Biochemical and Biophysical Research Communications **288,** 1238–1243 (2001)
doi:10.1006/bbrc.2001.5909, available online at http://www.idealibrary.com on IDEAL®

Three Functional Isoforms of GAR-2, a *Caenorhabditis elegans* G-Protein-Linked Acetylcholine Receptor, Are Produced by Alternative Splicing

SuJeong Suh,*[,1] Yang-Seo Park,†[,1] Yong-Seok Lee,* Tae-Ju Cho,†
Bong-Kiun Kaang,*[,2] and Nam Jeong Cho†[,2]

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

Received September 17, 2001

We have previously isolated a cDNA clone from *Caenorhabditis elegans* that encodes a novel form of G-protein-linked acetylcholine receptor, termed GAR-2. GAR-2 is similar to but pharmacologically distinct from muscarinic acetylcholine receptors. Here we report the identification of two *gar-2* cDNA clones that are different from the previous one. These newly identified cDNAs encode polypeptides of 664 and 627 amino acids, whereas the previous one encodes a polypeptide of 614 amino acids. The three GAR-2 isoforms, which differ only in the third intracellular loop, arise from alternative splicing. Electrophysiological analyses using the *Xenopus* oocyte system showed that all three GAR-2 isoforms couple to the activation of G-protein-gated inwardly rectifying K$^+$ (GIRK1) channel with similar drug specificity. Our results indicate that alternative splicing plays an important role in promoting molecular diversity of G-protein-linked acetylcholine receptors in *C. elegans*. © 2001 Academic Press

Key Words: gar-2; **G-protein-linked acetylcholine receptor; alternative splicing; *Caenorhabditis elegans.***

Acetylcholine (ACh) is known to be a major neurotransmitter in both vertebrates and invertebrates. Two types of receptors, nicotinic ACh receptor and muscarinic ACh receptor (mAChR), have been shown to play pivotal roles in cholinergic neurotransmission. Whereas nicotinic ACh receptors mediate fast neurotransmission by acting as ligand-gated ion channels, mAChRs mediate slow neurotransmission by interacting with G proteins. In mammals, five mAChR subtypes (m1–m5), encoded by separate genes, have been identified (1, 2). These subtypes share a high degree of amino acid sequence similarity, except for the third intracellular loop (i3 loop). It seems likely that this highly variable i3 loop is critical for subtype-specific signal transduction pathway.

In the nematode *Caenorhabditis elegans,* three genes (*gar-1, gar-2,* and *gar-3*) have been shown to encode G-protein-linked ACh receptors (3–5). GAR-3 appears to be a member of the mAChR family, which couples to the activation of phospholipases C and D (4, 6). On the other hand, GAR-1 and GAR-2 are considered to be different from the conventional mAChRs based on their amino acid sequence divergency and distinct drug specificity (3, 5). For example, GAR-1 and GAR-2 do not respond effectively to potent muscarinic drugs such as oxotremorine, atropine, and scopolamine.

Alternative splicing has been reported to generate diverse isoforms of G-protein-linked neurotransmitter receptors, including dopamine D2 receptor (7, 8), serotonin receptors (9), μ-opioid receptor (10–12), $GABA_B$ receptor (13, 14), and histamine H3 receptor (15). No splice variants of mammalian G-protein-linked ACh receptors (that is, mAChRs) exist, as the corresponding genes are intronless. However, all of the three *C. elegans* G-protein-linked ACh receptor genes have introns in the coding region (3–5), suggesting the possibility of alternative splicing. In fact, we have recently shown that GAR-1 exists in three alternatively spliced forms (16). In the current work we show that alternative splicing of the *gar-2* gene generates three functional GAR-2 isoforms.

Abbreviations used: ACh, acetylcholine; GIRK, G-protein-gated inwardly rectifying K$^+$ channel; i3 loop, third intracellular loop; mAChR, muscarinic acetylcholine receptor; RT-PCR, reverse transcriptase-polymerase chain reaction.

[1] These authors contributed equally to this work.

[2] To whom correspondence and reprint requests may be addressed. (B.-K.K.) Fax: 82-2-874-1206. E-mail: kaang@snu.ac.kr. (N.J.C.) Fax: 82-43-267-2306. E-mail: namjcho@chungbuk.ac.kr.

MATERIALS AND METHODS

Drugs. ACh chloride, atropine sulfate, (−)-scopolamine hydrochloride, oxotremorine free base, and arecoline hydrobromide were purchased from Sigma.

0006-291X/01 $35.00

Copyright © 2001 by Academic Press
All rights of reproduction in any form reserved.

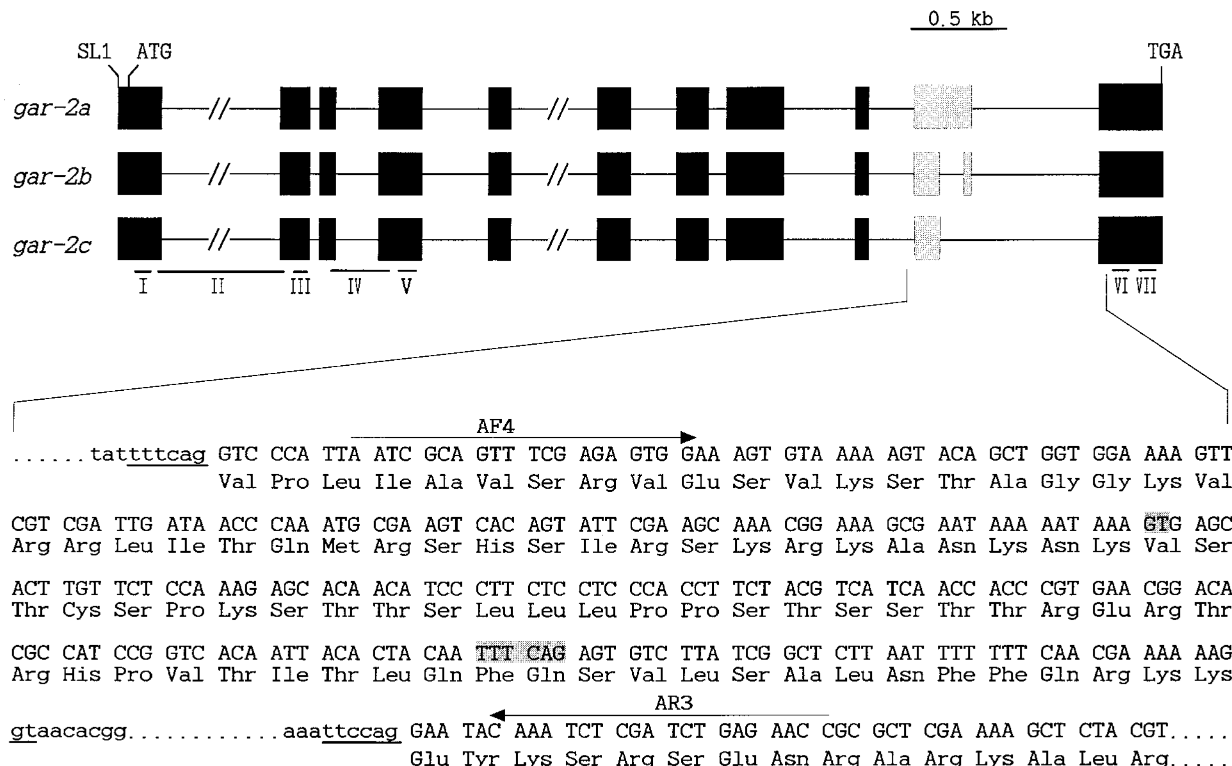

FIG. 1. (Top) Exon/intron arrangement of the three *gar-2* splice variants. Boxes represent exons and lines represent introns. Exons involved in alternative splicing are indicated by gray boxes. The positions of putative transmembrane domains (I–VII) are indicated. The first and fifth introns are unusually long (~4.6 and ~2.3 kb, respectively). SL1, spliced leader 1; ATG, translation initiation codon; TGA, translation termination codon. (Bottom) The sequences of the alternatively spliced exons and their flanking regions. The uppercase and lowercase letters denote exon and intron sequences of *gar-2a,* respectively. The 5′ splice donor and 3′ splice acceptor sequences are underlined, while the alternatively used 5′ splice donor and 3′ splice acceptor sequences within the 10th exon of *gar-2a* are shaded. The primers (AF4 and AR3) used for the RT-PCR analysis of the *gar-2* splice variants (see Fig. 2) are indicated by arrows. The nucleotide sequences of *gar-2a, gar-2b,* and *gar-2c* have been submitted to GenBank under Accession Nos. AY053364, AY053365, and AF272738, respectively.

Isolation of gar-2 cDNAs. *gar-2* cDNAs were isolated by reverse transcriptase-polymerase chain reaction (RT-PCR) as described by Lee *et al.* (5). Nucleotide sequences of the cDNAs were determined using the Cy5 AutoRead sequencing kit and ALFexpress DNA sequencer (Pharmacia).

RT-PCR analysis of gar-2 expression during development. Stage-specific RNAs were prepared from synchronized populations of animals as described by Hwang *et al.* (4). RT-PCR was performed as described previously (16), with primers flanking the alternatively spliced region: AF4 (5′-AATCGCAGTTTCGAGAGTGG-3′) and AR3 (5′-GGTTCTCAGATCGAGATTTG-3′) were used, respectively, as the forward and the reverse primer.

Electrophysiological analysis. Preparation of cRNAs, injection into oocytes of *Xenopus laevis,* and electrophysiological recordings were done as described in our previous reports (3, 5).

RESULTS

We have previously isolated a cDNA clone from *C. elegans* that encodes a novel form of G-protein-linked ACh receptor, termed GAR-2 (5). To examine whether other *gar-2* cDNAs exist in *C. elegans,* we further analyzed the RT-PCR products of *gar-2* by restriction enzyme digestion and nucleotide sequence determination. We identified two new cDNA clones that are different from the previous one. The newly identified cDNAs encode polypeptides of 664 and 627 amino acids, while the previous one encodes a polypeptide of 614 amino acids. We named these cDNAs, from the longest, *gar-2a, gar-2b,* and *gar-2c.* The three GAR-2 isoforms are identical, except for the i3 loop region. When compared to GAR-2c, which has been described in our previous report (5), GAR-2a has an insertion of 50 amino acids and GAR-2b has an insertion of 13 amino acids in the i3 loop region.

Comparison of the cDNA sequences with the corresponding genomic sequence reveals that the three cDNAs are splice variants of the *gar-2* gene. The *gar-2a* and *gar-2c* cDNAs consist of 11 exons, while the *gar-2b* cDNA consists of 12 exons (Fig. 1, top). Exon 10 of *gar-2a* is 276 bp long and possesses internal 5′ splice donor and 3′ splice acceptor sites (Fig. 1, bottom). In case of *gar-2b,* both internal splice sites are utilized to remove an in-frame intron (111 bp), thereby producing

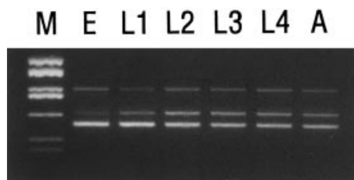

FIG. 2. RT-PCR analysis of the *gar-2* splice variants. RT-PCR was performed using the stage-specific RNA as a template. The positions and sequences of the primers (AF4 and AR3) are indicated in Fig. 1. The PCR products were analyzed on a 2.5% NuSieve agarose gel. The expected sizes of the PCR products for *gar-2a, gar-2b,* and *gar-2c* are 293, 182, and 143 bp, respectively. M, size markers (587, 458/434, 298, 267/257, 174, 102, and 80 bp); E, embryo; L1–L4, first to fourth larval stages; A, young adult.

two small exons (126 and 39 bp). In case of *gar-2c,* only the internal 5′ splice donor site is utilized, resulting in the exclusion of the downstream 150 bp segment from the exon 10. Thus, exon 10 of *gar-2c* is 126 bp long.

To examine the relative expression pattern of the *gar-2* splice variants during development, we prepared stage-specific RNAs and performed RT-PCR experiments with primers flanking the alternatively spliced region. Three RT-PCR products of expected size (293, 182, and 143 bp) were observed at all developmental stages examined (Fig. 2), implying that the three *gar-2* mRNAs are expressed throughout development. The *gar-2c* isoform was predominant at all developmental stages and the relative ratio of the three *gar-2* isoforms appeared to vary at different stages.

We have previously shown that GAR-2c couples to the activation of GIRK1 channel in *Xenopus* oocytes, probably via G_i protein (5). To explore the possibility that the structural variations caused by alternative splicing might affect G protein coupling specificity and/or efficacy of the receptor, we expressed each isoform in *Xenopus* oocytes and compared their electrophysiological properties. When coexpressed with GIRK1 channel, which is considered to be activated by G_i or G_o protein (17), all three GAR-2 isoforms evoked the GIRK current by ACh treatment with similar efficacy (Fig. 3). These GIRK currents appeared to be mediated by G_i protein but not by G_o protein in *Xenopus* oocytes, because none of the GAR-2 isoforms produced the transient Ca^{2+}-activated Cl^- current (data not shown), which is known to be stimulated by G_o or G_q protein (18, 19). Overall, these results suggest that the three GAR-2 isoforms have similar G protein coupling characteristics.

Next we compared the pharmacological properties of the three GAR-2 isoforms with those of pig m2 mAChR, which is also known to couple to the activation of the GIRK1 channel (20). Oxotremorine, a muscarinic agonist, failed to elicit the GIRK current in any of the oocytes expressing the GAR-2 isoforms (Fig. 4 and Table 1). In control experiments, oxotremorine strongly induced the current in oocytes expressing the m2 mAChR. Similarly, another agonist, arecoline, was not effective in activating the GIRK current in oocytes expressing the GAR-2 isoforms, whereas the drug produced the current in oocytes expressing the m2 mAChR.

We also compared the effects of antagonists on the GAR-2 isoforms. The presence of either atropine or scopolamine did not significantly inhibit the ACh-induced current mediated by the GAR-2 isoforms (Fig. 5 and Table 1). In control experiments, atropine or scopolamine almost completely blocked the current mediated by the m2 mAChR. Overall, the three alternatively spliced variants displayed similar pharmacological profiles.

DISCUSSION

Alternative splicing produces multiple receptor isoforms from single G-protein-linked neurotransmitter receptor genes (7–15), thereby increasing receptor diversity. Evidence reported in this study shows that alternative splicing of the *gar-2* gene produces three functional isoforms, all of which couple to the activation of GIRK1 channel. Together with our previous report showing the existence of three functional splice variants of GAR-1 (16), this study reveals that diverse G-protein-linked ACh receptor isoforms may act to mediate the slow form of cholinergic neurotransmission in *C. elegans.*

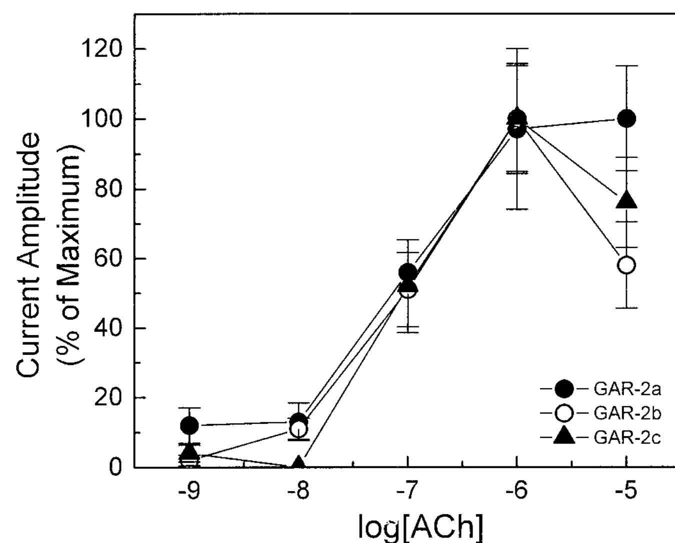

FIG. 3. Dose–response curves for induction of GIRK current by three GAR-2 isoforms. *Xenopus* oocytes were injected with cRNA (2.5 ng each) for GAR-2a, GAR-2b, or GAR-2c together with GIRK1 cRNA (2.5 ng). Recordings were done as described by Lee *et al.* (5). The amplitude of the ACh-induced current was normalized to its high K^+-induced current, and the average values with error bars (±SE) were plotted as percentage of the maximum current value obtained in each isoform. Each data point in the curves consists of recordings from 12 to 18 oocytes. The EC_{50} values were $\sim 10^{-7}$ M in all three isoforms.

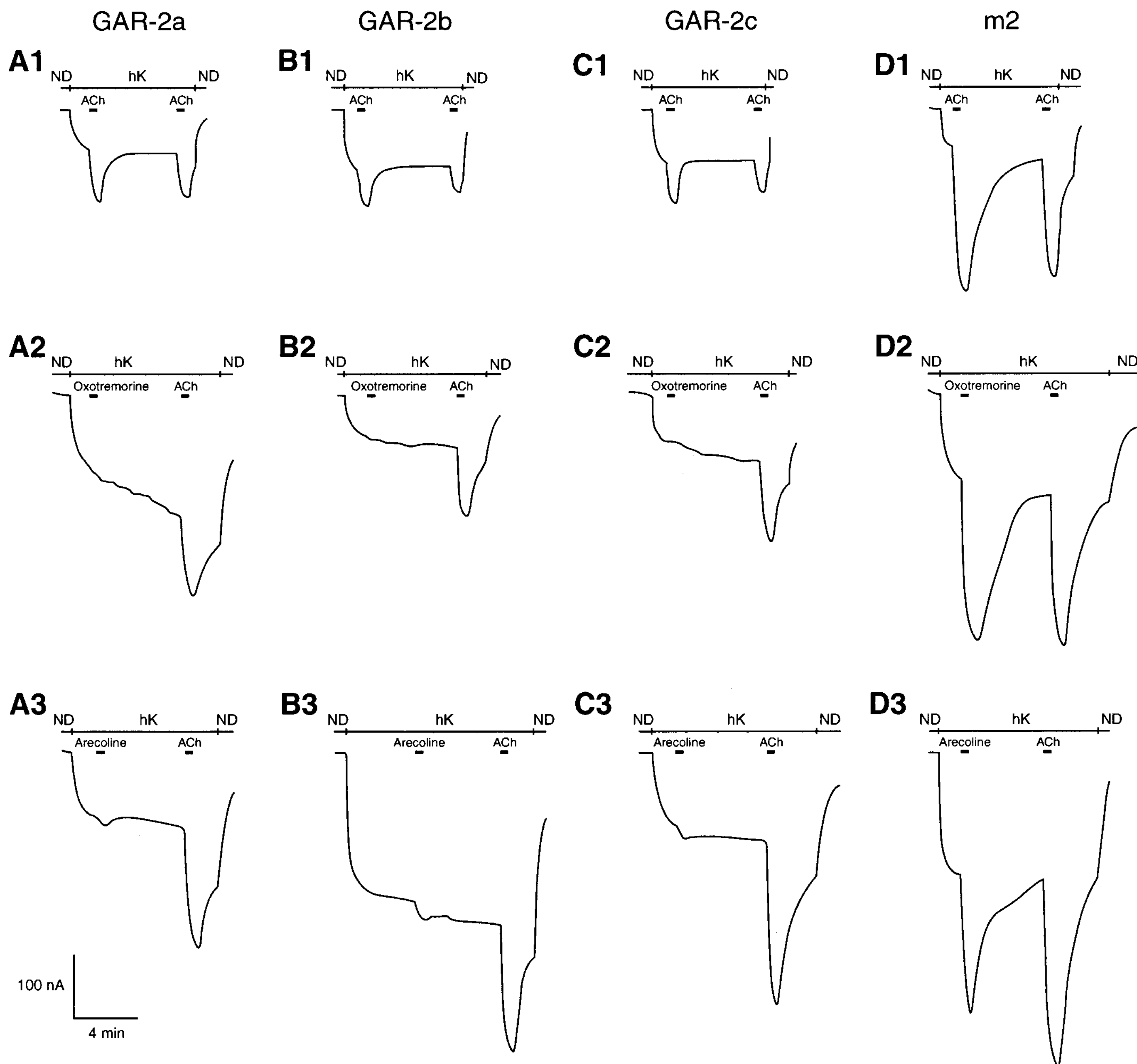

FIG. 4. Agonist specificity of the three GAR-2 isoforms. Oocytes were injected with cRNA (2.5 ng each) for GAR-2a (A1, A2, A3), GAR-2b (B1, B2, B3), GAR-2c (C1, C2, C3), or pig m2 mAChR (D1, D2, D3) together with GIRK1 cRNA (2.5 ng). All the current traces were recorded at a holding potential of −70 mV. The drugs (1 μM each) were present in the bathing solution as indicated by bars. ND, ND96 solution; hK, high K^+ solution.

For some G-protein-linked neurotransmitter receptors, such as dopamine D2 receptor (21–23) and histamine H3 receptor (15), alternative splicing in the i3 loop region has been reported to affect G protein coupling specificity and/or efficacy. We tested whether the three GAR-2 isoforms have different coupling characteristics, since alternative splicing of GAR-2 also occurs in the i3 loop region (Fig. 1). However, we could not detect any substantial difference among the isoforms: electrophysiological analyses using the *Xenopus* oocyte expression system indicated that all three isoforms may couple to the same class of G proteins, most likely of the G_i class, with similar efficacy. We cannot exclude, however, the possibility that the GAR-2 isoforms might interact with different subtypes of G proteins and, therefore, mediate distinct signaling events.

It is possible that the structural variations present in the GAR-2 isoforms may affect receptor sequestra-

TABLE 1

Effects of Agonists and Antagonists on GAR-2 Isoforms and Pig m2 mAChR

Drug	% of ACh ± SE (*n*)[a]			
	GAR-2a	GAR-2b	GAR-2c	m2
ACh[b]	94 ± 12 (10)	96 ± 13 (11)	91 ± 6 (8)	90 ± 2 (8)
Oxotremorine	0 ± 0 (9)	0 ± 0 (8)	0 ± 0 (5)	66 ± 4 (6)
Arecoline	4 ± 2 (10)	5 ± 3 (10)	13 ± 5 (10)	66 ± 9 (10)
Atropine	85 ± 4 (13)	87 ± 6 (13)	81 ± 3 (7)	2 ± 8 (7)
Scopolamine	80 ± 8 (10)	85 ± 10 (11)	84 ± 5 (8)	0 ± 0 (7)

[a] Data are represented as means ± SE values of percentage of ACh response in the same oocytes. (*n*) indicates the number of experiments. All drugs were treated at 1 μM.

[b] Oocytes were treated with ACh twice by a 5-min interval.

tion. Two splice variants of dopamine D2 receptor (D2L and D2S), which are identical except for an insertion of 29 amino acids in the i3 loop, have been shown to be differentially sequestrated (24). Furthermore, deletion mutagenesis experiments with mAChRs have shown that i3 loop is critically involved in the receptor sequestration (25, 26).

Recent studies with dopamine D2 receptor have indicated that the two splice variants of the receptor may be differentially localized. Khan *et al.* (27) reported that D2S is mainly localized in dopaminergic cell bodies and axons, while D2L is abundant in non-dopaminergic neurons. Usiello *et al.* (28) observed that D2L-deficient mice lack postsynaptic dopamine responses but retain presynaptic dopamine functions. These studies suggest that D2S may act as a presynaptic autoreceptor, whereas D2L may be a postsynaptic receptor. Presumably, the difference in the i3 loop present in the two splice variants of dopamine D2 receptor provides a structural motif for the different targeting of the receptors. Thus it will be of interest to explore the possibility that the GAR-2 splice variants are differentially localized and perform distinct synaptic functions.

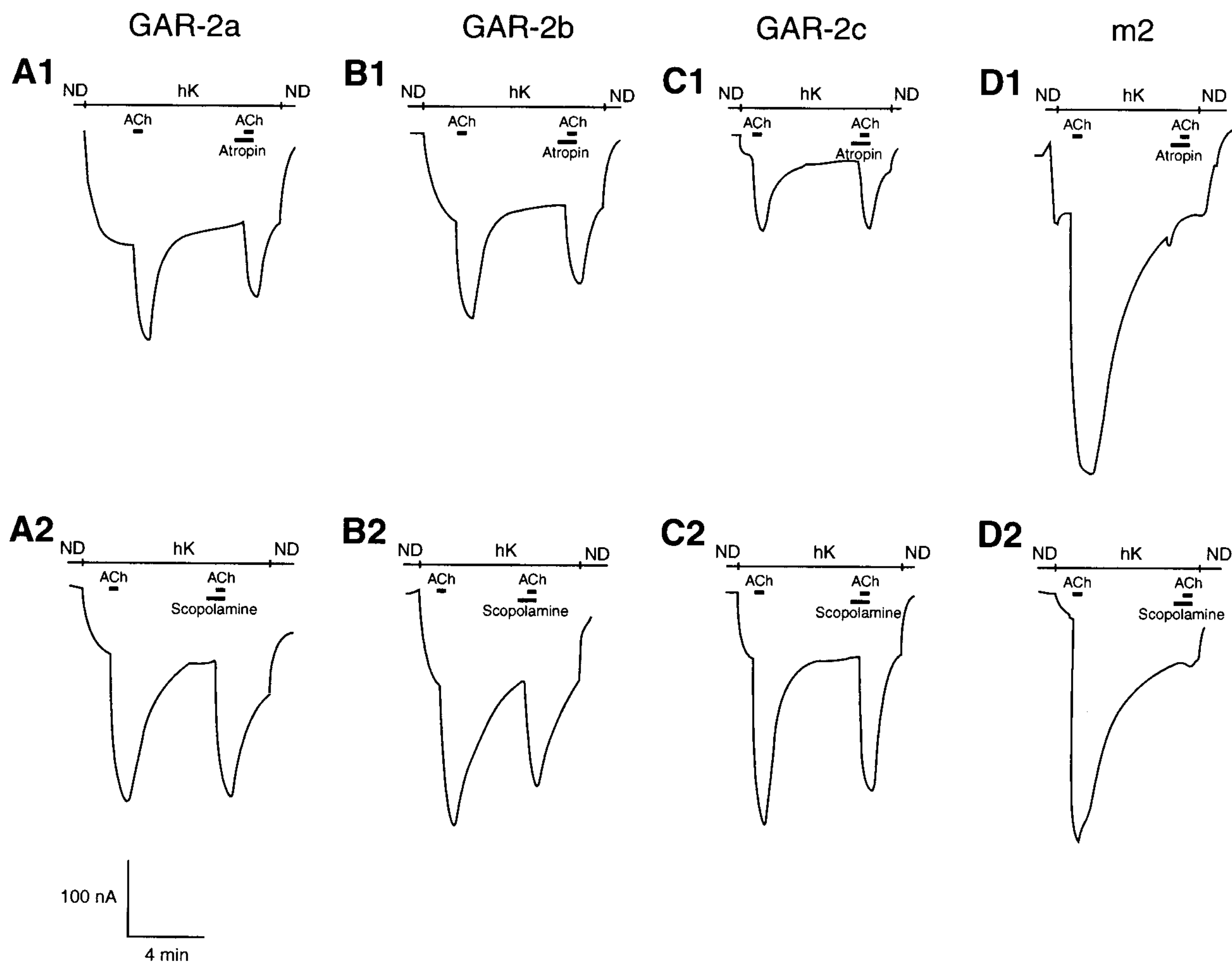

FIG. 5. Antagonist specificity of the three GAR-2 isoforms. Oocytes were injected with cRNA (2.5 ng each) for GAR-2a (A1, A2), GAR-2b (B1, B2), GAR-2c (C1, C2), or pig m2 mAChR (D1, D2) together with GIRK1 cRNA (2.5 ng). Recordings were performed as in Fig. 4. ACh and other antagonists were treated at 1 μM.

In conclusion, we have shown that three functional GAR-2 isoforms are produced by alternative splicing. The three alternatively spliced variants, which differ in the i3 loop, exhibited similar pharmacological profiles and signaling activities. While the physiological significance of alternative splicing of GAR-2 remains to be established, our study demonstrates that alternative splicing serves an important role in promoting molecular diversity of G-protein-linked ACh receptors in *C. elegans.*

ACKNOWLEDGMENTS

We thank Jae Sun Lee for his help with DNA sequencing. This work was supported by the Korean Ministry of Science and Technology under the Brain Science Research Program (B.-K.K.) and Grant 2000-2-21300-005-1 from the Basic Research Program of the Korea Science & Engineering Foundation (N.J.C.). S.J.S. and Y.-S.L. were supported by BK21 Research Fellowships from the Korean Ministry of Education.

REFERENCES

1. Bonner, T. I., Buckley, N. J., Young, A. C., and Brann, M. R. (1987) *Science* **237,** 527–532.
2. Bonner, T. I., Young, A. C., Brann, M. R., and Buckley, N. J. (1988) *Neuron* **1,** 403–410.
3. Lee, Y.-S., Park, Y.-S., Chang, D.-J., Hwang, J. M., Min, C. K., Kaang, B.-K., and Cho, N. J. (1999) *J. Neurochem.* **72,** 58–65.
4. Hwang, J. M., Chang, D.-J., Kim, U. S., Lee, Y.-S., Park, Y.-S., Kaang, B.-K., and Cho, N. J. (1999) *Receptors Channels* **6,** 415–424.
5. Lee, Y.-S., Park, Y.-S., Nam, S., Suh, S. J., Lee, J., Kaang, B.-K., and Cho, N. J. (2000) *J. Neurochem.* **75,** 1800–1809.
6. Min, D. S., Cho, N. J., Yoon, S. H., Lee, Y. H., Hahn, S.-J., Lee, K.-H., Kim, M.-S., and Jo, Y.-H. (2000) *J. Neurochem.* **75,** 274–281.
7. Giros, B., Sokoloff, P., Martres, M.-P., Riou, J.-F., Emorine, L. J., and Schwartz, J.-C. (1989) *Nature* **342,** 923–926.
8. Monsma, F. J., Jr., McVittie, L. D., Gerfen, C. R., Mahan, L. C., and Sibley, D. R. (1989) *Nature* **342,** 926–929.
9. Hamblin, M. W., Guthrie, C. R., Kohen, R., and Heidmann, D. E. A. (1998) *Ann. N.Y. Acad. Sci.* **861,** 31–37.
10. Bare, L. A., Mansson, E., and Yang, D. (1994) *FEBS Lett.* **354,** 213–216.
11. Zimprich, A., Simon, T., and Hollt, V. (1995) *FEBS Lett.* **359,** 142–146.
12. Pan, Y.-X., Xu, J., Bolan, E., Abbadie, C., Chang, A., Zuckerman, A., Rossi, G., and Pasternak, G. W. (1999) *Mol. Pharmacol.* **56,** 396–403.
13. Kaupmann, K., Huggel, K., Heid, J., Flor, P. J., Bischoff, S., Mickel, S. J., McMaster, G., Angst, C., Bittiger, H., Froestl, W., and Bettler, B. (1997) *Nature* **386,** 239–246.
14. Isomoto, S., Kaibara, M., Sakurai-Yamashita, Y., Nagayama, Y., Uezono, Y., Yano, K., and Taniyama, K. (1998) *Biochem. Biophys. Res. Commun.* **253,** 10–15.
15. Drutel, G., Peitsaro, N., Karlstedt, K., Wieland, K., Smit, M. J., Timmerman, H., Panula, P., and Leurs, R. (2001) *Mol. Pharmacol.* **59,** 1–8.
16. Park, Y.-S., Lee, Y.-S., Cho, N. J., and Kaang, B.-K. (2000) *Biochem. Biophys. Res. Commun.* **268,** 354–358.
17. Dascal, N., Schreibmayer, W., Lim, N. F., Wang, W., Chavkin, C., DiMagno, L., Labarca, C., Kieffer, B. L., Gaveriaux-Ruff, C., Trollinger, D., Lester, H. A., and Davidson, N. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 10235–10239.
18. Lubbert, H., Hoffman, B. J., Snutch, T. P., van Dyke, T., Levine, A. J., Hartig, P. R., Lester, H. A., and Davidson, N. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 4332–4336.
19. Quick, M. W., Simon, M. I., Davidson, N., Lester, H. A., and Aragay, A. M. (1994) *J. Biol. Chem.* **269,** 30164–30172.
20. Kubo, Y., Reuveny, E., Slesinger, P. A., Jan, Y. N., and Jan, L. Y. (1993) *Nature* **364,** 802–806.
21. Montmayeur, J.-P., and Borrelli, E. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 3135–3139.
22. Montmayeur, J.-P., Guiramand, J., and Borrelli, E. (1993) *Mol. Endocrinol.* **7,** 161–170.
23. Liu, L.-X., Monsma, F. J., Jr., Sibley, D. R., and Chiodo, L. A. (1996) *Synapse* **24,** 156–164.
24. Itokawa, M., Toru, M., Ito, K., Tsuga, H., Kameyama, K., Haga, T., Arinami, T., and Hamaguchi, H. (1996) *Mol. Pharmacol.* **49,** 560–566.
25. Lameh, J., Philip, M., Sharma, Y. K., Moro, O., Ramachandran, J., and Sadee, W. (1992) *J. Biol. Chem.* **267,** 13406–13412.
26. Tsuga, H., Kameyama, K., Haga, T., Honma, T., Lameh, J., and Sadee, W. (1998) *J. Biol. Chem.* **273,** 5323–5330.
27. Khan, Z. U., Mrzljak, L., Gutierrez, A., and Calle, A. D. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 7731–7736.
28. Usiello, A., Baik, J.-H., Rouge-Pont, F., Picetti, R., Dierich, A., LeMeur, M., Piazza, P. V., and Borrelli, E. (2000) *Nature* **408,** 199–203.