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Cloning and Expression of a G Protein-Linked Acetylcholine Receptor from *Caenorhabditis elegans*

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Abstract: We have isolated a cDNA clone from the nematode *Caenorhabditis elegans* that encodes a protein of greatest sequence similarity to muscarinic acetylcholine receptors. This gene codes for a polypeptide of 682 amino acids containing seven putative transmembrane domains. The amino acid identities, excluding a highly variable middle portion of the third intracellular loop, to the human m1–m5 receptors are 28–34%. When this cloned receptor was coexpressed with a G protein-gated inwardly rectifying K^+ channel (GIRK1) in *Xenopus* oocyte, acetylcholine was able to elicit the GIRK current. This acetylcholine-induced current was substantially inhibited by the muscarinic antagonist atropine in a reversible manner. However, another muscarinic agonist oxotremorine and antagonists scopolamine and pirenzepine had little or negligible effects on this receptor. Taken together, these results suggest that the cloned gene encodes a G protein-linked acetylcholine receptor that is most similar to but pharmacologically distinct from muscarinic acetylcholine receptors. **Key Words:** G protein-linked acetylcholine receptor—Muscarinic acetylcholine receptor—*Caenorhabditis elegans*—G protein-gated inwardly rectifying K^+ channel—*Xenopus* oocyte—Electrophysiology.
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Acetylcholine (ACh) is believed to play a key role in various nervous functions such as contraction of skeletal muscles, emotion, perception, cognition, learning, and memory. ACh has been known to exert its effects by binding two kinds of postsynaptic integral membrane proteins, termed nicotinic and muscarinic receptors. Nicotinic receptors are cation ion channels, whereas muscarinic receptors mediate the regulation of ion channels by activating signal-transducing G proteins and intracellular effector systems.

Muscarinic acetylcholine receptors (mAChRs), like other members of the G protein-linked receptor superfamily, have a predicted structure of seven transmembrane domains. Since Kubo et al. (1986) reported the cloning of a cDNA for pig mAChR, mAChR genes have been isolated from a variety of organisms including humans. In mammals, five mAChR subtypes (m1–m5) have been identified (Bonner et al., 1987, 1988). The biochemical responses mediated by the five mAChR subtypes are known to be different. The m1, m3, and m5 subtypes are shown to preferentially stimulate phosphatidylinositol metabolism by pertussis toxin-insensitive mechanisms, whereas the m2 and m4 subtypes preferentially inhibit adenylyl cyclase by pertussis toxin-sensitive mechanisms (Peralta et al., 1988; Jones et al., 1992). The amino acid sequence comparison between the receptor subtypes reveals a substantial variation in the third intracellular loop (i3 loop) region, which is considered to be the site of G protein recognition. Thus, it is believed that the receptor subtypes mediate different biochemical responses via coupling to different G proteins. Electrophysiological studies also showed that mAChR subtypes modulate different kinds of ion channels. For instance, the m1 subtype, when expressed in *Xenopus* oocyte, activates a Ca^{2+}-dependent Cl^- channel, whereas the m2 subtype regulates a K^+ channel that is directly stimulated by a G protein (Kubo et al., 1986, 1993; Fukuda et al., 1987).

We have begun to study the cholinergic system, using the nematode *Caenorhabditis elegans* as an experimental animal. *C. elegans* is suitable for this study because its nervous system is very simple and because a variety of genetic and molecular biological techniques are readily applicable. Previous studies have shown that *C. elegans* mAChRs bind *N*-[^{3}H]methylscopolamine ([^{3}H]NMS) with high affinity and that ligand binding properties of *C. elegans* mAChRs are generally similar to those of mammalian counterparts (Culotti and Klein, 1983; You et al., 1996). In this study, we identified a novel G protein-

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Abbreviations used: ACh, acetylcholine; CFTR, cystic fibrosis transmembrane conductance regulator; GIRK, G protein-gated inwardly rectifying K^+ channel; hK, high K^+; 5-HT, 5-hydroxytryptamine; i3 loop, third intracellular loop; mAChR, muscarinic acetylcholine receptor; m1–m5 and hm1–hm5, five mAChR subtypes and five human mAChR subtypes, respectively; NMS, *N*-methylscopolamine.

linked ACh receptor in *C. elegans,* which is different from the known mAChRs in ligand binding properties.

MATERIALS AND METHODS

Drugs

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

Isolation of the *C. elegans* G protein-linked ACh receptor cDNA clone

C. elegans (N2, wild-type strain) was grown either on nematode growth medium plates (Brenner, 1974) or in liquid culture (Sulston and Brenner, 1974) using *E. coli* strain OP50 as a food source.

The cDNA clone was obtained by PCR, using single-stranded DNA, prepared from a *C. elegans* mixed-stage cDNA library (Stratagene), as a template. The primers were designed based on the genomic sequence of a *C. elegans* cosmid clone in the databank (C15B12; accession no. U23529) (Wilson et al., 1994). PCR was performed in a total volume of 50 μl containing 1.5 m*M* $MgCl_2$, 1× reaction buffer (Amersham), 200 μ*M* of each dNTP, 2.5 U *Taq* polymerase (Amersham), 1 μ*M* forward primer (MF no. 1), 1 μ*M* reverse primer (MR no. 1), and 20 ng of template DNA. The PCR condition was as follows: an initial denaturation at 95°C for 5 min, 30 cycles of amplification at 95°C for 1 min, 55°C for 2 min, and 72°C for 3 min, and a final extension at 72°C for 7 min. The PCR products were ligated into pCRII vector (Invitrogen) and the nucleotide sequences of the cDNA clones were determined by the dideoxynucleotide chain termination method, using the Cy5 AutoRead sequencing kit and ALFexpress DNA sequencer (Pharmacia). We identified one clone (pCMR10), which contains the identical nucleotide sequence to the corresponding genomic sequence (Wilson et al., 1994). This gene was named *gar-1* (for G protein-linked ACh receptor). Primer sequences are as follows: MF no. 1, 5′-ATCGATTGCTTCTCATCTTG-3′; MR no. 1, 5′-ATTTTGGATCCAGATCTATGCAACT-3′ (the shaded sequence G was changed from the sequence A to create a *Bam*HI site).

Genomic Southern blot analysis

Genomic DNA was isolated from *C. elegans* as described by Emmons et al. (1979). The genomic DNA (5 μg) was digested with restriction enzymes, electrophoresed through a 0.8% (wt/vol) agarose gel, and transferred to a nitrocellulose filter by using 20× saline–sodium citrate as described by Sambrook et al. (1989). The blot was hybridized to a ^{32}P-labeled *C. elegans* G protein-linked ACh receptor probe. The incubation was performed at 37°C for 16 h in a hybridization buffer [6× saline–sodium citrate, 30% (vol/vol) formamide, 0.5% (wt/vol) sodium dodecyl sulfate, 5× Denhardt's solution, and 0.1 mg/ml salmon sperm DNA]. The blot was washed twice (10 min each) at 37°C in 2× saline–sodium citrate/0.5% (wt/vol) sodium dodecyl sulfate and twice (20 min each) at 50°C in 0.2× saline–sodium citrate/0.1% (wt/vol) sodium dodecyl sulfate. The probe DNA was labeled with [^{32}P]dCTP (3,000 Ci/mmol, Amersham), using a random primed DNA labeling kit (Boehringer Mannheim).

Preparation of stage-specific RNA and RT-PCR

Gravid worms were treated with alkaline hypochlorite solution (Emmons et al., 1979) and hypochlorite-resistant embryos were then hatched in M9 buffer overnight at 20°C. Hatched L1 larvae were placed on nematode growth medium plates and allowed to develop to various developmental stages before harvest for RNA preparation. Harvested worms were frozen in liquid nitrogen and ground to a powder using a mortar and pestle. RNA was prepared from the frozen worm powder by use of the Tri reagent (Sigma) following the procedure recommended by the manufacturer.

RT-PCR was performed by using the RNA PCR kit (Takara). The first strand of cDNA was synthesized from 0.9 μg of each stage-specific total RNA in a 5-μl solution containing 5 m*M* $MgCl_2$, 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 1 m*M* of each dNTP, 5 U RNase inhibitor, 0.125 μ*M* oligo(dT) primer, and 1.25 U of avian myeloblastosis virus (AMV) reverse transcriptase XL. The incubation was performed at 30°C for 10 min, followed by 42°C for 1 h. The reaction was terminated by heating at 99°C for 5 min. For PCR, 3 μl of the RT reaction mixture was added to a 12-μl solution containing 1× Ex *Taq* buffer, 0.25 μ*M* forward primer (MF no. 1), 0.25 μ*M* reverse primer (MR no. 1), and 0.075 U of Takara Ex *Taq* polymerase. The PCR condition was as follows: an initial denaturation at 94°C for 2 min, 41 cycles of amplification at 94°C for 30 s, 57°C for 30 s, and 72°C for 2 min, and a final extension at 72°C for 8 min. The PCR product (2 μl) was analyzed by gel electrophoresis.

cRNA preparation and expression in *Xenopus* oocytes

The cRNAs for the *C. elegans* G protein-linked ACh receptor, pig cardiac mAChR (Fukuda et al., 1987), human β_2-adrenergic receptor (Kobilka et al., 1987), mouse 5-HT_{2C} (previously termed 5-HT_{1C}) receptor (Lubbert et al., 1987), human cystic fibrosis transmembrane conductance regulator (CFTR) (Bear et al., 1991), and rat G protein-gated inwardly rectifying K^+ channel (GIRK1) (Dascal et al., 1993) were prepared by in vitro transcription, using the cRNA Megascript kit (Ambion) (Uezono et al., 1993). The resulting cRNAs were identified on a gel and quantified spectrophotometrically.

Stages IV and V oocytes were removed from female *Xenopus laevis* (Xenopus I Inc.) and then defolliculated by incubation for 1.5–2 h with 2 mg/ml collagenase (type IA, Sigma) in OR-Mg solution (82 m*M* NaCl, 20 m*M* $MgCl_2$, 2 m*M* KCl, 5 m*M* HEPES, pH 7.5). Oocytes were microinjected with cRNAs and incubated for 2–3 days at 18°C in ND96 solution (96 m*M* NaCl, 2 m*M* KCl, 1.8 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 5 m*M* HEPES, pH 7.5) supplemented with 2.5 m*M* sodium pyruvate and 50 μg/ml gentamicin.

Electrophysiology

Two-electrode voltage clamp was performed with a Geneclamp 500 amplifier (Axon Instruments). The voltage electrode and the current electrode were pulled, using a horizontal puller (Sutter Instruments), to have resistances of 1–5 MΩ. The electrodes were filled with 3 *M* KCl. The oocytes were placed in a chamber perfused with Ca^{2+}-free ND96. The holding potential was set at −80 mV. In the case of recording GIRK current, the solution was changed to high-K^+ (hK) solution (96 m*M* KCl, 2 m*M* NaCl, 1.8 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 5 m*M* HEPES, pH 7.5). Drugs dissolved in Ca^{2+}-free ND96 or hK solution were applied to the recording chamber via bath perfusion lines. The data were collected on a chart recorder and stored on video cassette recorder tape through Digidata (Instrutech) for later analysis.

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                                                     atcgattgcttctcatcttgaaaaaagcttccgtctagcag   -1
ATG CCG AAC TAC ACG GTA CCT CCA GAT CCA GCA GAC ACT AGC TGG GAT TCT CCA TAC AGC ATT CCA GTT CAG ATC   75
Met Pro Asn Tyr Thr Val Pro Pro Asp Pro Ala Asp Thr Ser Trp Asp Ser Pro Tyr Ser Ile Pro Val Gln Ile   25
GTT GTA TGG ATA ATT ATC ATT GTT CTA AGC TTG GAG ACT ATC ATT GGA AAT GCT ATG GTC GTG ATG GCC TAC CGG  150
Val Val Trp Ile Ile Ile Ile Val Leu Ser Leu Glu Thr Ile Ile Gly Asn Ala Met Val Val Met Ala Tyr Arg   50
ATT GAG AGA AAC ATC AGT AAA CAG GTA AGC AAC CGA TAT ATT GTT TCC CTC GCC ATA TCA GAT CTA ATC ATT GGC  225
Ile Glu Arg Asn Ile Ser Lys Gln Val Ser Asn Arg Tyr Ile Val Ser Leu Ala Ile Ser Asp Leu Ile Ile Gly   75
ATC GAA GGA TTT CCG TTT TTT ACG GTC TAC GTT CTG AAT GGG GAC CGG TGG CCT CTA GGA TGG GTG GCC TGT CAA  300
Ile Glu Gly Phe Pro Phe Phe Thr Val Tyr Val Leu Asn Gly Asp Arg Trp Pro Leu Gly Trp Val Ala Cys Gln  100
ACT TGG CTG TTT CTT GAC TAC ACG CTA TGT CTT GTG TCG ATT TTA ACG GTT CTT CTG ATT ACA GCC GAT AGA TAT  375
Thr Trp Leu Phe Leu Asp Tyr Thr Leu Cys Leu Val Ser Ile Leu Thr Val Leu Leu Ile Thr Ala Asp Arg Tyr  125
CTC TCA GTG TGT CAC ACT GCA AAG TAC TTA AAA TGG CAG AGC CCA ACA AAA ACG CAA CTT TTG ATT GTT ATG TCC  450
Leu Ser Val Cys His Thr Ala Lys Tyr Leu Lys Trp Gln Ser Pro Thr Lys Thr Gln Leu Leu Ile Val Met Ser  150
TGG TTG CTA CCA GCA ATT ATT TTT GGT ATC ATG ATT TAT GGT TGG CAA GCA ATG ACG GGA CAA TCT ACA AGT ATG  525
Trp Leu Leu Pro Ala Ile Ile Phe Gly Ile Met Ile Tyr Gly Trp Gln Ala Met Thr Gly Gln Ser Thr Ser Met  175
TCT GGC GCT GAA TGC TCT GCT CCG TTC TTG AGC AAT CCC TAT GTG AAC ATG GGA ATG TAT GTG GCC TAT TAC TGG  600
Ser Gly Ala Glu Cys Ser Ala Pro Phe Leu Ser Asn Pro Tyr Val Asn Met Gly Met Tyr Val Ala Tyr Tyr Trp  200
ACG ACA CTG GTT GCG ATG CTA ATT CTT TAC AAG GGT ATT CAT CAA GCA GCC AAA AAT CTT GAG AAA AAA GCC AAA  675
Thr Thr Leu Val Ala Met Leu Ile Leu Tyr Lys Gly Ile His Gln Ala Ala Lys Asn Leu Glu Lys Lys Ala Lys  225
GCC AAA GAG AGG AGA CAT ATT GCG TTG ATT CTG AGT CAG CGG CTG GGA ACG CAG GTC GGA GTT TCT TTA ATG CTC  750
Ala Lys Glu Arg Arg His Ile Ala Leu Ile Leu Ser Gln Arg Leu Gly Thr Gln Val Gly Val Ser Leu Met Leu  250
CAG TCA AAA GCG GAG AAG GAA AAA GCT GAA GAA GCT CAG AAA GAT AGT GGA TAT ACA AGC AAT CAG GCT GGG GGA  825
Gln Ser Lys Ala Glu Lys Glu Lys Ala Glu Glu Ala Gln Lys Asp Ser Gly Tyr Thr Ser Asn Gln Ala Gly Gly  275
TCT CTA AAC ACC GAG AAT GAC CAA AAC CTG GGA GTT ATT GAA GAA GAG AGA AGT GGA TTT CTC TCG AGA AGA GAA  900
Ser Leu Asn Thr Glu Asn Asp Gln Asn Leu Gly Val Ile Glu Glu Glu Arg Ser Gly Phe Leu Ser Arg Arg Glu  300
AGC AAC GAA AGC TAC TAT CCT GGT CCT CAT CCA ACT GCG GCC AAC TCT AGA AGG TGT AGT GAA ATG GAA AAA GTT  975
Ser Asn Glu Ser Tyr Tyr Pro Gly Pro His Pro Thr Ala Ala Asn Ser Arg Arg Cys Ser Glu Met Glu Lys Val  325
TCC TTA TTG AGT GAA AGT GAC GGA GTG CCG AGT ACA AGG CCT GCA AAA TCT TAT GGC AGA CTT TCG TTA AGA TCT 1050
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Asn Asn Asp Ser Asp Thr Thr Ser Val Ile Leu Gln Arg Ser Arg Lys Tyr Lys Lys Asn Lys Arg Pro Arg Ser  425
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Ile Glu Glu Ser Val Pro Asp Asp Asp Gln Thr Glu Thr Ile Glu Val Lys Arg Thr Asp Arg Trp Val Val Ser  475
ATG AAA AAA CGA ATT GCA AGA GCA TTG ATA CGT CGA CGA AGT ACG ACG AGG CCA GAA AGA GGA AGT AGC AGC AAC 1500
Met Lys Lys Arg Ile Ala Arg Ala Leu Ile Arg Arg Arg Ser Thr Thr Arg Pro Glu Arg Gly Ser Ser Ser Asn  500
AGC GAT GAT TCT TCG TCT GAA GTT GAG GGT GAA GAG AAA CCA GAA GTC AGA AAC AAC GGC CTC AAA ATT CCC CAG 1575
Ser Asp Asp Ser Ser Ser Glu Val Glu Gly Glu Glu Lys Pro Glu Val Arg Asn Asn Gly Leu Lys Ile Pro Gln  525
CTA ACC GTT AAC AAC GAA AAT CGC GGA GAA ACG TCT TCA CAG CCC GGA CGA GAC CGT CTT GCA CCA CCC AAT AAA 1650
Leu Thr Val Asn Asn Glu Asn Arg Gly Glu Thr Ser Ser Gln pro Gly Arg Asp Arg Leu Ala Pro Pro Asn Lys  550
ACT GAT ACA TTT TTA AGC GCC TCG GGA GTC TCC AGA AAA ATT TCG ACA ATC TCC ACG GTC ATC ACA AGG GAG AAG 1725
Thr Asp Thr Phe Leu Ser Ala Ser Gly Val Ser Arg Lys Ile Ser Thr Ile Ser Thr Val Ile Thr Arg Glu Lys  575
GTC ATT TCT TCA ATT TTT GCA CCG ATT GCC GTC TTC AAC AGA GGA AGA AAA CAG ACA AAA GCT GAA AAG AGG GCT 1800
Val Ile Ser Ser Ile Phe Ala Pro Ile Ala Val Phe Asn Arg Gly Arg Lys Gln Thr Lys Ala Glu Lys Arg Ala  600
CAT AAA GCA TTC AGA ACA ATC ACA TTT ATC GTA GGA TTT TTC GCA ATT CTT TGG TCA CCG TAT TAC ATA ATG GCA 1875
His Lys Ala Phe Arg Thr Ile Thr Phe Ile Val Gly Phe Phe Ala Ile Leu Trp Ser Pro Tyr Tyr Ile Met Ala  625
ACA GTG TAT GGT TTC TGT AAA GGC GAA TGC ATA CCA TCA TTT CTG TAC ACT CTA TCG TAT TAC ATG TGT TAC CTG 1950
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Asn Ser Ser Gly Asn Pro Phe Ala Tyr Ala Leu Ala Asn Arg Gln Phe Arg Ser Ala Phe Met Arg Met Phe Arg  675
GGG AAC TTT AAC AAA GTT GCA TAG atctggattcaaaat                                                    2064
Gly Asn Phe Asn Lys Val Ala ***                                                                     682
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FIG. 1. Nucleotide sequence and deduced amino acid sequence of the *C. elegans* G protein-linked ACh receptor. Putative transmembrane domains are shown by shaded background. The positions of introns, identified by comparison with the corresponding genomic sequence (Wilson et al., 1994), are indicated by arrowheads. The PCR primers used are underlined. This sequence has been submitted to GenBank under accession no. AF075245.

RESULTS

In an attempt to isolate a cDNA clone encoding *C. elegans* mAChR or its homologue, we performed a PCR, using *C. elegans* cDNA library as a template. The primers were designed based on the sequence information of a possible mAChR gene reported by the *C. elegans* genome sequencing consortium (Wilson et al., 1994) (see Materials and Methods). A cDNA clone, which contains an open reading frame of 682 amino acids, was identified by restriction enzyme mapping and sequence analysis (Fig. 1). Comparison of this cDNA sequence with the corresponding genomic sequence indicated that the *C. elegans* gene contains 16 introns in the coding region. Seven putative transmembrane domains, which is a characteristic feature of G protein-linked receptors, could be identified by hydropathy profile (Fig. 1). Other features of G protein-linked receptors were also found in this protein, for example, a potential *N*-glycosylation site (Asn^{3}) and two cysteine residues engaged in a disulfide linkage in the second and third extracellular segments (Fig. 2).

Database searches indicated that the cloned receptor is most similar to mAChRs. We compared the amino acid sequence of this receptor with those of five human mAChR subtypes (hm1–hm5; Fig. 2). Excluding the highly variable i3 loop except 20 amino acids on each side, the overall amino acid sequence identities to hm1, hm2, hm3, hm4, and hm5 are estimated to be 32, 34, 28,

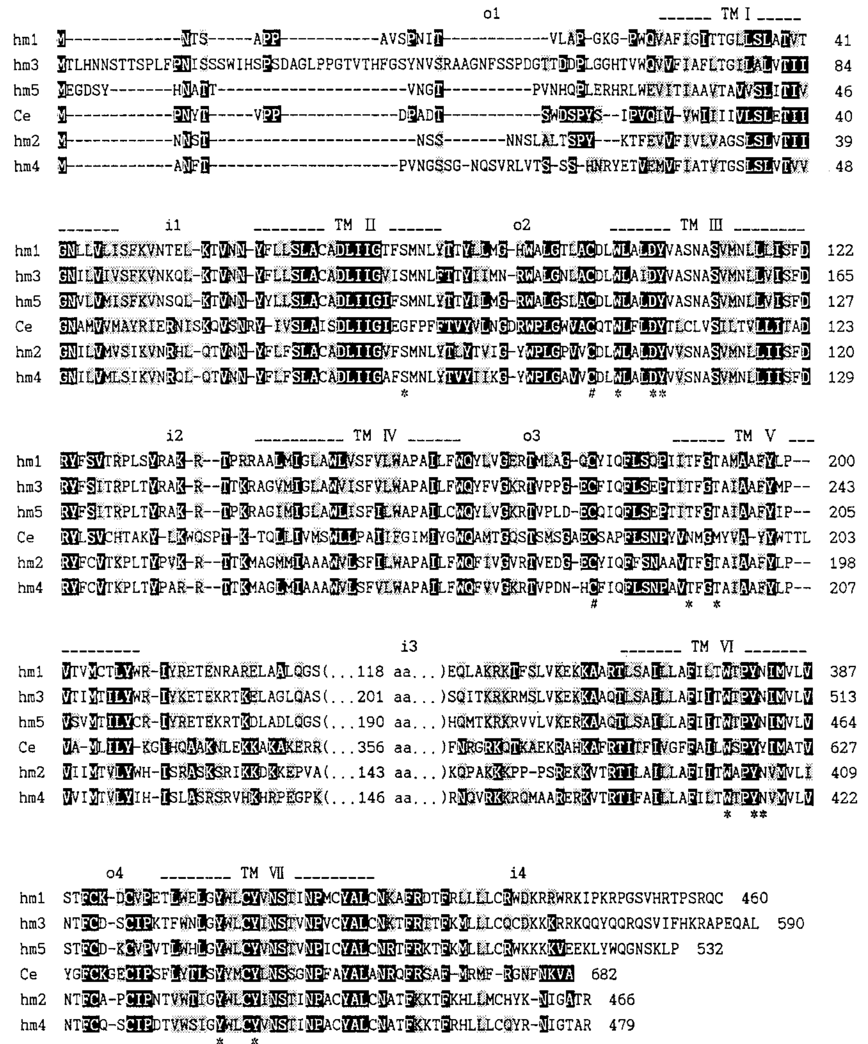

FIG. 2. Amino acid sequence comparison of the *C. elegans* G protein-linked ACh receptor and human mAChR subtypes (hm1–hm5). Seven putative transmembrane domains (TM I–TM VII), extracellular segments (o1–o4), and intracellular segments (i1–i4) are indicated. Identical amino acid residues are shown by black background and similar amino acid residues are shaded in gray. Only the N-terminal and the C-terminal portions of i3 loop are shown. #Cysteine residues engaged in a disulfide bond. *Amino acid residues reported to be important for muscarinic ligand binding (Wess et al., 1995). Some of these residues (four of 11) are absent in the *C. elegans* receptor. Gaps (−) are inserted to align amino acids. The amino acid sequences of human mAChRs are taken from Peralta et al. (1987) and Bonner et al. (1988).

32, and 32%, respectively. The amino acid sequence similarities of the same region to hm1, hm2, hm3, hm4, and hm5 are about 52, 52, 46, 51, and 50%, respectively. Site-directed mutagenesis analyses of mAChRs have identified several conserved amino acid residues that are important for muscarinic ligand binding (Wess et al., 1995). The sequence comparison revealed that the *C. elegans* receptor lacks some of these residues (Fig. 2). This sequence difference may partly explain the observation that the *C. elegans* receptor and mAChRs displayed distinct ligand binding properties (see Fig. 6).

To examine whether *C. elegans* contains multiple genes for the cloned receptor, genomic Southern blot analysis was performed using the cloned cDNA as a probe at moderate stringency. All the strongly hybridizing bands were of expected size (Fig. 3), suggesting that this receptor may be encoded by a single gene. A few weakly hybridizing bands (an ~6-kb *Eco*RI fragment, an ~3.5-kb *Hin*dIII fragment, and an ~3-kb *Eco*RV fragment) were also observed that may represent either other subtypes of the receptor or other closely related receptors.

We performed RT-PCR experiments to examine at which stage the cloned receptor gene transcript is present. A band of expected size (~2 kb) was observed at all developmental stages (Fig. 4), including the dauer stage (data not shown), indicating that the gene transcript is present throughout the developmental stages.

To determine whether the cloned cDNA encodes a functional receptor, we expressed the cDNA in *Xenopus* oocytes. The receptor cRNA was injected into oocytes either alone or together with GIRK1 cRNA. Replacement of Ca^{2+}-free ND96 solution by hK solution elicited an inward current (I_{hK}). Amplitude of I_{hK} in water-injected oocytes (57.0 ± 4.6 nA, n = 5) (Fig. 5A) was not significantly different from that in oocytes injected with the receptor cRNA alone (49.0 ± 4.8 nA, n = 5) (Fig. 5B). The current amplitude was larger in oocytes injected with GIRK1 alone (120.8 ± 9.4 nA, n = 15)

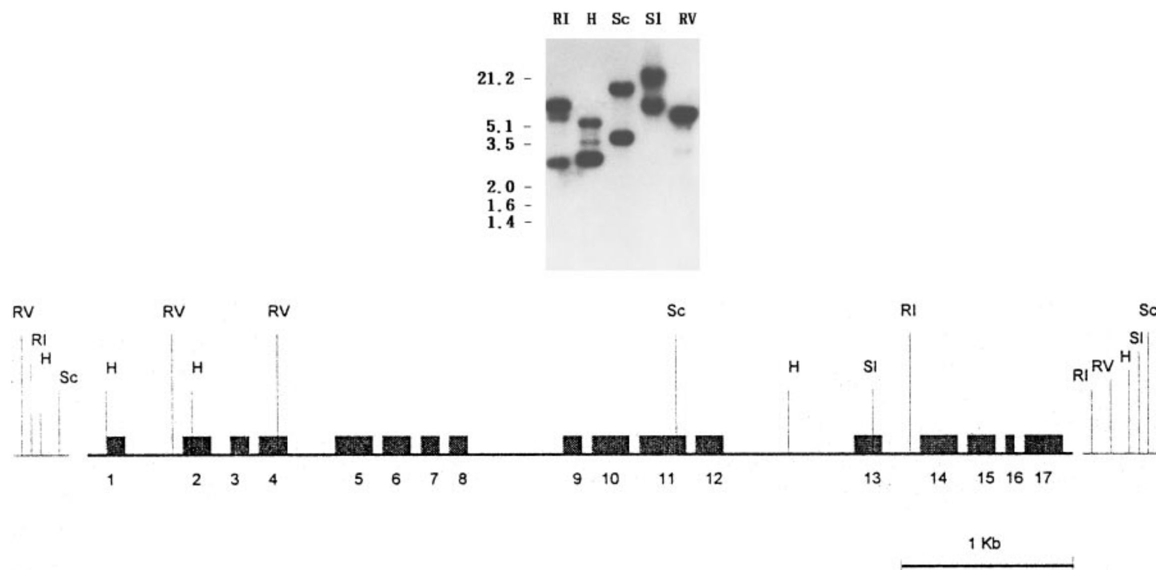

FIG. 3. Top: Genomic Southern blot analysis of the *C. elegans* G protein-linked ACh receptor gene. Genomic DNA (5 μg each) was digested with restriction enzymes, size-fractionated on a 0.8% (wt/vol) agarose gel, blotted onto a nitrocellulose filter, and hybridized with the *C. elegans* receptor cDNA. Positions of molecular weight markers are shown in kilobases. Fragments as small as 0.5 kb were not visible. RI, *Eco*RI; H, *Hin*dIII; Sc, *Sac*I; SI, *Sal*I; RV, *Eco*RV. **Bottom:** Structure and restriction map of the *C. elegans* receptor gene. Exons are indicated by black boxes. Enzyme sites on the thin line (on both ends of the map) are not drawn to scale. Expected sizes are as follows: RI (8.3 and 2.7 kb), H (0.5, 2.8, and 5.3 kb), Sc (3.7 and 15.0 kb), SI (>18 and 7.0 kb), and RV (4.7, 0.5, and 6.3 kb). The genomic sequence information, reported by the *C. elegans* genome sequencing consortium (Wilson et al., 1994), was obtained from the database (accession no. U23529).

(Fig. 5C). When oocytes injected with both the receptor and GIRK1 cRNAs were treated with 1 μM ACh, an additional increase (295.4 ± 56.3 nA, n = 21) in the inward current was observed (Fig. 5D). In contrast, water-injected oocytes and oocytes injected with the receptor cRNA alone or GIRK1 cRNA alone were not affected by treatment with 1 μM ACh. These results demonstrate that the cloned cDNA encodes a functional ACh receptor. The results also suggest that the cloned receptor couples to a G protein, possibly of the G_i/G_o class, as the GIRK1 channel is known to be regulated by many G_i/G_o-linked receptors.

We compared the pharmacological properties of the *C. elegans* G protein-linked ACh receptor with those of pig cardiac mAChR (m2), which is also known to couple to the activation of the GIRK1 channel. Oocytes expressing pig cardiac mAChR and GIRK1 produced the inward current (415.0 ± 79.3 nA, n = 9) by treatment with 1 μM ACh. The muscarinic antagonist atropine (1 μM) decreased the ACh-induced GIRK current mediated by the *C. elegans* receptor by 76.3 ± 6.2% (n = 13) in a reversible manner (Fig. 5D), whereas atropine blocked most of the current (90.2 ± 7.5%, n = 3) mediated by the pig cardiac mAChR (data not shown). Other potent antagonists, scopolamine (1 μM) and pirenzepine (1 μM), reduced the current mediated by the *C. elegans* receptor by only 15.0 ± 7.0% (n = 5) and 30.5 ± 16.0% (n = 3), respectively (Fig. 6A_2 and B_2). By contrast, they were able to reduce the current mediated by the pig cardiac mAChR by 97.6 ± 2.4% (n = 3) and 83.8 ± 6.0% (n = 3), respectively (Fig. 6A_1 and B_1). Although carbachol (1 μM), an ACh analogue, could induce the GIRK current in the oocytes expressing the *C. elegans* receptor (76.0 ± 9.9 nA, n = 5) (data not shown), the potent muscarinic agonist oxotremorine (1 μM) failed to elicit any significant response (n = 4) (Fig. 6C_2). These data show that the cloned receptor is pharmacologically different from the pig cardiac mAChR and other previously known mAChRs.

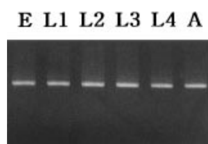

FIG. 4. RT-PCR for the *C. elegans* G protein-linked ACh receptor gene transcript. The first strand of cDNA was synthesized from the total RNA isolated from each developmental stage and used as a template for the subsequent PCR. The RT-PCR products were electrophoresed on a 1.0% (wt/vol) agarose gel. E, embryo; L1, first larval stage; L2, second larval stage; L3, third larval stage; L4, fourth larval stage; A, adult.

We next explored the possibility that the cloned *C. elegans* receptor couples to other G proteins. A transient endogenous Ca^{2+}-activated Cl^- current (1.24 ± 0.23 μA, n = 3) was generated, presumably via G_o/G_q, by activation of 5-HT_{2C} receptor (Fig. 7A_1). By contrast, the cloned *C. elegans* receptor did not produce the current (n = 6) (Fig. 7A_2), suggesting that the cloned receptor does not couple to G_o/G_q in *Xenopus* oocyte. In other experiments, the receptor cRNA was coinjected into oocytes with CFTR cRNA to see whether the receptor couples to G_s. These oocytes did not show any response to ACh (n

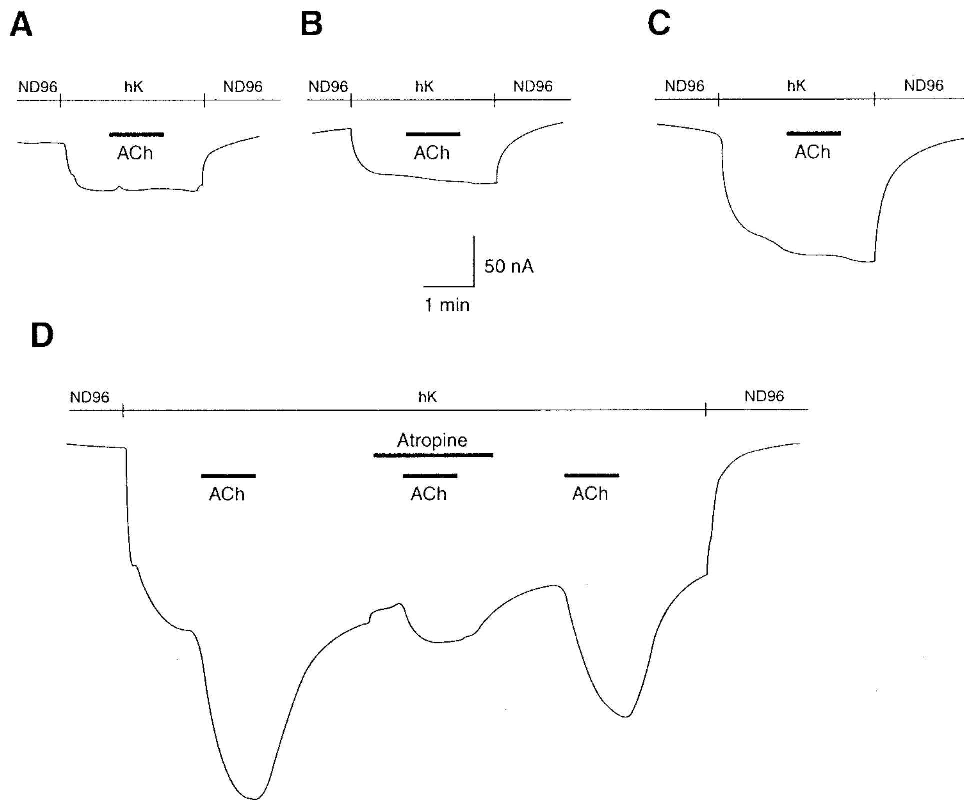

FIG. 5. Functional expression of the *C. elegans* G protein-linked ACh receptor in *Xenopus* oocytes and its coupling to a G protein. Oocytes were injected with the following cRNAs: distilled water (**A**), the receptor cRNA (2.5 ng; **B**), GIRK1 cRNA (2.5 ng; **C**), and the receptor cRNA and GIRK1 cRNA (2.5 ng each; **D**). All the current traces were recorded at a holding potential of −80 mV. Drugs were present in the bathing solution during the times indicated by bars. Only oocytes injected with both the receptor and GIRK1 cRNAs gave rise to an increase in the inward current on application of ACh (1 μM). The muscarinic antagonist atropine (1 μM) reduced this current in a reversible manner.

= 10) (Fig. 7B$_2$), whereas oocytes injected with both β_2-adrenergic receptor cRNA and CFTR cRNA produced the CFTR current by treatment with isoproterenol (662.0 ± 86.0 nA, n = 5) (Fig. 7B$_1$). These results imply that the cloned receptor does not couple to G_s.

DISCUSSION

We have isolated a cDNA clone from *C. elegans* that encodes a G protein-linked ACh receptor. The isolated cDNA is presumed to synthesize a polypeptide that contains seven putative transmembrane domains, which is a characteristic feature of the G protein-linked receptor superfamily. When expressed in *Xenopus* oocytes with GIRK1, this protein evoked the GIRK current in response to ACh. Thus, this receptor is functionally similar to mAChRs. Database searches also indicated that this *C. elegans* protein is more homologous to mAChRs than any other known proteins. However, this protein proved different in drug specificity from mAChRs. Scopolamine (1 μM) reduced the ACh-induced activation of the *C. elegans* receptor by only 15%, whereas it almost completely blocked that of pig cardiac mAChR. In addition, oxotremorine (1 μM) caused little, if any, activation of the *C. elegans* receptor. Conceivably, this *C. elegans* receptor may represent an ancient form of mAChR or a novel type of ACh receptor.

Nucleotide sequence analysis showed that the *C. elegans* G protein-linked ACh receptor gene contains 16 introns. No intron has been found in the coding region of mammalian mAChR genes, although *Drosophila* mAChR gene has been reported to have three introns in the i3 loop region (Shapiro et al., 1989). This observation is in contrast to the general tendency that the genes of lower organisms have shorter and fewer introns than those of higher organisms. In the evolution of the cholinergic nervous system of higher organisms, introns may have been removed from the ACh receptor genes.

We performed electrophysiological recording assays using the *Xenopus* oocyte expression system to examine the coupling specificity of the *C. elegans* receptor to various G proteins. In *Xenopus* oocytes, an endogenous Cl^- channel is activated by Ca^{2+} released from the internal stores by inositol 1,4,5-trisphosphate when G_o/G_q-mediated phosphatidylinositol metabolism is stimulated by receptors such as 5-HT$_{2C}$ receptor (Lubbert et al., 1987; Quick et al., 1994). CFTR is a Cl^- channel that is regulated by cyclic AMP-dependent protein kinase, and activation of G_s-linked receptors induces the CFTR

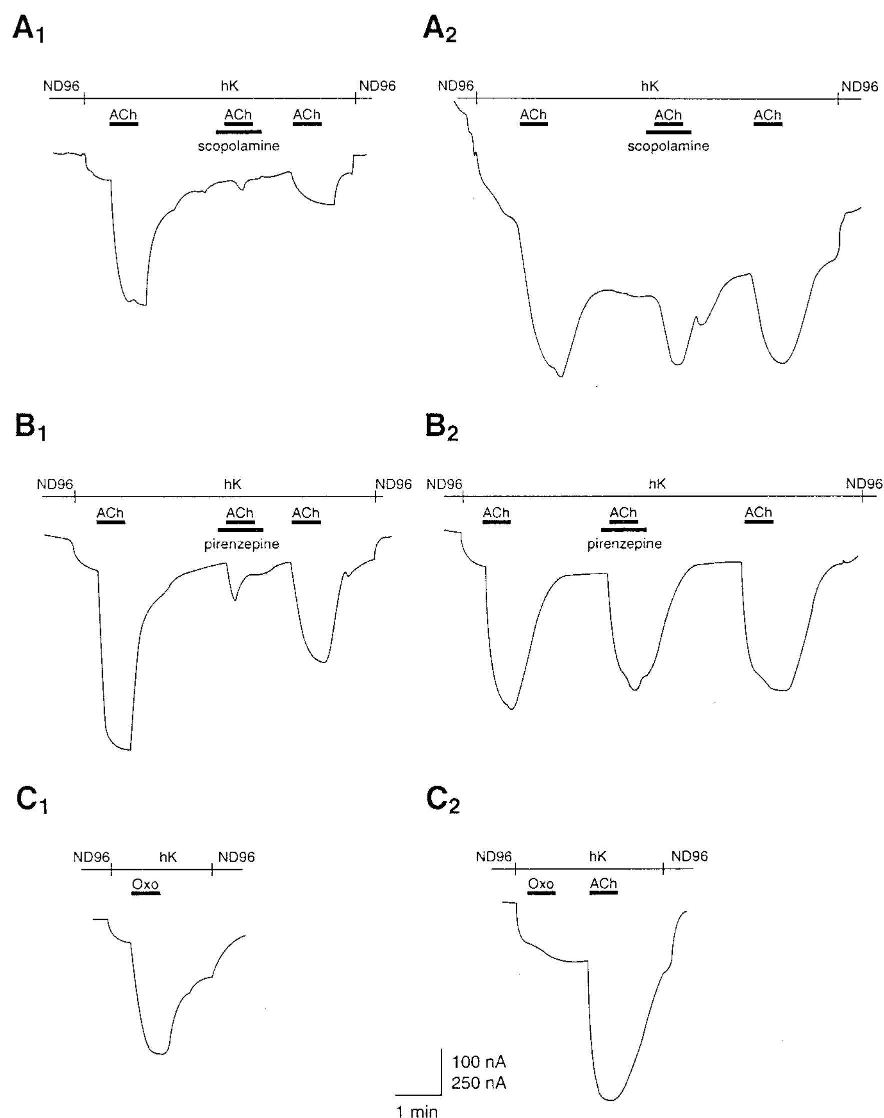

FIG. 6. Comparison of the *C. elegans* G protein-linked ACh receptor and pig cardiac mAChR (m2) with respect to drug specificity. GIRK1 cRNA (2.5 ng) was injected with pig cardiac mAChR cRNA (2.5 ng) (**A_1**, **B_1**, and **C_1**) or with the *C. elegans* receptor cRNA (2.5 ng) (**A_2**, **B_2**, and **C_2**). Recording and drug application were done as described in the legend to Fig. 5. The responses of the *C. elegans* receptor to the muscarinic drugs scopolamine (1 μM), pirenzepine (1 μM), and oxotremorine (1 μM) were much weaker than those of the pig cardiac mAChR. The vertical scale bar indicates 250 nA for B_2 and 100 nA for the other current traces. Oxo, oxotremorine.

current (Uezono et al., 1993). GIRK1 channel is known to be activated by G_i/G_o-linked receptors (Dascal et al., 1993). Therefore, if CFTR or GIRK1 cRNA were introduced with a receptor cRNA into the oocytes, the coupling specificity of the receptor to G proteins could be determined. Our electrophysiological data showed that the activation by ACh of the cloned receptor in the oocytes generates only GIRK current, not CFTR or endogenous Ca^{2+}-activated Cl^- current, suggesting that the cloned receptor couples to G_i, not to G_s or to G_o/G_q.

Previous ligand binding studies indicated the presence of high-affinity [^{3}H]NMS binding sites in *C. elegans* (Culotti and Klein, 1983; You et al., 1996). We asked whether the cloned receptor binds [^{3}H]NMS with high affinity. However, we failed to detect significant levels of [^{3}H]NMS binding sites when the cloned receptor gene was expressed in heterologous systems such as COS-1 cells, CHO-K1 cells, and *Xenopus* oocytes. These results are consistent with the electrophysiological data showing the negligible effect of scopolamine on the *C. elegans* receptor. Thus, it is likely that the gene responsible for the high-affinity [^{3}H]NMS binding sites in *C. elegans* is distinct from the one we cloned in this study. Amino acid sequence comparison revealed that each of the five mAChR subtypes is $<$35% identical to the *C. elegans* receptor, further suggesting that the *C. elegans* receptor does not belong to any known subtype of mAChRs and, therefore, may constitute a new class of G protein-linked ACh receptors.

In summary, we have identified a novel G protein-linked ACh receptor that behaves differently from conventional mAChRs. The finding of the G protein-linked ACh receptor in this study may indicate that ACh receptor system is more diverse than we understand at present. Further study on this receptor is needed to establish its functional role in the cholinergic nervous system, and it will be of considerable interest to see whether this receptor homologue is also present in other organisms.

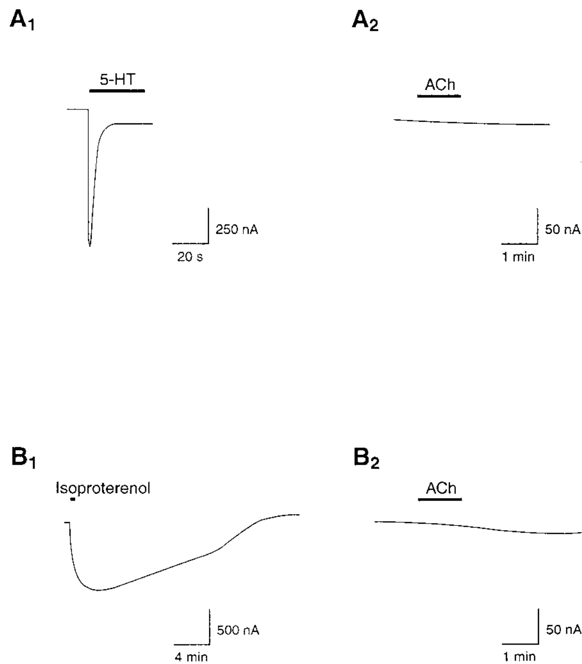

FIG. 7. The *C. elegans* G protein-linked ACh receptor did not stimulate the endogenous Ca^{2+}-activated Cl^- channel or the CFTR channel. Oocytes were injected with the following cRNAs: 5-HT_{2C} receptor cRNA (100 pg; **A$_1$**), the *C. elegans* receptor cRNA (2.5 ng; **A$_2$**), CFTR cRNA (2.5 ng) and β_2-adrenergic receptor cRNA (2.5 ng; **B$_1$**), and CFTR cRNA (2.5 ng) and the *C. elegans* receptor cRNA (2.5 ng; **B$_2$**). Recording and drug application were done as described in the legend to Fig. 5. The *C. elegans* receptor evoked neither the transient endogenous Cl^- current nor the CFTR current on application of ACh (1 μM).

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