga, K., Ichiyama, A., Kangawa, K., Matsuo, H., Hirose, T. & Numa, S. (1986) *FEBS Lett.* **209,** 367–372.
4. Peralta, E. G., Ashkenazi, A., Winslow, J. W., Smith, D. H., Ramachandran, J. & Capon, D. J. (1987) *EMBO J.* **6,** 3923–3929.
5. Peralta, E. G., Winslow, J. W., Peterson, G. L., Smith, D. H., Ashkenazi, A., Ramachandran, J., Schimerlik, M. I. & Capon, D. J. (1987) *Science* **236,** 600–605.
6. Bonner, T. I., Buckley, N. J., Young, A. C. & Brann, M. R. (1987) *Science* **237,** 527–532.
7. Bonner, T. I., Young, A. C., Brann, M. R. & Buckley, N. J. (1988) *Neuron* **1,** 403–410.
8. Braun, T., Schofield, P. R., Shivers, B. D., Pritchett, D. B. & Seeburg, P. H. (1987) *Biochem. Biophys. Res. Commun.* **149,** 125–132.
9. Shapiro, R. A., Scherer, N. M., Habecker, B. A., Subers, E. M. & Nathanson, N. M. (1988) *J. Biol. Chem.* **263,** 18397–18403.
10. Dixon, R. A. F., Kobilka, B. K., Strader, D. J., Benovic, J. L., Dohlman, H. G., Frielle, T., Bolanowski, M. A., Bennett, C. D., Rands, E., Diehl, R. E., Mimford, R. A., Slater, E. E., Sigal, I. S., Caron, M. G., Lefkowitz, R. J. & Strader, C. D. (1986) *Nature (London)* **321,** 75–79.
11. Kobilka, B. K., Dixon, R. A. F., Frielle, T., Dohlman, H. G., Bolanowski, M. A., Sigal, I. S., Yang-Feng, T. L., Francke, U., Caron, M. G. & Lefkowitz, R. J. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 46–50.
12. Nathans, J. (1987) *Annu. Rev. Neurosci.* **10,** 163–194.
13. Dohlman, H. G., Caron, M. G. & Lefkowitz, R. J. (1987) *Biochemistry* **26,** 2657–2664.
14. Bonner, T. I. (1989) *Trends NeuroSci.* **12,** 148–151.
15. O'Dowd, B. F., Hnatowich, M., Regan, J. W., Leader, M. W., Caron, M. G. & Lefkowitz, R. J. (1988) *J. Biol. Chem.* **263,** 15985–15992.
16. Kobilka, B. K., Kobilka, T. S., Daniel, K., Regan, J. W., Caron, M. G. & Lefkowitz, R. J. (1988) *Science* **240,** 1310–1316.
17. Kubo, T., Bujo, H., Akiba, I., Nakai, J., Mishina, M. & Numa, S. (1988) *FEBS Lett.* **241,** 119–125.
18. Shapiro, R. A. & Nathanson, N. M. (1989) *Biochemistry*, in press.
19. Pittman, R. M. (1971) *Comp. Gen. Pharmacol.* **2,** 347–371.
20. Greenspan, R. J. (1980) *J. Comp. Physiol.* **137,** 83–92.
21. Gorczyca, M. & Hall, J. C. (1984) *J. Neurogenet.* **1,** 289–313.
22. Greenspan, R. J., Finn, J. A. & Hall, J. C. (1980) *J. Comp. Neurol.* **189,** 741–774.
23. Gorczyca, M. & Hall, J. C. (1987) *J. Neurosci.* **7,** 1361–1369.
24. Wolfgang, W. J. & Forte, M. A. (1989) *Dev. Biol.* **131,** 321–330.
25. Hall, L. M. C. & Spierer, P. (1986) *EMBO J.* **5,** 2949–2954.
26. Itoh, N., Slemmon, R., Hawke, D. H., Williamson, R., Morita, E., Itakura, K., Roberts, E., Shively, J. E., Crawford, G. D. & Salvaterra, P. M. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 4081–4085.
27. Hermans-Borgmeyer, I., Zopf, D., Ryseck, R.-P., Hovemann, B., Betz, H. & Gundelfinger, E. D. (1986) *EMBO J.* **5,** 1503–1508.
28. Wadsworth, S. C., Rosenthal, L. S., Kammermeyer, K. L., Potter, M. B. & Nelson, D. J. (1988) *Mol. Cell. Biol.* **8,** 778–785.
29. Bossey, B., Ballivet, M. & Spierer, P. (1988) *EMBO J.* **7,** 611–618.
30. Haim, N., Nahum, S. & Dudai, Y. (1979) *J. Neurochem.* **32,** 543–552.
31. Schwarz, T. L., Tempel, B. L., Papazian, D. M., Jan, N. Y. & Jan, L. Y. (1988) *Nature (London)* **331,** 137–142.
32. Langer-Sofer, P. R., Levine, M. L. & Ward, D. C. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 4381–4385.
33. Munson, P. J. & Rodbard, D. (1980) *Anal. Biochem.* **107,** 220–239.
34. Peralta, E. G., Ashkenazi, A., Winslow, J. W., Ramachandran, J. & Capon, D. J. (1988) *Nature (London)* **334,** 434–437.
35. Fukuda, K., Higashida, H., Kubo, T., Maeda, A., Akiba, I., Bujo, H., Mishina, M. & Numa, S. (1988) *Nature (London)* **335,** 355–358.
36. Ashkenazi, A., Winslow, J. W., Peralta, E. G., Peterson, G. L., Schimerlik, M. I., Capon, D. J. & Ramachandran, J. (1987) *Science* **238,** 672–675.
37. Ashkenazi, A., Peralta, E. G., Winslow, J. W., Ramachandran, J. & Capon, D. J. (1989) *Cell* **56,** 487–493.
38. Stein, R., Pinkas-Kramarski, R. & Sokolovsky, M. (1988) *EMBO J.* **7,** 3031–3035.
39. Conklin, B. R., Brann, M. R., Buckley, N. J., Ma, A. L., Bonner, T. I. & Axelrod, J. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8698–8702.
40. Venter, J. C., Eddy, B., Hall, L. M. & Fraser, C. M. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 272–276.
41. Keller, E. B. & Noon, W. A. (1985) *Nucleic Acids Res.* **13,** 4971–4981.
42. Chen, C.-N., Denome, S. & Davis, R. L. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 9313–9317.
43. Bloomquist, B. T., Shortridge, R. D., Schneuwly, S., Perdew, M., Montell, C., Steller, H., Rubin, G. & Pak, W. L. (1988) *Cell* **54,** 723–733.