The GAR-3 Muscarinic Receptor Cooperates With Calcium Signals to Regulate Muscle Contraction in the Caenorhabditis elegans Pharynx

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ABSTRACT

Muscarinic acetylcholine receptors regulate the activity of neurons and muscle cells through G-protein-coupled cascades. Here, we identify a pathway through which the GAR-3 muscarinic receptor regulates both membrane potential and excitation-contraction coupling in the Caenorhabditis elegans pharyngeal muscle. GAR-3 signaling is enhanced in worms overexpressing gar-3 or lacking GPB-2, a G-protein β-subunit involved in RGS-mediated inhibition of G\textsubscript{i/o} and G\textsubscript{q}α-linked pathways. High levels of signaling through GAR-3 inhibit pharyngeal muscle relaxation and impair feeding—but do not block muscle repolarization—when worms are exposed to arecoline, a muscarinic agonist. Loss of gar-3 function results in shortened action potentials and brief muscle contractions in the pharyngeal terminal bulb. High levels of calcium entry through voltage-gated channels also impair terminal bulb relaxation and sensitize worms to the toxic effects of arecoline. Mutation of gar-3 reverses this sensitivity, suggesting that GAR-3 regulates calcium influx or calcium-dependent processes. Because the effects of GAR-3 signaling on membrane depolarization and muscle contraction can be separated, we conclude that GAR-3 regulates multiple calcium-dependent processes in the C. elegans pharyngeal muscle.

Organisms throughout the animal kingdom employ muscarinic acetylcholine receptors to regulate the excitability of neurons and muscle cells. In mammals, muscarinic receptors acting through G proteins of the G\textsubscript{q} family enhance the slow, tonic contraction of smooth muscle. G\textsubscript{q} proteins activate phospholipase C\β, stimulating the production of inositol triphosphate (IP\textsubscript{3}) and diacyl glycerol (DAG) and triggering the release of calcium from intracellular stores. In the heart, receptors coupled to G\textsubscript{q ij} family proteins reduce heart rate and contractility in response to signals from the parasympathetic nervous system. Muscarinic receptors can also increase neuronal excitability and are thought to inhibit neurotransmitter release in the central nervous system (for review see Caulfield and Birdsall 1998). However, the physiological relevance of muscarinic signaling is sometimes difficult to pinpoint. For example, knockout mice lacking any one of the five mammalian muscarinic receptor subtypes are viable and generally healthy (Gomez\textsubscript{a} et al. 1999, 2001; Matsui et al. 2000; Hamilton et al. 2001; Yamada et al. 2001). In addition, a wide variety of signaling events has been associated with muscarinic receptor activation, but the importance of these responses in regulating behavior is difficult to demonstrate. Here, we characterize a muscarinic receptor in the pharynx of Caenorhabditis elegans and show that increased or decreased signaling through this receptor has profound effects on feeding behavior. By analyzing genetic interactions, we also begin to identify effectors in this muscarinic pathway.

C. elegans is a soil-dwelling nematode that takes in food through a neuromuscular organ, the pharynx. When the radially arranged pharyngeal muscles contract, the lumen opens and bacteria, suspended in water, are sucked into the pharyngeal corpus (Seymour et al. 1983; Avery and Horvitz 1989). Relaxation of the pharyngeal muscle closes the lumen, expelling the water and trapping the bacteria. Each muscle contraction (or “pump”) also shifts the position of grinder plates in the terminal bulb of the pharynx. Bacteria in the terminal bulb are broken open by the grinder and then passed posteriorly into the intestine during the subsequent pump (Doncaster 1982). A second, less-frequent muscle contraction, isthmus peristalsis, transports bacteria from the corpus to the terminal bulb (Avery and Horvitz 1989).

Acetylcholine is essential for the survival of C. elegans and is crucial for pharyngeal muscle activity (Rand and Russell 1984; Avery and Horvitz 1990). Nicotinic acetylcholine receptors in the pharynx are necessary for rapid pumping (Raizen et al. 1995; McKay et al. 2004), but the function of muscarinic receptors is not well understood. Arecoline, a muscarinic agonist, has profound effects on some C. elegans mutants. Worms carrying mutations in the gpb-2 gene are hypersensitive to arecoline: they cannot grow in the presence of arecoline concentrations that have little effect on wild-type worms.

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(Avery et al. 1993; Brundage et al. 1996). ghb-2 encodes a G-protein β-subunit homologous to mammalian Gβs. GPB-2 has been shown to interact with the RGS proteins EAT-16 and EGL-10, which increase the rate of GTP hydrolysis by Gα and Gα, respectively. In the absence of GPB-2, the RGS proteins are unable to terminate signaling through their target Go-subunits, leading to abnormalities in locomotion, defecation, egg laying, and feeding (Chase et al. 2001; Robatzek et al. 2001; van der Linden et al. 2001). Arecoline is likely to act through Gα, because mutations in egl-30, which encodes the C. elegans Gα, suppress the hypersensitivity of ghb-2 mutants (Brundage et al. 1996). However, neither the muscarinic receptor assumed to be upstream of Gα nor the downstream effectors in the pathway have been identified.

We have attempted to define the elements of the arecoline-stimulated signaling cascade in the pharynx. We find that the muscarinic receptor encoded by gar-3 is an important arecoline receptor and that high levels of signaling through GAR-3 prevent pharyngeal muscle relaxation without blocking repolarization. This effect is related to the level of calcium entry into the pharyngeal muscle through an L-type calcium channel. We have also found that loss of gar-3 function enhances pharyngeal muscle repolarization and relaxation. Because the effects of GAR-3 signaling cannot be explained as a simple excitation of pharyngeal muscle membrane, we propose that GAR-3 activates a previously unidentified signaling cascade that regulates both membrane potential and gar-3 that GAR-3 activates a previously unidentified signaling body movement and improve picture quality. The effect of gar-3 muscle through an L-type calcium channel. We have also adult worms that had been kept on DA837/5 m arecoline. We find that the muscarinic receptor encoded by gar-3(vu78) V gar-3(lg1201) V gpb-2(sa603) I gpb-2(ad541) I gar-3 (vu78) V JD298 ghb-2(ad541) I gar-3(lg1201) V JD292 ghb-2 (sa603) I gar-3(vu78) V JD217 gsb-2(vu78) V JD250 gsb-3(lg1201) V DA465 ent-2(ad465) II JD285 ent-2(ad465) II gar-3(lg1201) V JD105 avr-15(ad1051) V JD308 avr-15(ad1051) gar-3(lg1201) V JD258 ghb-2(ad541) I egl-19(n582) IV MT1212 egl-19(n582) IV JD303 egl-19(n582) gar-3(lg1201) V MT6129 egl-19(n2368sd) IV JD301 egl-19(n2368sd) IV gar-3(lg1201) V JD783 itr-1(sa73) IV JD265 ghb-2(ad541) I itr-1(sa73) IV CB540 unc-68(e540) V and JD291 unc-68(e540) gar-3(lg1201) V.

**Chemicals:** Chemicals used were the creatinine sulfate complex of serotonin (5-hydroxytryptamine; Sigma, St. Louis) and arecoline hydrobromide (Sigma).

**Assays of arecoline sensitivity:** Ability to grow in the presence of arecoline was assessed on plates seeded with *E. coli* strain D1837 (Davis et al. 1995); 100 mm arecoline in M9 buffer (Lewis and Flemming 1995) was applied to seeded plates at a 1/20 volume ratio (e.g., 0.6 ml for a 12-ml plate) to achieve a final concentration of 5 mm. Plates were not used until at least 24 hr after arecoline application to allow for diffusion of the drug throughout the agar.

Eggs were harvested as described (Lewis and Flemming 1995). Approximately 100 eggs were placed onto each of four DA837/no-drug plates and four DA837/5 mm arecoline plates. Adult worms were counted and removed daily until few or no viable larval worms remained. Assays were terminated at 3 days (0 mm arecoline) or 4 days (5 mm arecoline), except in tests of the growth rate of egl-19(n2368sd) mutants. Because egl-19(n2368) mutant worms grow slowly on DA837 plates, worms in the “no-drug” condition were counted through day 5 and worms exposed to arecoline were counted through day 7. The mean number of adult worms surviving drug exposure was divided by the mean number of adults on plates without arecoline (~100 per plate), generating a survival ratio. Error bars represent the SE of this ratio. For assays of transgenic worms, only adults expressing green fluorescent protein (GFP) were counted (40–60 per no-drug plate). All results shown are representative of at least two independent experiments.

Pharyngeal stuffing was assessed in L3 and L4 larvae kept for 24 hr on DA837/5 mm arecoline plates. Worms were transferred to 200 μl of Dent’s saline (Avery et al. 1995) on a glass coverslip and cut just behind the terminal bulb of the pharynx. Removing the majority of the body of the worm effectively immobilizes the pharynx without adding paralytic agents. Dissected pharynxes were scored immediately on an inverted compound microscope. Video images were collected from adult worms that had been kept on DA837/5 mm arecoline plates for 21 hr and then moved to unseeded 5 mm arecoline plates for 3 hr to allow the pharynx to empty. Images in Figure 1B were collected with a Hitachi KP160 CCD camera and Adobe Premiere 6.5 software. We used an *unc-29(e1072)* mutant overexpressing gar-3 in the image in Figure 3B to reduce body movement and improve picture quality. The effect of gar-3 overexpression was the same in wild-type and *unc-29* mutant backgrounds. These images were collected with a Panasonic WV-BP550 video camera. Still images were extracted from videotapes using Scion Image Beta 3 release software (Scion, Frederick, MD).

**Extracellular recordings (electropharyngeograms):** Electropharyngeograms (EPGs) were recorded as previously described (Avery et al. 1995) except that the bath solution was M9 with 10 mm serotonin. Recordings were made with a Patch-PC501A amplifier (Warner Instruments, Hamden, CT), a Digidata 1322A acquisition system (Axon Instruments, Union City, CA), and Axoscope 8.1 software (Axon Instruments). All experiments were performed in M9 buffer with Dent’s saline (Avery et al. 1995) and their F2 progeny were transferred to plates buffer (H9251). The wild-type strain was *C. elegans* varietv Bristol, strain N2. Mutant strains used were DA541 ghb-2 (ad541) I, JT603 ghb-2(sa603) I, JD222 ghb-2(ad541) I, gar-3 (vu78) V, JD298 ghb-2(ad541) I, gar-3(lg1201) V, JD292 ghb-2 (sa603) I, gar-3(vu78) V, JD217 gsb-2(vu78) V, JD250 gsb-3(lg1201) V, DA465 ent-2(ad465) II, JD285 ent-2(ad465) II, gar-3(lg1201) V, JD105 avr-15(ad1051) V, JD308 avr-15(ad1051) gar-3(lg1201) V, JD258 ghb-2(ad541) I, egl-19(n582) IV, MT1212 egl-19(n582) IV, JD303 egl-19(n582) gar-3(lg1201) V, MT6129 egl-19(n2368sd) IV, JD301 egl-19(n2368sd) IV, gar-3(lg1201) V, JD783 itr-1(sa73) IV, JD265 ghb-2(ad541) I, itr-1(sa73) IV, CB540 unc-68(e540) V, and JD291 unc-68(e540) gar-3(lg1201) V.

**General methods and strains:** Worms were cultured and handled as described by Sulston and Hodgkin (1988) with the following modifications: worms were routinely grown on NGMSR plates (Avery 1993) without nystatin. All worms were maintained at 20°C on Escherichia coli strain H101 (Boyer and Roulland-Dussoix 1969). The wild-type strain was *C. elegans* variety Bristol, strain N2. Mutant strains were used: DA541 ghb-2 (ad541) I, JT603 ghb-2(sa603) I, JD222 ghb-2(ad541) I, gar-3 (vu78) V, JD298 ghb-2(ad541) I, gar-3(lg1201) V, JD292 ghb-2 (sa603) I, gar-3(vu78) V, JD217 gsb-2(vu78) V, JD250 gsb-3(lg1201) V, DA465 ent-2(ad465) II, JD285 ent-2(ad465) II, gar-3(lg1201) V, JD105 avr-15(ad1051) V, JD308 avr-15(ad1051) gar-3(lg1201) V, JD258 ghb-2(ad541) I, egl-19(n582) IV, MT1212 egl-19(n582) IV, JD303 egl-19(n582) gar-3(lg1201) V, MT6129 egl-19(n2368sd) IV, JD301 egl-19(n2368sd) IV, gar-3(lg1201) V, JD783 itr-1(sa73) IV, JD265 ghb-2(ad541) I, itr-1(sa73) IV, CB540 unc-68(e540) V, and JD291 unc-68(e540) gar-3(lg1201) V.
survival were mapped using TCI transposon mapping (Williams 1995). Because both vu78 and the gar-3 open reading frame mapped to the same region of chromosome V, we sequenced the gar-3 coding region from worms carrying the vu78 mutation and identified a G-to-A transition in a coding exon that converts alanine 108 to a threonine. The lgi1201 allele of gar-3 was isolated from a deletion library by EleGene. The deletion breakpoints are 5‘-AAAAATGCAAAACATATA GTA//TTATGCATCGATTTATAT-3‘. Sequencing consistently demonstrated the insertion of a single A residue between the breakpoints.

Statistical analysis: For pumping rates and EPG parameters, data sets were compared using the Mann-Whitney two-tailed U-test. For comparison of survival in the presence of arecoline, we used a one-tailed t-test. We determined the difference between the survival ratios for two different strains and calculated the SE of the difference. The difference divided by the SE of the difference gives a value for t, which was used to calculate the P value.

Sequence comparisons: Human muscarinic receptor sequences were aligned with GAR-3 using the ClustalW program, and the shaded alignment was generated with GeneDoc (Pittsburgh Supercomputing Center; http://www.psc.edu/biomed/genedoc/).

Generation of gar-3::GFP and gar-3 overexpression constructs: The gar-3::GFP expression construct was generated by overlap-extension PCR using wild-type genomic DNA and vector pPD95.77 as templates (a gift from A. Fire, J. Ahnn, G. Seydoux, and S. Xu). The 5‘ forward primer is located ∼6000 bp upstream of the gar-3 ATG. Primers used were: gar-3Pro, 5‘-ggg aca cag ctc ctt tcc aat caa-3‘; gfp-gar-3F, 5‘-cag aca cca tta ttc cag atg ttg gcc aaa gga ccc aaa g-3‘; gfp-gar-3R, 5‘-ctt ggg gtc ctt cgg cca aca tct aat ggg aag g-3‘; and gfp-R, 5‘-cag aca cca tca tcc cgg gca atg atc ctg g-3‘. The final PCR product was purified on a QIAGEN (Valencia, CA) spin column and injected into wild-type hermaphrodites using standard methods (Mello and Fire 1995) at a concentration of ∼10 ng/μl. Fluorescent images were collected at ×600 using OpenLab software (Improvement, Lexington, MA) and cropped using Adobe Photoshop 6.0 (Adobe, Mountain View, CA).

The gar-3 overexpression construct consists of two overlapping PCR products amplified from wild-type genomic DNA. The two fragments recombine in vivo to generate a full-length gar-3 construct. Primers used were gar-3Pro (above); gar-3-1038R, 5‘-ggt agg tgg tta cta atg cga aca tct g-3‘; gar-3-43F, 5‘-cgg cag aca cca cca ttc cag atg-3‘; and gar-3-5124R, 5‘-gct gcc aac gaa tga gca atg aat cg-3‘. The two PCR products were purified as above and mixed with a ttx-3::GFP marker (Hobert et al. 1997) to final concentrations of 25 ng/μl for each fragment. The mixture was injected into wild-type and C1072 unc-29(e1072) I worms. Transgenic worms were identified by GFP expression in AIY.

Measurement of pumping rate: Motions of the terminal bulb grinder plates were used to count pumps. At least 20 gravid adult worms of approximately the same size and age were placed on a lawn of E. coli HB101 and allowed to acclimate for at least 1 hr. Counts were made at room temperature (∼22°–25°). Each worm was observed for 30 sec and each recorded number of pumps was doubled to generate a "pumps per minute" number.

RESULTS

gpb-2 mutants respond to arecoline with enhanced pharyngeal muscle contraction: gpb-2 mutants, which have lost the RGS-mediated brake on Gα and Gqα signaling, are hypersensitive to the muscarinic agonist arecoline (Brundage et al. 1996). Unlike wild-type worms, young larvae carrying mutations in gpb-2 cannot grow in the presence of 5 nm arecoline (Figure 1A). Previously, we confirmed that arecoline acts through muscarinic receptors by showing that the muscarinic antagonist atropine improves the survival of gpb-2 mutants exposed to arecoline (Robatzek et al. 2001). Close examination reveals that the pharynxes of arecoline-treated gpb-2 mutants exhibit exaggerated muscle contractions and fail to close between pumps, while pharynxes from arecoline-treated wild-type worms close quickly after each contraction (Figure 1B). All regions of a gpb-2 mutant pharynx (the corpus, isthmus, and terminal bulb) can exhibit this phenotype, but the phenomenon is most striking in the corpus. Failure of the pharyngeal lumen to fully open and close disrupts the transport of bacteria along the length of the pharynx and into the intestine. As a result, the pharynx becomes stuffed with food. After a 24-hr exposure to arecoline, wild-type pharynxes are rarely stuffed with bacteria. In contrast, the lumens of almost all gpb-2(ad541) pharynxes are packed with bacteria (Figure 1C).

To further define the observed defect of pharyngeal function, we recorded EPGs from arecoline-exposed gpb-2 mutants. In a normal EPG, depolarization of the pharyngeal muscle is represented by a large positive R1 spike, which is followed by a plateau phase of relatively stable membrane potential. The plateau ends with a large negative R1 spike, representing repolarization of the corpus muscle (Raizen and Avery 1994). A smaller R2 spike, reflecting terminal bulb repolarization, often follows the R1 spike. EPGs recorded from gpb-2 mutant pharynxes after 24 hr of arecoline exposure display E and R spikes and appear normal (Figure 1D). That is, the pharyngeal muscle depolarizes and repolarizes normally, but the muscle does not fully relax. We conclude that in gpb-2 mutants, where signaling through Gα and Gqα pathways cannot be impeded, stimulation of muscarinic receptors by arecoline causes excessive muscle contraction, but does not block repolarization.

Mutation of gar-3 improves pharyngeal function in arecoline-exposed gpb-2 mutants: gar-3 encodes a protein with strong homology to vertebrate muscarinic acetylcholine receptors (Hwang et al. 1999; Figure 2A; also see supplementary information at http://www.genetics.org/supplemental/ for complete alignment). We created a reporter construct by fusing 6 kb of sequence upstream of the gar-3 ATG to GFP and observed GFP expression in the pharyngeal muscle, in pharyngeal neuron I3, and in neurons of the extrapharyngeal nervous system (Figure 2B). Within the pharyngeal muscle, the intensity of staining is highest in the terminal bulb, lower in the isthmus, and variable in the metacorpus.
Figure 1.—gpb-2 mutant pharynxes are hypersensitive to arecoline. (A) Ratio of worms that survive to adulthood in the presence (solid) or absence (shaded) of 5 mM arecoline. gpb-2 mutants are prevented from growing by 5 mM arecoline, but two gar-3 mutations restore growth. See MATERIALS AND METHODS for explanation of the calculation. (B) Sequential images of the pharynxes of wild-type and gpb-2(ad541) mutant adults exposed to arecoline. The time at which each image was collected is shown in milliseconds. In a wild-type pharynx (left), the lumen begins in the closed position (0), opens partially at 132 msec, is maximally open at 231 msec, and closes fully by 267 msec. The lumen is outlined in black. In contrast, the gpb-2 mutant pharynx (right) begins in a partially open position (the two-headed arrow spans the lumen and is the same size in all four panels). The muscles contract and open the lumen further at 99 and 165 msec, but then fail to fully relax. At 267 msec, the muscle has returned to its original, partially contracted position. Animals were removed from food 3 hr before images were collected, allowing the gpb-2 mutant pharynx to empty of bacteria. Bars, 20 μm. (C) Arecoline-induced failure of relaxation disrupts pharyngeal transport in gpb-2 mutants, causing pharynxes to become stuffed with food. The pharynxes of third and fourth stage (L3 and L4) larvae exposed to 5 mM arecoline for 24 hr were categorized as “severely stuffed” (the pharyngeal lumen is distended with bacteria to its fully open position), “partially stuffed” (bacteria have accumulated in the pharynx but the lumen is not fully open), or “not stuffed.” (D) EPG recordings from wild-type and gpb-2(ad541) worms exposed to 5 mM arecoline for 24 hr. In both traces, excitation (E) and repolarization (R1 and R2) spikes are visible (arrowheads), indicating that each pharynx is depolarizing and repolarizing normally. Because the horizontal axes are relatively compressed in these traces, the R1 and R2 spikes are very close together.

Activity of gpb-2 mutants (Figure 1A). gar-3(vu78), which was isolated in a screen for suppressors of arecoline sensitivity in a gpb-2 mutant background, carries a point mutation that converts a highly conserved alanine in the second transmembrane domain to a threonine (Figure 3A). gar-3(lg1201), which was isolated from a deletion library by PCR screening, carries a 1.5-kb deletion that removes two exons and disrupts splicing. Both gar-3 mutations improve the rate of survival of gpb-2(ad541) gar-3(vu78) double mutants from <10% to >60% (Figure 1A).

Because the ad541 allele is a point mutation in the final exon of gpb-2, we also verified that gar-3 mutations reduce the arecoline sensitivity of other gpb-2 mutants. sa603 is a putative null allele of gpb-2, harboring an early stop codon (Robatzek et al. 2001). gpb-2(sa603) mutants are unable to survive arecoline exposure, but almost 30% of gpb-2(sa603); gar-3(vu78) double mutants are viable in the presence of 5 mM arecoline (Figure 1A).

Mutation of gar-3 also suppresses the defect in food transport exhibited by gpb-2(ad541) mutants. gpb-2(ad541); gar-3(vu78) double-mutant worms are far less likely than gpb-2 single mutants to have stuffed pharynxes after arecoline exposure (Figure 1C). We conclude that arecoline acts through GAR-3 to promote pharyngeal mus-
Figure 2.—gar-3 encodes a muscarinic acetylcholine receptor expressed in neurons and in the pharyngeal muscle. (A) Alignment of the N-terminal portion of the GAR-3 amino acid sequence with human muscarinic receptors (the GAR-3 sequence data are available in the GenBank databases under accession no. AF139093). The vu78 point mutation is indicated by an arrowhead. The lg1201 deletion is underlined. The deletion also disrupts the intron-exon junctions identified by Hwang et al. (1999) so that sequences 3′ to the breakpoints are likely mistranslated. A complete alignment is available in the supplementary information at http://www.genetics.org/supplemental/. (B) A gar-3 reporter is expressed in neurons of the extrapharyngeal nervous system; in the pharyngeal muscles of the metacorpus, isthmus, and terminal bulb; and in pharyngeal neuron I3 (observed by muscle staining). Bar, 50 μm.

In the absence of GPB-2, signaling through GAR-3 cannot be downregulated and the pharynx fails to relax.

Overexpression of gar-3 renders wild-type pharynxes sensitive to arecoline: Wild-type worms carrying extra copies of the promoter and coding regions of gar-3 exhibit impaired growth in the presence of arecoline, when compared with worms carrying only a GFP transformation marker (Figure 3A). Worms overexpressing gar-3 also have stuffed pharynxes after arecoline exposure (Figure 3B), indicating impaired pharyngeal transport. As in gpb-2 mutants, we can directly observe failure of pharyngeal muscle relaxation (Figure 3C). Overexpression of gar-3 thus mimics the loss of gpb-2 function, confirming that increased signaling through GAR-3 causes extended contraction of the pharyngeal muscle and prevents normal feeding and growth.

Figure 3.—Overexpression of gar-3 causes arecoline hypersensitivity. (A) Ratio of worms that survive to adulthood in the presence (solid) or absence (shaded) of 5 mM arecoline. Transgenic worms carry extrachromosomal arrays containing either a GFP marker alone (tx-3::GFP) or the marker plus extra copies of wild-type gar-3 [gar-3(+); tx-3::GFP] and are identified by GFP expression. Worms carrying extra copies of gar-3 are inhibited from growing by 5 mM arecoline, while worms carrying only a GFP marker are essentially unaffected. (B) Arecoline impedes relaxation and disrupts pharyngeal transport in worms carrying gar-3 transgenes. See Figure 1C for procedure and description of categories. (C) Sequential images of the pharynx of an adult worm expressing a gar-3 transgene and exposed to arecoline (as in Figure 1B). The pharynx is empty, but the lumen of the corpus is open (edges of the corpus lumen are accentuated with black lines). The corpus muscles contract at 66 and 198 msec (arrows), expanding the lumen, but relaxation of the muscles leaves the lumen open. Bar, 20 μm.

Loss of gar-3 function alters the duration of pharyngeal muscle action potentials: Loss of gar-3 function protects gpb-2 mutant worms from the effects of arecoline, but GAR-3 signaling also regulates pumping in a wild-type background. gar-3 loss-of-function mutants pump more rapidly than wild-type worms, and the mo-
sections of the grinder plates suggest that their terminal bulb contractions are abnormally brief. We analyzed EPG traces to determine whether the duration of pharyngeal muscle action potentials is normal in gar-3 mutants. We first measured the total length of each action potential, from the beginning of the excitatory E spike to the end of the R2 spike, which represents terminal bulb repolarization. The average duration of an action potential is shorter in gar-3(lg1201) mutants than in wild-type worms (Table 1, \( P < 0.001 \)). When we examined the interval between the E spike and the R1 spike (corpus repolarization), we found that the difference in total action potential duration can be attributed to shortened action potentials in the pharyngeal terminal bulb [Table 1; E to R1 duration is not significantly different in wild-type and gar-3(lg1201) mutants, but R1 to R2 durations are different \( (P < 0.001) \)]. The R2 spike occurs only 40 msec after corpus repolarization in gar-3(lg1201) mutant worms, while the gap between R1 and R2 spikes in wild-type worms averages 70 msec (Figure 4). Thus, loss of gar-3 function appears to specifically speed up the repolarization of the terminal bulb muscle.

The duration of a pharyngeal muscle action potential is influenced by at least two pharyngeal neurons: excitatory motor neuron MC and inhibitory motor neuron M3 (NiCARIS and Avery 2003). To determine whether the effect of gar-3(lg1201) on action potential duration is mediated by MC, we constructed eat-2; gar-3 double mutants. eat-2 encodes a subunit of the pharyngeal muscle nicotinic receptor, which mediates fast depolarization in response to MC firing (Raizen et al. 1995; McKay et al. 2004). eat-2(ad465) mutants pump slowly and have action potentials longer than those of wild-type worms. eat-2(ad465); gar-3(lg1201) double mutants have action potentials significantly shorter than those of eat-2(ad465) single mutants [Table 1, total action potential duration \( (P < 0.001) \)]. As in wild-type worms and gar-3 single mutants, the difference in action potential duration is due to early terminal bulb repolarization [R1 to R2 duration is shorter in eat-2; gar-3 double mutants than in eat-2 single mutants \( (P < 0.001) \)]. The fact that loss of gar-3 function causes brief action potentials in eat-2 mutants suggests that gar-3 has effects on action potential duration that are independent of fast neurotransmission from MC.

We have also examined the interaction between GAR-3 function and motor neuron M3. M3 fires during the plateau phase of the action potential and releases glutamate, which binds ligand-gated chloride channels on the pharyngeal muscle surface (Raizen and Avery 1994; Dent et al. 1997). The influx of chloride ions hastens the repolarization of the muscle membrane. M3 function can be eliminated either by laser ablation of the neuron itself or by mutations in avr-15, which encodes the \( \alpha \)-subunit of the chloride channel. Both modifications delay repolarization and result in long action potentials. Interestingly, terminal bulb repolarization lags behind corpus repolarization in both wild-type and avr-15 mutant worms, suggesting that a mechanism other than M3 activity ensures that the terminal bulb does not repolarize before the corpus. Double-mutant worms carrying mutations in both avr-15 and gar-3 do not have conspicuously brief pharyngeal pumps. Consistent with this observation, EPG analysis reveals that overall pump duration does not differ between avr-15(ad1051) and avr-15(ad1051); gar-3(lg1201) worms (Table 1; \( P = 0.6 \)). However, the interval between corpus repolarization (R1 spike) and terminal bulb repolarization (R2 spike) is shorter in avr-15; gar-3 double mutants than in avr-15 single mutants (Table 1; \( P < 0.001 \)). We can draw two conclusions from this result: first, that distinct processes regulate corpus repolarization and terminal

### Table 1

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<th>Genotype</th>
<th>Pump rate per minute</th>
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<td></td>
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<td>244.8 ± 4.8</td>
</tr>
<tr>
<td>avr-15(ad1051); gar-3(lg1201)</td>
<td>187.6 ± 4.5</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>egl-19(n2368sd)</td>
<td>151.1 ± 4.6</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>egl-19(n2368sd); gar-3(lg1201)</td>
<td>188.8 ± 5.1</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>egl-19(n582)</td>
<td>134.2 ± 3.0</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>egl-19(n582); gar-3(lg1201)</td>
<td>178.9 ± 2.5</td>
<td>Yes</td>
<td></td>
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</tbody>
</table>

Action potential segment durations were measured on EPG recordings. Pumping rate was assessed in intact worms on lawns of E. coli HB101 bacteria. Contractions of the terminal bulb were categorized as brief or not on the basis of direct observation of the terminal bulb grinder plates. All results are presented as mean ± SEM.
bulb repolarization and, second, that GAR-3 signaling specifically affects the terminal bulb and is, to some degree, independent of M3.

**Loss of gar-3 increases pumping rate independently of pump duration:** In addition to shortening action potential duration, gar-3 mutations affect the rate of pharyngeal pumping. gar-3 loss-of-function mutants pump more frequently than wild-type worms when eating *E. coli* strain HB101 (Boyer and Roulland-Dussoix 1969) and *eat-2(ad465); gar-3(lg1201)* double mutants pump more frequently than *eat-2(ad465)* single mutants (Table 1; *P* < 0.001 for both comparisons). Rapid pumping in both cases could be a simple consequence of shortened action potentials. However, pumping rate is considerably faster in *avr-15(ad1051); gar-3(lg1201)* mutants than in *avr-15(ad1051)* single mutants, although total pump duration does not differ in the two strains (Table 1, *P* < 0.001). Loss of gar-3 function therefore seems to affect pharyngeal activity in a second way, by either increasing the excitability of the pharyngeal muscle or shortening the refractory period between pumps.

**gar-3 signaling is linked to intracellular calcium levels:** Muscarinic receptors in vertebrate tissues influence intracellular calcium levels in a number of ways. In smooth muscle, receptors of the M3 subtype raise cytoplasmic calcium concentrations through phospholipase C activation, generation of IP3, and the release of calcium from intracellular stores (Berridge 1993). Activation of smooth muscle muscarinic receptors also opens nonspecific cation channels, which may permit calcium influx (Benham et al. 1985; Lee et al. 1993). In contrast, myocardial muscarinic receptors coupled to Gα have inhibitory effects on L-type calcium channels (Valenzuela et al. 1997). In neurons, muscarinic signaling can increase or decrease the activity of voltage-gated calcium channels (Bernheim et al. 1992; Meza et al. 1999). Because manipulation of intracellular calcium plays a key role in muscarinic signaling in many tissues, we examined the role of calcium in gar-3 signaling.

The electrical activity of the *C. elegans* pharyngeal muscle is distinct from both smooth and cardiac muscle of vertebrates. The pharyngeal muscle has rapid action potentials, like the vertebrate heart. However, fast depolarization is predominantly mediated by an L-type voltage-gated calcium channel encoded by *egl-19*. Calcium influx through EGL-19 is likely to contribute to excitation-contraction coupling as well as membrane depolarization, although the role of intracellular calcium stores has not been well characterized. Consistent with a dual role of calcium influx, a gain-of-function mutation in *egl-19* causes extended depolarization and contraction of the terminal bulb (Lee et al. 1997). This mutation, *egl-19(n2368)*, is thought to disrupt voltage-dependent inactivation in the EGL-19 channel. We have found that *egl-19(n2368)* mutants are hypersensitive to arecoline, exhibiting growth inhibition (Figure 5A). Mutation of gar-3 reduces the arecoline hypersensitivity of *egl-19 (n2368)* mutants, suggesting that GAR-3 signaling is di-

![Figure 4](image1.png)

**Figure 4.**—Loss of gar-3 function hastens terminal bulb repolarization. Representative EPGs from wild-type and gar-3 (lg1201) worms. The interval between the E-phase spike (pharyngeal muscle depolarization) and the R1 spike (corpus repolarization) is approximately the same in both traces, but the interval between the R1 spike and the R2 spike (terminal bulb repolarization) is shorter in gar-3(lg1201) mutants.

![Figure 5](image2.png)

**Figure 5.**—Effect of calcium channel activity on arecoline sensitivity. (A) Ratio of worms that survive to adulthood in the presence (solid) or absence (shaded) of 5 mM arecoline. *egl-19(n2368)* mutants carry a gain-of-function mutation in the egl-19 calcium channel gene and are unable to grow in the presence of arecoline. Loss of gar-3 function suppresses this effect. (B) Ratio of worms that survive to adulthood in the presence (solid) or absence (shaded) of 5 mM arecoline. Loss of egl-19 function causes a small but significant increase in the survival of *gpb-2(ad541)* mutants exposed to arecoline (P < 0.005, asterisk). See MATERIALS AND METHODS for explanation of the calculation of statistical significance.
rectly linked to EGL-19 activity or that the two pathways converge. Conversely, a reduction of function mutation, egl-19(n582), slightly reduces the sensitivity of gpb-2 (ad541) mutants to arecoline (Figure 5B). The n582 mutation seems to generally reduce EGL-19 channel activity (Lee et al. 1997). Thus, the level of calcium influx through EGL-19 affects arecoline sensitivity, as does the level of GAR-3 signaling.

To explore the possibility that GAR-3 acts via EGL-19, we constructed egl-19; gar-3 double mutants and examined pharyngeal pumping. In contrast to loss-of-function mutations in gar-3, which cause rapid pumping, both loss- and gain-of-function mutations in egl-19 reduce the rate of pharyngeal pumping. Mutation of gar-3 increases pumping rate and causes contractions to appear brief in both egl-19 loss-of-function and egl-19 gain-of-function backgrounds (Table 1; both increases in pumping rate are significant: P < 0.001). Mutating gar-3 increases pumping rate to a similar degree in wild-type, egl-19 gain-of-function, and egl-19 loss-of-function backgrounds. Taken together, these results suggest that GAR-3 may alter EGL-19 channel activity in a manner that is not affected by either of the mutations we examined or has effects on calcium-dependent processes downstream of EGL-19.

**Examination of possible GAR-3 effectors:** Because GAR-3 seems to act through EGL-30 Gα₃ to influence calcium signals and muscle contraction, we have attempted to identify effector molecules likely to function downstream of EGL-30. The best-known function of Gα₃ proteins is to stimulate the production of IP₃ and DAG by phospholipase Cβ (Berridge and Irvine 1984). In mammals, IP₃ is crucial for smooth muscle contraction (Berridge 1993). IP₃ binds to receptors on the sarcoplasmic reticulum, triggering calcium release. High levels of intracellular calcium allow calmodulin to activate the myosin light chain kinase that facilitates muscle contraction (Adelstein et al. 1980). *C. elegans* possesses an IP₃-receptor homolog, *itr-1*, which is expressed in the pharynx and has effects on feeding (Walker et al. 2002). We attempted to determine whether *itr-1* plays a role in GAR-3 signaling by examining the arecoline sensitivity of ggb-2(ad541); *itr-1(sa73) double mutants. These double-mutant animals are very unhealthy and many of their eggs fail to develop, perhaps because loss of ggb-2 function enhances the ovulation defects exhibited by *itr-1* mutants (Cladnin et al. 1998; Dal Santo et al. 1999). Mutation of *itr-1* did not decrease the arecoline sensitivity of ggb-2(ad541) mutants in our survival assay (a total of ~40 adult worms grew from 600 ggb-2(ad541); *itr-1(sa73) eggs placed on no-drug plates, while no adults grew from 600 eggs placed on 5 mM arecoline plates), but the extremely low rate of survival of ggb-2; *itr-1* mutant larvae makes interpretation of this result difficult. Therefore, the role of IP₃-mediated calcium release in GAR-3 signaling may require further study.

**Contraction of mammalian cardiac muscle also requires calcium release from intracellular stores, but uses a mechanism distinct from that used by smooth muscle.** Calcium release in cardiac muscle is accomplished by the ryanodine receptor, which mediates calcium-induced calcium release from the sarcoplasmic reticulum. Ryanodine receptor activity is required for cardiac function, suggesting that calcium influx through plasma membrane voltage-gated channels is insufficient to trigger contraction (Kiriazis and Kranias 2000). The *C. elegans* homolog of the ryanodine receptor, UNC-68, is expressed in pharyngeal muscle, but may play an unexpected role in muscle contraction. Maryon et al. (1998) have found that *unc-68* null mutants retain the ability to pump, suggesting that calcium release through UNC-68 is not essential for excitation-contraction coupling. Furthermore, the terminal bulb muscles of *unc-68* mutants exhibit sporadic failure to relax, although the muscles of the anterior isthmus sometimes fail to contract (Maryon et al. 1998). We have found that neither *unc-68(e540) nor unc-68(e540); gar-3(g1201) mutants can grow in the presence of 5 mM arecoline (data not shown). This result is consistent with the idea that calcium release through UNC-68 does not have the same function in the *C. elegans* pharynx as in the mammalian heart, but does not increase our understanding of GAR-3 signaling pathways.

**DISCUSSION**

We have demonstrated that signaling through the GAR-3 muscarinic receptor affects two different aspects of pharyngeal muscle function. Excessive GAR-3 signaling results from arecoline exposure in worms overexpressing gar-3 or lacking the GPB-2-mediated brake on G-protein signaling. Under these conditions, the pharyngeal muscle depolarizes and repolarizes in an apparently normal fashion, but the muscle fails to relax to the closed position. Failure of the pharyngeal lumen to close interferes with the effective transport of bacteria into the intestine and prevents worms from growing. Because we have observed pharyngeal muscle contraction in the face of muscle membrane repolarization, we conclude that GAR-3 signaling alters the relationship between excitation and contraction in the pharyngeal muscle (E-C coupling).

However, the phenotypes of gar-3 loss-of-function mutants directly implicate GAR-3 signaling in the control of pharyngeal muscle membrane potential. Loss of gar-3 function speeds up the repolarization of the terminal bulb muscles and shortens action potentials. Furthermore, mutating gar-3 increases pharyngeal pumping rate without changing action potential duration in the arm-15 mutant background. This increased pumping rate could represent a regulatory response to low GAR-3 activity or might reflect an increase in the excitability of the pharyngeal muscle membrane and an additional GAR-3-mediated effect on membrane potential. Thus,
GAR-3 activity in the pharyngeal muscle has at least two distinct components, one affecting E-C coupling and the other affecting membrane potential. Our data also suggest a role for calcium in gar-3 signaling. The level of calcium influx during an action potential, determined by the activity of EGL-19, is an important factor in arecoline sensitivity. gar-3 interacts genetically with egl-19, and GAR-3 signaling may directly influence EGL-19 activity. However, the GAR-3 pathway may also converge with intracellular calcium signaling after calcium influx through EGL-19. We believe that we can account for all our observations by proposing that GAR-3 signaling alters the kinetics of several calcium-dependent processes in the pharyngeal muscle, influencing both E-C coupling and membrane potential (Figure 6). For example, calcium channels often undergo calcium-dependent inactivation (Eckert and Chad 1984). GAR-3 activity may increase the concentration of calcium necessary to inactivate EGL-19 channels. Signaling through GAR-3 may also alter the calcium sensitivity of contractile proteins: adrenergic receptor activation in the mammalian heart causes phosphorylation of troponin I, decreasing the sensitivity of myofilaments to calcium ions. In addition, GAR-3 signaling may affect the clearance of calcium from the cytoplasm, just as myocardial adrenergic signaling increases the efficiency of calcium reuptake into the sarcoplasmic reticulum (Bers 2002).

We have not yet delineated the full signaling cascade linking GAR-3 activation muscle contraction, but have begun to identify important signaling molecules. EGL-30 Gα is almost certainly involved in transmitting signals from GAR-3. Previous studies of arecoline sensitivity in gpb-2 mutants implicated EGL-30 in the muscarinic pathway (Brundage et al. 1996). Furthermore, we have found that the RGS protein EAT-16, which has been shown to specifically inhibit EGL-30 signaling, is required to keep GAR-3 signaling in check. eat-16 mutants are unable to grow in the presence of arecoline, and, when exposed, exhibit the same phenotype of extended pharyngeal muscle contraction as gpb-2 mutants (data not shown). Downstream of EGL-30, the GAR-3 signaling cascade may use a mechanism not yet observed in other muscarinic systems. While Gα proteins often act through IP3-mediated calcium release in vertebrate systems, we have not observed any role for the IP3 receptor in arecoline sensitivity. We are currently exploring different lines of investigation to identify additional components of the GAR-3 signaling cascade.

Finally, we note that GAR-3 may be one of several pharyngeal targets of arecoline. Mutations in gar-3 only partially suppress the arecoline sensitivity of gpb-2 loss-of-function mutations. Furthermore, mutation of gar-3 is less effective at suppressing arecoline sensitivity in gpb-2(sa603) null mutants than in gpb-2(ad541) point mutants. In a previous study using a slightly different method (Robatzek et al. 2001), we found that atropine, a muscarinic antagonist, was equally effective at suppressing arecoline hypersensitivity in these two mutant strains. We have also observed that gar-3 mutations do not eliminate pharyngeal stuffing in adult gpb-2 worms, suggesting that arecoline acts on additional targets in adult worms to impair pharyngeal function.

In addition, arecoline has effects on nonpharyngeal tissues that may not be mediated by GAR-3. Arecoline causes numerous behavioral abnormalities in gpb-2 mutant worms, including sporadically uncoordinated motion, apparent failure of defection, and cessation of egg laying. We also note that mutations in egl-8, which encodes a phospholipase Cβ, partially suppress the arecoline sensitivity of gpb-2 mutants, although EGL-8 expression has not been detected in the pharyngeal muscle by immunostaining or by GFP reporter construct (Lackner et al. 1999; Miller et al. 1999).

In summary, we have shown that the GAR-3 muscarinic receptor plays a key role in regulating pharyngeal muscle activity. The muscarinic agonist arecoline acts through GAR-3 to extend pharyngeal muscle contraction without preventing membrane repolarization. Conversely, loss of gar-3 function shortens the action potentials of the pharyngeal terminal bulb, leading to brief muscle contractions. Calcium influx through the EGL-
19 voltage-gated calcium channel also contributes to muscle contraction and arecoline sensitivity. We therefore believe that GAR-3 regulates intracellular calcium levels or manages the activity of calcium-dependent processes to optimize pharyngeal pumping. In this way, the GAR-3 signaling pathway differs substantially from muscarinic cascades in vertebrate smooth muscle, instead serving a function similar to that of adrenergic signaling in the vertebrate heart. Rather than simply facilitating muscle contraction, GAR-3 signaling may subtly adjust the kinetics of pharyngeal muscle function, perhaps allowing the worm to optimize feeding behavior.

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LITERATURE CITED


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