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Characterization of GAR-2, a Novel G Protein-Linked Acetylcholine Receptor from *Caenorhabditis elegans*

Yong-Seok Lee, *Yang-Seo Park, †Seunghee Nam, SuJeong Suh, †Junho Lee, Bong-Kiun Kaang, and *Nam Jeong Cho

*Molecular Neurobiology Laboratory, Institute of Molecular Biology and Genetics, School of Biological Sciences, College of Natural Sciences, Seoul National University; †Department of Biology, Yonsei University, Seoul; and *School of Life Sciences, Chungbuk National University, Cheongju, Korea*

Abstract: We have previously identified two G protein-linked acetylcholine receptors (GARs), GAR-1 and GAR-3, in the nematode *Caenorhabditis elegans.* Whereas GAR-3 is a homologue of muscarinic acetylcholine receptors (mAChRs), GAR-1 is similar to but pharmacologically distinct from mAChRs. In the current work we isolated a new type of GAR using *C. elegans* genome sequence information. This receptor, named GAR-2, consists of 614 amino acid residues and has seven putative transmembrane domains. Database searches indicate that GAR-2 is most similar to GAR-1 and closely related to GAR-3/mAChRs. The overall amino acid sequence identities to GAR-1 and GAR-3 are ~32 and ~23%, respectively. When GAR-2 was coexpressed with the G protein-activated inwardly rectifying K^+ (GIRK1) channel in *Xenopus* oocytes, acetylcholine was able to evoke the GIRK current in a dose-dependent fashion. Oxotremorine, a classical muscarinic agonist, had little effect on the receptor, indicating that GAR-2 is pharmacologically different from mAChRs but rather similar to GAR-1. GAR-2 differs from GAR-1, however, in that it showed virtually no response to muscarinic antagonists such as atropine, scopolamine, and pirenzepine. Expression studies using green fluorescent protein reporter gene fusion revealed that GAR-2 is expressed in a subset of *C. elegans* neurons, distinct from those expressing GAR-1. Together with our previous reports, this study demonstrates that diverse types of GARs are present in *C. elegans.* **Key Words:** GAR-2—G protein-linked acetylcholine receptor—Muscarinic acetylcholine receptor—*Caenorhabditis elegans*—*Xenopus* oocyte expression—Green fluorescent protein fusion.
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Acetylcholine (ACh) is known to be a major neurotransmitter in both vertebrates and invertebrates. In humans, ACh has been implicated in various nervous functions such as contraction of skeletal muscles, emotion, perception, cognition, learning, and memory. Two distinct types of cholinergic neurotransmission exist: a rapid form mediated by nicotinic ACh receptors (nAChRs) and a slow form mediated by muscarinic ACh receptors (mAChRs). Whereas nAChRs are pentameric proteins that act as ligand-gated ion channels, mAChRs are single polypeptides with seven transmembrane domains that mediate signal transduction by activating G proteins and intracellular effector molecules.

The functional roles of ACh in the nervous system are diverse, and this functional diversity stems largely from the molecular diversity of both nAChRs and mAChRs. Gene cloning studies have shown that vertebrate nAChRs are encoded by multiple α and non-α subunit genes. Various combinations of these subunits could yield many subtypes of nAChRs with different channel properties (McGehee, 1999). For vertebrate mAChRs, five subtypes (m1–m5) are encoded by separate genes (Bonner et al., 1987, 1988). The mAChR subtypes exhibit tissue-specific expression and mediate distinct biochemical responses (Kubo et al., 1986*a*,*b*; Peralta et al., 1987, 1988).

Caenorhabditis elegans is considered to possess most of the fundamental components of vertebrate synapses and thus may serve as a model for investigating the molecular mechanisms underlying synaptic function. Genetic analyses have identified many *C. elegans* genes that are involved in cholinergic neurotransmission. Two

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Address correspondence and reprint requests to Dr. B.-K. Kaang at Molecular Neurobiology Laboratory, Institute of Molecular Biology and Genetics, School of Biological Sciences, College of Natural Sciences, Seoul National University, San 56-1 Silim-dong Kwanak-gu, Seoul 151-742, Korea. E-mail: kaang@snu.ac.kr or Dr. N. J. Cho at School of Life Sciences, Chungbuk National University, Cheongju 361-763, Korea. E-mail: namjcho@chungbuk.ac.kr

Drs. Y.-S. Lee and Y.-S. Park contributed equally to this work.

Abbreviations used: ACh, acetylcholine; CFTR, cystic fibrosis transmembrane conductance regulator; GAR, G protein-linked acetylcholine receptor; GFP, green fluorescent protein; GIRK, G protein-activated inwardly rectifying K^+; hK, high K^+; HSN, hermaphrodite-specific neuron; 5-HT, 5-hydroxytryptamine; i3 loop, third intracellular loop; mAChR, muscarinic acetylcholine receptor; nAChR, nicotinic acetylcholine receptor; PVM, nucleus periventricularis magnocellularis; SL1 and 2, spliced leader 1 and 2, respectively.

tightly linked genes responsible for ACh synthesis and packaging into synaptic vesicles have been identified by screening mutants that are resistant to acetylcholinesterase inhibitors. One gene, *cha-1,* encodes choline acetyltransferase (Rand and Russell, 1984; Alfonso et al., 1994), and the other gene, *unc-17,* encodes a putative vesicular ACh transporter (Alfonso et al., 1993). Three genes for acetylcholinesterase have been identified (Culotti et al., 1981; Johnson et al., 1981, 1988). Null mutants in *cha-1, unc-17,* or acetylcholinesterase genes are lethal (Johnson et al., 1988; Rand, 1989; Alfonso et al., 1993), indicating that cholinergic neurotransmission is essential for survival. Several genes that encode nAChR subunits have been reported (Treinin and Chalfie, 1995; Fleming et al., 1997). Mutations in these genes result in various phenotypes, including uncoordinated movement, resistance to the nematocide levamisole, and neuronal degeneration. About 40 genes have been predicted to encode nAChR subunits by computer analysis of the *C. elegans* genome sequence (Bargmann, 1998), suggesting that nAChRs are encoded by an extensively large gene family. Although pharmacological studies indicate the existence of mAChRs in *C. elegans* (Culotti and Klein, 1983; You et al., 1996), mutations in the receptor genes have not been identified. As a consequence, the functional and physiological roles of mAChRs are poorly understood.

In an effort to elucidate the molecular mechanisms by which the ACh signal is processed through G proteins and second messenger systems in *C. elegans,* we attempted to clone the genes for G protein-linked ACh receptors (GARs). Sequence analysis of the entire genome revealed that at least three genes are expected to code for GARs (*C. elegans* Sequencing Consortium, 1998). We recently isolated two of them, *gar-1* and *gar-3* (Hwang et al., 1999; Lee et al., 1999). The *gar-3* gene encodes a *C. elegans* mAChR that couples to the activation of phosphatidylinositol hydrolysis and displays virtually identical ligand binding specificity to vertebrate mAChRs (Hwang et al., 1999). On the other hand, *gar-1* encodes a GAR whose pharmacological profile is distinct from that of mAChRs (Lee et al., 1999).

In this article we report the cloning and functional characterization of a novel GAR, termed GAR-2. We show that like GAR-1, GAR-2 couples to activation of the G protein-activated inwardly rectifying K^+ (GIRK1) channel. However, pharmacological analyses indicate that GAR-2 is different from GAR-1, as well as from other previously known GARs. Furthermore, expression studies indicate that GAR-1 and GAR-2 are expressed in nonoverlapping populations of *C. elegans* neurons.

MATERIALS AND METHODS

Drugs

ACh chloride, (±)-isoproterenol hydrochloride, 5-hydroxytryptamine (5-HT) creatinine sulfate, oxotremorine free base, carbamylcholine chloride (carbachol), atropine sulfate, (−)-scopolamine hydrochloride, and pirenzepine dihydrochloride were purchased from Sigma.

Isolation of *gar-2* cDNA

The wild-type N2 strain of *C. elegans* was maintained and grown on NGM agar plates seeded with *Escherichia coli* strain OP50. Total RNA was isolated from a mixed population of *C. elegans* using the Tri reagent (Sigma). RT-PCR was performed using the RNA PCR kit (Takara) by following the procedure recommended by the manufacturer. PCR primers used are as follows: AF3, 5′-GCTGAGATCTGTGAAGAATGGCGGTTGCA-3′ (the underlined sequence CT was changed from the sequence GG to create a *Bgl*II site); and AR1, 5′-TAATAGATCTATGCGATCAAACTCTTCG-3′ (a *Bgl*II site created is underlined). The PCR products were digested with *Bgl*II and ligated into pBluescript that had been digested with *Bam*HI. The nucleotide sequences of the PCR products were determined by the dideoxynucleotide chain termination method, using the Cy5 AutoRead sequencing kit and ALFexpress DNA sequencer (Pharmacia). To determine the 5′ end of the *gar-2* cDNA, RT-PCR was performed using spliced leader 1 (SL1) or 2 (SL2) as a forward primer and a gene-specific primer AR2 as a reverse primer. The RT-PCR products were ligated into pBluescript and sequenced as described above. The AR2 sequence is 5′-GTGTCGTCCAATAGTATGAG-3′.

Electrophysiological analysis

The cRNAs for GAR-2, pig m2 mAChR, rat GIRK1 channel, human β_2-adrenergic receptor, human cystic fibrosis transmembrane conductance regulator (CFTR), and mouse 5-HT_{2C} receptor were prepared by in vitro transcription using the Megascript kit (Ambion). Preparation of *Xenopus* oocytes and electrophysiological recordings were performed as described by Lee et al. (1999).

Expression of GAR-1::GFP and GAR-2::GFP

To examine the expression pattern of GAR-1, we constructed a fusion gene between the upstream region of *gar-1* and the green fluorescent protein (GFP) reporter gene. To obtain the *gar-1* upstream region, a cosmid DNA (C15B12; kindly provided by Dr. A. Coulson) containing the *gar-1* gene was amplified with primers MF-5UT and MR-5UT. The PCR product, containing ~ 2.6 kb of the upstream region and the first 16 codons of *gar-1,* was digested with *Pst*I and *Bam*HI and then inserted into the *C. elegans* GFP expression vector pPD95.75 (kindly provided by Dr. A. Fire). This construct was named pPD-gar1, and the junction sequences were confirmed by DNA sequencing.

To examine the expression pattern of GAR-2, we constructed a fusion gene between *gar-2* and the GFP reporter gene. This construct, which we named pPD-gar2I, contains ~ 2.7 kb of the upstream region, ~ 4.6 kb of the first intron, and the first 65 codons of *gar-2.* To obtain this sequence, a cosmid DNA (F47D12; kindly provided by Dr. A. Coulson) was amplified with two primer pairs, AF-5UT/AR-5UT and AF-5UT/AR5. The PCR product with AF-5UT/AR-5UT was digested with *Pst*I and *Bgl*II, and the PCR product with AF-5UT/AR5 was digested with *Bgl*II and *Bam*HI. The two digested PCR products were inserted into the *C. elegans* GFP expression vector pPD95.75 at the *Pst*I and *Bam*HI site. The junction and exon sequences were confirmed by DNA sequencing.

Primers used are as follows: MF-5UT, 5′-TGTTCTGCAGTCGTGCCCGCTCATC-3′ (a *Pst*I site created is underlined); MR-5UT, 5′-TGGAGGATCCCAGCTAGTGTCTGC-3′ (a *Bam*HI site created is underlined); AF-5UT, 5′-CAAATGTCCACTGCAGCTGA-3′ (a *Pst*I site is underlined); AR-5UT, 5′-TGGAGGATCCCAGCTAGTGTCTGC-3′ (a

*Bam*HI site created is underlined); and AR5, 5′-ATTGGATCCTCGAATGCTGTGTACACCGGGATA-3′ (a *Bam*HI site created is underlined). PCR was performed in a total volume of 50 μl containing 1× plaque-forming unit (pfu) DNA polymerase buffer, 200 μ*M* each deoxynucleotide triphosphate, 2.5 units of cloned pfu DNA polymerase (Stratagene), 50 pmol of each primer, and 100 ng of cosmid DNA. Cycling conditions were 95°C for 45 s, 55°C for 45 s, and 72°C for 6 min (16 min for the PCR with AF-5UT/AR5) for 25 cycles, followed by a 20-min extension at 72°C. pPD-gar1 or pPD-gar2I DNA was microinjected with a plasmid DNA containing the dominant-selectable marker *rol-6* into the gonads of *C. elegans*, and the transformed progeny were analyzed by confocal (Bio-Rad) or conventional (Carl Zeiss) fluorescence microscopy.

RESULTS

Cloning of *gar-2* cDNA

Recently, the complete genome sequence of *C. elegans* has been reported (*C. elegans* Sequencing Consortium, 1998). Three genes (C15B12.5, F47D12.2, and C53A5.12) have been predicted to encode putative GARs by computer analysis of the genome sequence. We have previously reported that C15B12.5 encodes a novel GAR (GAR-1) (Lee et al., 1999), whereas C53A5.12, together with Y40H4A.1, encodes a homologue of mAChRs (GAR-3) (Hwang et al., 1999). From the sequence analysis of a cosmid clone F47D12 (GenBank accession no. U22831), we hypothesized that F47D12.2 and F47D12.1, predicted to be two separate genes by computer analysis, may encode a single polypeptide that is similar to GAR-1. To verify this, we performed RT-PCR using F47D12.2-specific oligonucleotide (AF3) as a forward primer and F47D12.1-specific oligonucleotide (AR1) as a reverse primer (Fig. 1, top). From the several PCR products obtained, we were able to isolate a cDNA clone whose nucleotide sequence matches the corresponding genomic sequence. This cDNA, which we designated *gar-2*, is expected to encode a polypeptide of 614 amino acids with an estimated molecular mass of 69,959 daltons. Comparison of the cDNA sequence with the corresponding genomic sequence reveals that the *gar-2* gene spans ~11 kb and contains 10 introns in the coding region (Fig. 1, bottom).

GAR-2 contains seven putative transmembrane domains and two conserved cysteine residues (Cys^{79} and Cys^{160}) for possible disulfide linkage formation, indicating that GAR-2 has structural features of G protein-linked receptors. GAR-2, however, differs from most G protein-linked receptors in that it carries an extremely short amino-terminal extracellular domain of five amino acid residues and lacks consensus sites for N-linked glycosylation at the amino terminus (Fig. 1, top). To examine whether the proposed ATG initiation codon is authentic, we attempted to determine the 5′ end sequence of the *gar-2* mRNA. It has been known that many *C. elegans* mRNAs are *trans*-spliced at their 5′ ends to a 22-nucleotide spliced leader, SL1 or SL2. The *gar-2* gene possesses a potential 3′ splice acceptor sequence (TTGCAG) 43 bp upstream of the putative ATG initiation codon, raising the possibility of *trans*-splicing. To test this possibility, we performed RT-PCR using SL1 or SL2 as a forward primer and a *gar-2*-specific oligonucleotide (AR2) as a reverse primer. When SL1 was used, a band of the expected size of 612 bp was observed. Sequence analysis of this PCR product confirmed that *gar-2* has an SL1 sequence at the 5′ end (Fig. 1, top). This also strongly suggests that the proposed ATG codon is the start signal for protein synthesis.

Amino acid sequence comparison of GAR-2 with other GARs

Database searches indicate that GAR-2 is most similar to GAR-1. The overall amino acid sequence identity to GAR-1 is ~32% (Fig. 2). When a highly variable third intracellular loop (i3 loop) region is excluded, the identity goes up to 45%. GAR-2, like GAR-1, shows a relatively low degree of sequence identity to GAR-3, the *C. elegans* mAChR. The amino acid sequence identity between GAR-2 and GAR-3, excluding the i3 loop region, is ~28%. The amino acid sequence identities of GAR-2 to human mAChR subtypes, excluding the i3 loop region, are 27–35%: 32% to human m1, 35% to human m2, 27% to human m3, 33% to human m4, and 30% to human m5. It was noted that GAR-1 and GAR-2 exhibit a high degree of sequence identity in the membrane-proximal segments of the i3 loop. As these segments of mAChRs are known to be important for proper G protein recognition and activation (Wess et al., 1995), it is possible that GAR-1 and GAR-2 share a certain degree of functional similarity.

Site-directed mutagenesis analyses of mAChRs have shown that 11 amino acid residues are critically involved in muscarinic ligand binding (Wess et al., 1995). Indeed, these amino acid residues are conserved among mAChRs, including GAR-3 (Fig. 2). Of the 11 conserved amino acid residues, five are different in GAR-2; four are different in GAR-1 (Fig. 2). Four of the five different amino acid residues in GAR-2 are identical to the four different ones in GAR-1, suggesting that ligand binding properties of GAR-2 may be similar to those of GAR-1. The phylogenetic tree shown in Fig. 3 also indicates that GAR-2 is more closely related to GAR-1 than to mAChRs. Taken together, GAR-1 and GAR-2 may share a common ancestor and constitute a novel family of GARs distinct from the mAChR family.

GAR-2 couples to activation of GIRK1 channel in *Xenopus* oocytes

GAR-1 has been shown to couple to the GIRK1 channel (Lee et al., 1999), which is known to be activated by the G_i/G_o family in *Xenopus* oocytes. Here we tested whether *gar-2* cDNA encodes a functional receptor that can stimulate the GIRK1 channel. We expressed GAR-2 and GIRK1 by injecting their cRNAs into *Xenopus* oocytes. Replacement of ND96 solution with high potassium (hK) solution generated an inward current (I_{hK}) in oocytes (Fig. 4A). The amplitude of I_{hK} in water-injected

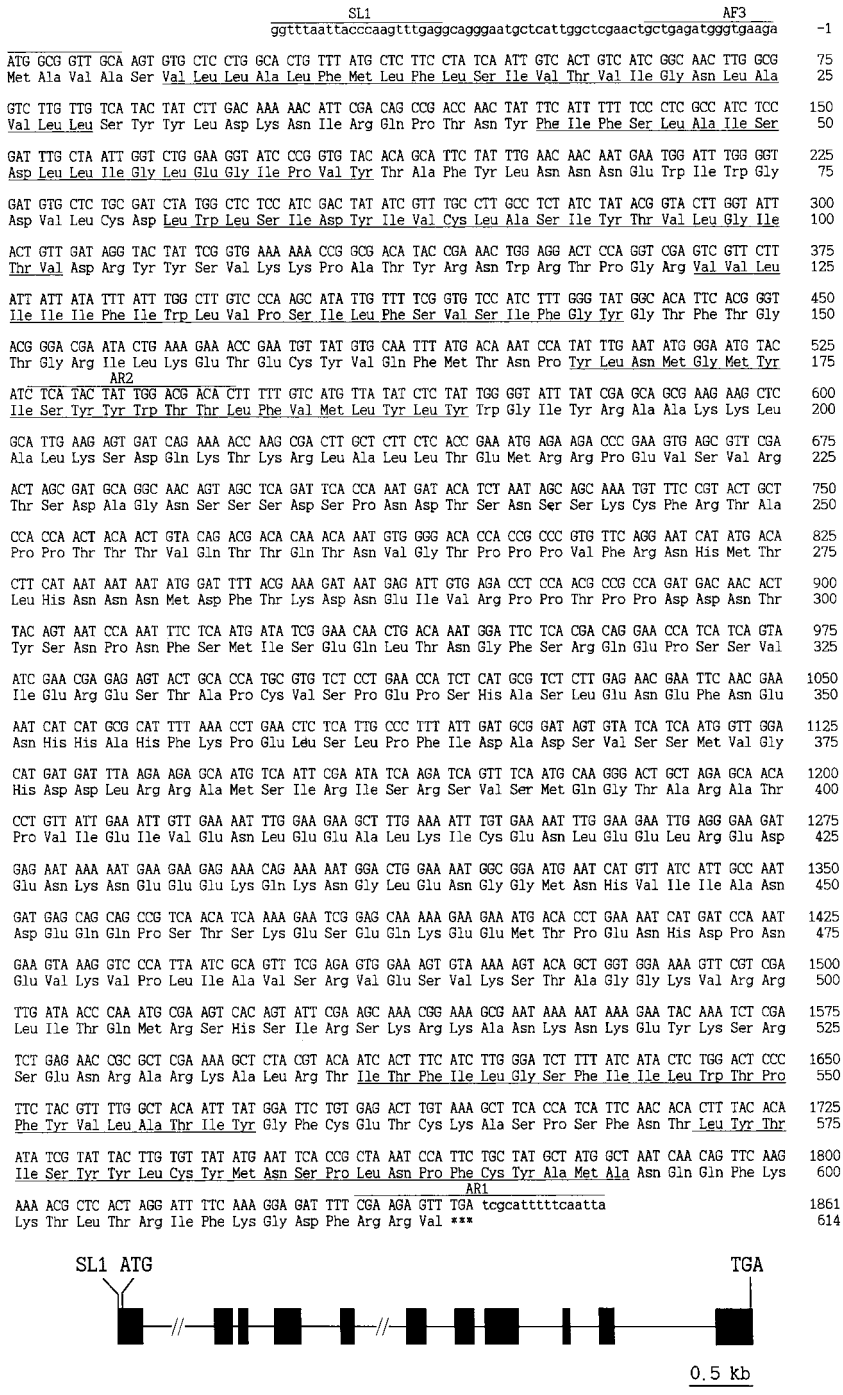

FIG. 1. Top: Nucleotide sequence and deduced amino acid sequence of the *C. elegans* GAR-2. Sequences of SL1 and primers (AF3, AR2, and AR1) used for RT-PCR experiments are indicated. Seven putative transmembrane domains are underlined. This sequence has been deposited in GenBank under accession no. AF272738. **Bottom:** Genomic organization of the *gar-2* gene. Black boxes indicate exons; lines indicate introns. The first and fifth introns are unusually long (~4.6 and ~2.3 kb, respectively). ATG, translation initiation codon; TGA, translation termination codon.

oocytes (89.0 ± 6.4 nA, n = 5) was similar to that in oocytes expressing GAR-2 alone (93.0 ± 9.7 nA, n = 5). The current amplitude was larger in oocytes expressing GIRK1 alone (235.0 ± 20.0 nA, n = 3). Treatment with 1 μM ACh of oocytes expressing both GAR-2 and GIRK1 gave rise to an additional increase of inward current (484.3 ± 55.8 nA, n = 14). This GAR-2/GIRK1-mediated current was reproducibly evoked by a repeated application of ACh. In control experiments, ACh had little if any effect on the inward current in oocytes expressing either GAR-2 alone (n = 5) or GIRK1 alone (n = 5). As shown in Fig. 4A, the ACh-induced, GAR-2/GIRK1-mediated inward current increased in a dose-dependent manner (EC_{50} of ~35 nM), although it decreased at a concentration of ACh >10 μM. These results indicate that GAR-2 activates the GIRK1 channel in *Xenopus* oocytes, probably via the G_i/G_o family of G proteins.

FIG. 2. Amino acid sequence comparison of the three *C. elegans* GARs. Amino acid residues that are identical to GAR-2 are highlighted in gray. Putative transmembrane domains (I–VII) are indicated. Amino acid residues reported to be important for muscarinic ligand binding (Wess et al., 1995) are denoted by asterisks. Cysteine residues that are likely to be engaged in disulfide bond formation are indicated by arrows. Sequences were aligned using the Clustal X program. The amino acid sequences of GAR-1 and GAR-3 were taken from Lee et al. (1999) and Hwang et al. (1999), respectively.

We also examined whether GAR-2 couples to other G proteins, besides the G_i/G_o family. We focused on the CFTR, a Cl^- channel activated by cyclic AMP-dependent protein kinase after stimulation of G_s (Uezono et al., 1993), and the *Xenopus* endogenous Cl^- channel activated by inositol 1,4,5-trisphosphate after stimulation of G_q or G_o (Lubbert et al., 1987). When GAR-2 cRNA was coinjected into oocytes with CFTR cRNA, 1 μM ACh treatment elicited only a weak CFTR current (71.4 ± 14.4 nA, n = 18) and virtually no transient Cl^- current (20.0 ± 11.7 nA, n = 18; Fig. 4B). By contrast, activation of the β_2-adrenergic receptor with 1 μM isoproterenol generated a robust CFTR current (1,009.1 ± 114.9 nA, n = 11). Activation of the 5-HT_{2C} receptor by 1 μM 5-HT also produced a distinctive transient Cl^- current (2,787.5 ± 413.4 nA, n = 4). These analyses indicate that GAR-2 does not couple to G_s, G_q, or G_o effectively. Taken together, these results demonstrate that the cloned *gar-2* cDNA encodes a functional ACh receptor that couples to a G protein, most likely of the G_i family, in *Xenopus* oocytes.

Pharmacological comparison of GAR-2 with m2 mAChR

We compared the pharmacological properties of the GAR-2 receptor with those of pig cardiac mAChR (m2), which is also known to couple to the activation of the GIRK1 channel (Kubo et al., 1993). Oocytes expressing

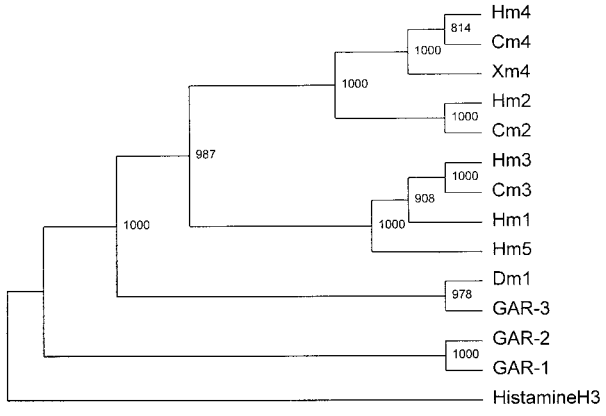

FIG. 3. Phylogenetic tree of GARs. The three *C. elegans* GARs and some representative mAChRs were aligned using Clustal X with default parameters. The phylogenetic tree was drawn using TREEVIEW (Page, 1996). The tree was rooted by using the human histamine H3 receptor as an outgroup. Bootstrap values obtained with 1,000 replicates are shown at the nodes. The protein sequences were taken from public databases with the indicated accession numbers: Hm, human mAChR (Hm1, X52068; Hm2, M16404; Hm3, U29589; Hm4, M16405; and Hm5, M80333); Cm, chicken mAChR (Cm2, P30372; Cm3, P49578; and Cm4, P17200); Xm, *Xenopus* mAChR (Xm4, P30544); Dm, *Drosophila* mAChR (Dm1, M27495); and HistamineH3, human histamine H3 receptor (AF140538).

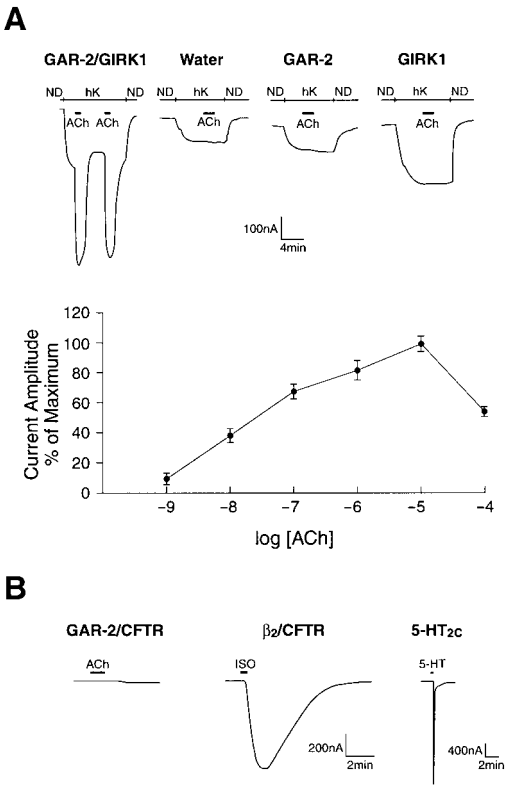

FIG. 4. Functional expression of GAR-2 in *Xenopus* oocytes. **A:** GIRK1 current activated by GAR-2 (**upper panel**) and dose–response curve of the current (**lower panel**). Oocytes were injected with a mixture of GAR-2 cRNA and GIRK1 cRNA (2.5 ng each), water, GAR-2 cRNA (2.5 ng), or GIRK1 cRNA (2.5 ng). ND, ND96 solution. For the dose–response plot, the peak amplitude of the GIRK current was measured and normalized to the maximal current value. Data in the dose–response plot are average ± SE (error bars) values of at least three separate recordings from different oocytes. **B:** GAR-2 did not stimulate the CFTR channel or the transient endogenous Cl^- current in *Xenopus* oocytes. Oocytes were injected with a mixture of GAR-2 cRNA (2.5 ng) and CFTR cRNA (5 ng), a mixture of β_2-adrenergic receptor cRNA (250 pg) and CFTR cRNA (5 ng), or 5-HT_{2C} cRNA (250 pg). Drugs were present in the bathing solution during the times indicated by bars. ISO, isoproterenol. All the current traces were recorded at a holding potential of −70 mV.

pig m2 mAChR and the GIRK1 channel produced the inward current (310.0 ± 21.7 nA, n = 14) by treatment with 1 μM ACh (Fig. 5A2 and B2). The potent muscarinic agonist oxotremorine (1 μM) failed to elicit the GIRK current in oocytes expressing GAR-2 and the GIRK1 channel (n = 5; Fig. 5A1, center), whereas treatment with oxotremorine of oocytes expressing m2 mAChR and the GIRK1 channel induced the current (n = 4) as with ACh (Fig. 5A2, center). Furthermore, the GIRK current produced by another muscarinic agonist, carbachol (1 μM), was only 39.2 ± 2.3% (n = 4) of that produced by ACh in GAR-2 (Fig. 5A1, right), whereas this value was much higher in the m2 receptor (81.5 ± 1.7%, n = 5; Fig. 5A2, right). Next we examined the effects of antagonists on the ACh-induced GIRK current activated by the two receptors. Atropine (1 μM), scopolamine (1 μM), and pirenzepine (1 μM) were not able to reduce the current mediated by GAR-2 (Fig. 5B1). By contrast, all these antagonists inhibited the GIRK current mediated by pig m2 mAChR by 98.7 ± 0.8 (n = 5), 97.4 ± 0.8 (n = 5), and 61.4 ± 5.7% (n = 5), respectively (Fig. 5B2). These data show that GAR-2 exhibits phar-

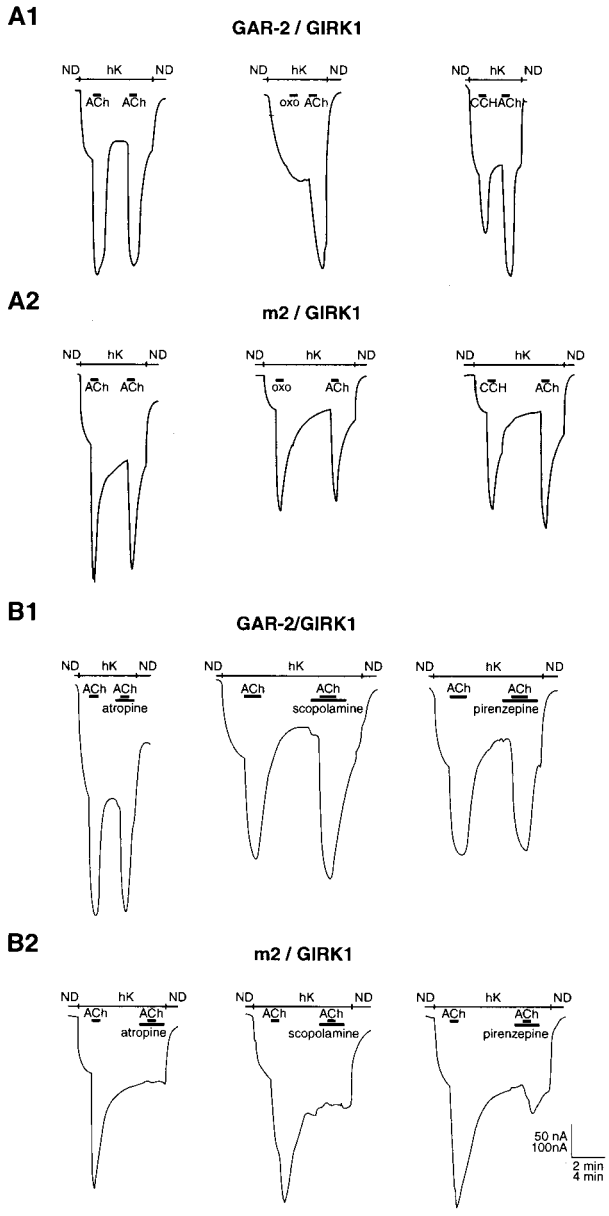

FIG. 5. Pharmacological comparison of GAR-2 and pig m2 mAChR. **A:** Specificity of the agonists oxotremorine (OXO) and carbachol (CCH). **B:** Specificity of the antagonists atropine, scopolamine, and pirenzepine. GIRK1 cRNA (2.5 ng) was injected either with GAR-2 cRNA (2.5 ng; **A1** and **B1**) or with pig m2 mAChR cRNA (2.5 ng; **A2** and **B2**). Drugs were present in the bathing solution during the times indicated by bars. ND, ND96 solution. All the current traces were recorded at a holding potential of −70 mV. The scale bar indicates 50 nA and 2 min for B2 and 100 nA and 4 min for the other traces.

macological characteristics that are different from those of mammalian mAChRs.

Expression pattern of GAR-2::GFP

We examined the expression pattern of GAR-2 during development using a GAR-2::GFP construct (see Materials and Methods). GAR-2 was expressed in a subset of neurons from the folding stage during embryogenesis through adulthood (data not shown). This observation is consistent with our RT-PCR results showing the presence of *gar-2* mRNA throughout development (data not shown). We found that GAR-2 was expressed in some head neurons with ciliated endings (Fig. 6C and D), which are putative sensory neurons, in many cells of the ventral cord (Fig. 6F and H), which are considered to be motor neurons, and in the hermaphrodite-specific neuron (HSN) motor neurons (Fig. 6J), which innervate vulval muscles in the hermaphrodite.

We also examined the expression pattern of GAR-1::GFP and observed that GAR-1 was expressed in a subset of neuronal cells different from those expressing GAR-2. GAR-1 was expressed in some head neurons with ciliated endings (Fig. 6A) and in the nucleus periventricularis magnocellularis (PVM) neuron (Fig. 6B).

DISCUSSION

We have previously reported the identification of two GARs in *C. elegans*, termed GAR-1 and GAR-3. Whereas GAR-3 is a *C. elegans* mAChR (Hwang et al., 1999), GAR-1 is a new type of GAR that is pharmacologically different from conventional mAChRs (Lee et al., 1999). Here we characterized a third type of GAR, designated GAR-2. Amino acid sequence analysis suggests that like GAR-1, GAR-2 does not belong to any known mAChR subtypes and therefore may constitute a novel family of GARs. Taken together, three different types of GARs appear to mediate the slow form of cholinergic neurotransmission in *C. elegans.*

By comparing electrophysiology, pharmacology, and expression of the three GARs, we found some similarities and differences among them. First, both GAR-1 and GAR-2, when expressed in *Xenopus* oocytes, activate the GIRK1 channel. On the other hand, GAR-3 activates the endogenous Cl^- channel in *Xenopus* oocytes. These results imply that the signal transduction pathway mediated by GAR-3 is distinct from those mediated by GAR-1 and GAR-2. Second, GAR-1 and GAR-2 are different from GAR-3 in that muscarinic ligands (such as oxotremorine, atropine, scopolamine, and pirenzepine) do not work on GAR-1 and GAR-2 as effectively as on GAR-3. GAR-2 is still distinguishable from GAR-1 because GAR-2 is not inhibited at all by muscarinic antagonists (atropine, scopolamine, and pirenzepine) that block GAR-1 to some extent. Third, RT-PCR experiments show that all the three GARs are expressed throughout development, from embryonic to adult stage. GFP expression studies indicate, however, that GAR-1 and GAR-2 are expressed in different types of neuronal cells.

Unlike other GARs, GAR-2 contains no consensus site for N-linked glycosylation at its amino terminus. Nevertheless, GAR-2 was able to activate the GIRK1 channel in response to ACh. This observation suggests that N-linked glycosylation may not be required for the physiological function of GARs. Indeed, it has been shown that elimination of potential N-linked glycosylation sites from the m2 mAChR by site-directed mutagenesis does not affect cell surface localization, ligand binding, stability, or functional coupling of the receptor (van Koppen and Nathanson, 1990). The lack of N-linked glycosylation is not unique to the *C. elegans* receptor but is also found in some mammalian G protein-linked receptors. Human and rat α_{2B}-adrenergic receptors, for example, have been shown to possess no putative site for N-linked glycosylation (Lomasney et al., 1990; Zeng et al., 1990).

Database searches indicate that most *C. elegans* genes for G protein-linked receptors contain introns in the coding region, whereas the genes for human G protein-linked receptors are predominantly intronless (Gentles and Karlin, 1999). This seems to be also true for GARs. Nucleotide sequence comparison between the cDNA and the genomic DNA indicates that the *gar-2* gene contains 10 introns in the coding region. Two other GAR genes in *C. elegans, gar-1* and *gar-3,* are also shown to have introns in the coding region (Hwang et al., 1999; Lee et al., 1999). By contrast, no intron has been found in the coding region of human GAR (that is, mAChR) genes. Although the neurophysiological significance of this genomic divergence between *C. elegans* and humans is not fully understood, the introns of the *C. elegans* receptor genes appear to play two important roles. First, the introns may contain information essential for proper cell-specific expression of the receptors. When we compared the expression patterns of the two GFP constructs with and without the first intron of *gar-2,* more neurons were observed to express the GFP construct with the first intron. For example, the GFP construct without the first intron, which contains ~2.7 kb of the upstream region and the first eight codons of *gar-2,* was not expressed in the motor neurons of the ventral cord (data not shown). In the case of *gar-1,* however, the introns may not be involved in cell-specific expression: inclusion of all the introns of *gar-1* did not significantly alter the expression pattern (data not shown). Second, the introns of the *C. elegans* receptor genes seem to be crucial for generating multiple receptor isoforms from single genes. We have previously shown that three functional GAR-1 isoforms are generated by alternative use of introns of the *gar-1* gene (Park et al., 2000). Our preliminary data also indicate that three and two mRNA species are synthesized from the *gar-2* and *gar-3* genes, respectively. It seems likely that alternative splicing serves as a general mechanism for diversifying GARs in *C. elegans.*

The expression of GAR-2 in various cell types (Fig. 6) may reflect the involvement of the receptor in diverse behaviors of *C. elegans.* For instance, the expression of

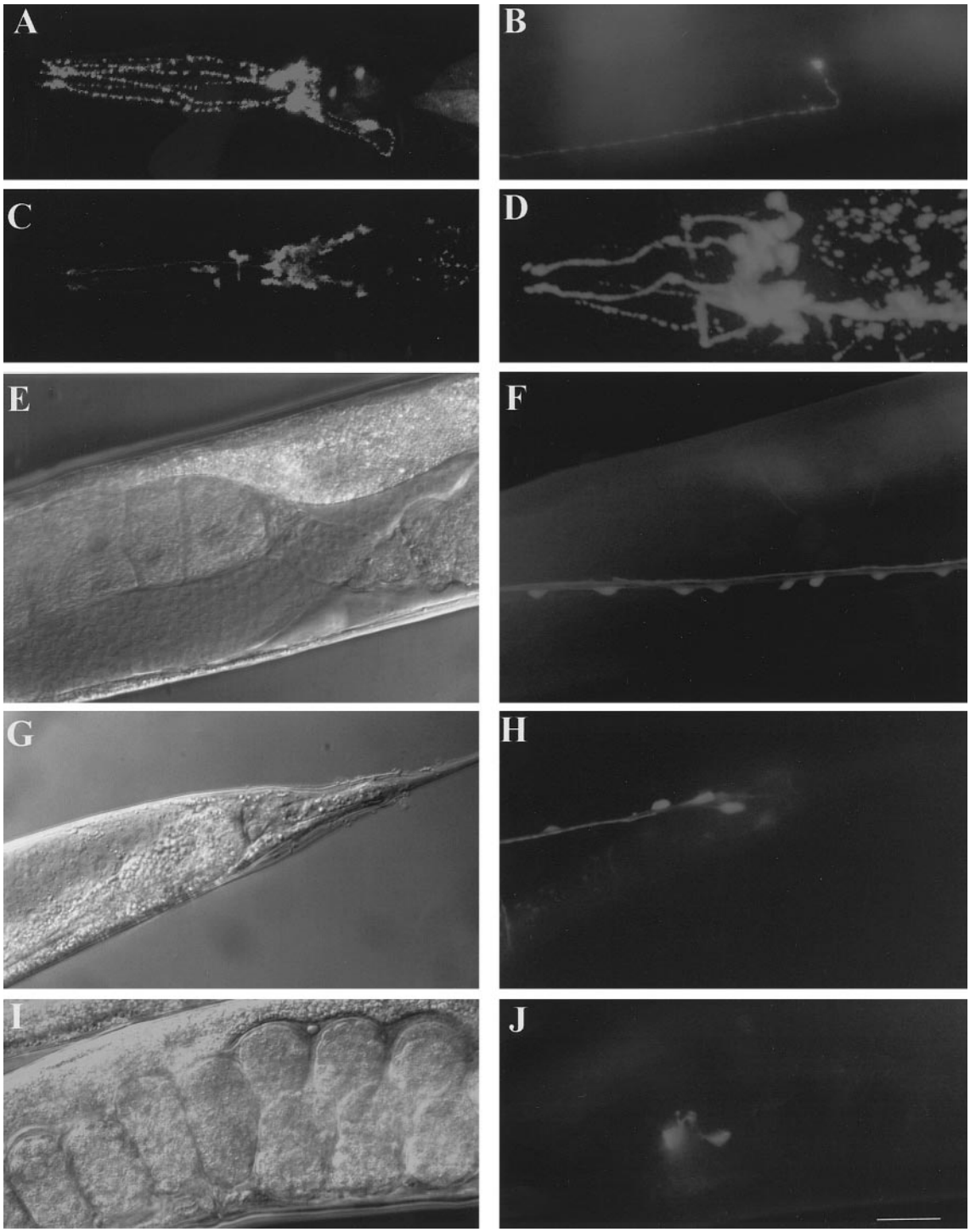

FIG. 6. Expression patterns of GAR-1 (A and B) and GAR-2 (C–J). **A:** Confocal image of GAR-1::GFP expression in the head region of a larva. **B:** GAR-1 expression in the PVM neuron of an adult. **C:** Confocal image of GAR-2::GFP expression in the head region of a larva. **D:** Confocal image of GAR-2::GFP expression in the head region of an adult. The animal is laid on the slide 90° twisted from that in C. **E** and **F:** GAR-2 expression in the ventral region of an adult shown by (E) Nomarski optics image and (F) GFP fluorescence image. **G** and **H:** GAR-2 expression in the tail region of a larva shown by (G) Nomarski optics image and (H) GFP fluorescence image. **I** and **J:** GAR-2 expression in the HSN motor neurons of an adult shown by (I) Nomarski optics image and (J) GFP fluorescence image. Bar = 10 μm.

GAR-2 in the HSN motor neurons (Fig. 6J) may indicate that GAR-2 participates in egg-laying behavior because these neurons play an important role in egg laying (Trent et al., 1983; Desai and Horvitz, 1989). We plan to determine the roles of GAR-2 in *C. elegans* behaviors by generating and analyzing loss-of-function mutants in *gar-2*.

Many neurotransmitter receptors act not only as postsynaptic signal transducers but also as presynaptic signal modulators. Our GFP expression data indicate that GAR-1 and GAR-2 might be present in some putative sensory neurons. As many synaptic inputs on sensory neurons are considered to be presynaptic, it is possible that GAR-1 and GAR-2 function as autoreceptors and/or heteroreceptors; that is, these receptors are located at the presynaptic site of the sensory neurons and regulate the release of neurotransmitters. In vertebrates, several lines of evidence have shown that mAChRs can work presynaptically. In the electric organ of *Torpedo ocellata* activation of mAChRs results in the inhibition of ACh release (Michaelson et al., 1979), suggesting the role of mAChRs as negative feedback regulators. Axoplasmic transport of mAChRs in dog splenic nerve (Laduron, 1980) also supports the presynaptic function of the receptors. In addition, immunocytochemistry indicates the presynaptic localization of mAChRs in the rat striatum and hippocampus (Hersch et al., 1994; Rouse et al., 1999). The detailed molecular mechanisms underlying presynaptic control of neurotransmitter release are not known, and the *C. elegans* system might provide a convenient model for the study.

This study, combined with our previous reports (Hwang et al., 1999; Lee et al., 1999), shows that three types of GARs (GAR-1, GAR-2, and GAR-3) are present in *C. elegans*. Whereas GAR-3 appears to be the mAChR homologue, homologues of GAR-1 and GAR-2 have not been reported in other organisms. It is not clear at present whether GAR-1 and GAR-2 are uniquely found in *C. elegans*. It is possible that the existence of these receptors may have been overlooked in other species because potent mAChR-specific agents such as atropine, scopolamine, and oxotremorine are not very effective on these receptors. Further investigation is needed to explore this possibility.

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