DOI: 10.1534/genetics.103.020230

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

Katherine A. Steger and Leon Avery[1]

Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9148

Manuscript received July 17, 2003
Accepted for publication February 13, 2004

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 $G_o\alpha$- and $G_q\alpha$-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_q family enhance the slow, tonic contraction of smooth muscle. G_q proteins activate phospholipase Cβ, stimulating the production of inositol triphosphate (IP_3) and diacyl glycerol (DAG) and triggering the release of calcium from intracellular stores. In the heart, receptors coupled to $G_{i/o}$ 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 (Gomeza *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 1962). 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

[1]*Corresponding author:* Avery Lab, UTSW Medical Center, 6000 Harry Hines Blvd., Room NA5.300, Dallas, TX 75390-9148.
E-mail: leon@eatworms.swmed.edu

(Avery 1993; Brundage *et al.* 1996). *gpb-2* encodes a G-protein β-subunit homologous to mammalian $G\beta_5$. GPB-2 has been shown to interact with the RGS proteins EAT-16 and EGL-10, which increase the rate of GTP hydrolysis by $G_q\alpha$ and $G_o\alpha$, respectively. In the absence of GPB-2, the RGS proteins are unable to terminate signaling through their target Gα-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 G_q, because mutations in *egl-30*, which encodes the *C. elegans* $G_q\alpha$, suppress the hypersensitivity of *gpb-2* mutants (Brundage *et al.* 1996). However, neither the muscarinic receptor assumed to be upstream of G_q 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 excitation-contraction coupling through effects on intracellular calcium signaling.

MATERIALS AND METHODS

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° on *Escherichia coli* strain HB101 (Boyer and Roulland-Dussoix 1969). The wild-type strain was *C. elegans* variety Bristol, strain N2. Mutant strains used were DA541 *gpb-2 (ad541) I*, JT603 *gpb-2(sa603) I*, JD222 *gpb-2(ad541) I*; *gar-3 (vu78) V*, JD283 *gpb-2(ad541) I*; *gar-3(lg1201) V*, JD292 *gpb-2 (sa603) I*; *gar-3(vu78) V*, JD217 *gar-3(vu78) V*, JD250 *gar-3(lg1201) V*, DA465 *eat-2(ad465) II*, JD285 *eat-2(ad465) II*; *gar-3(lg1201) V*, JD105 *avr-15(ad1051) V*, JD308 *avr-15(ad1051) gar-3(lg1201) V*, JD258 *gpb-2(ad541) I*; *egl-19(n582) IV*, MT1212 *egl-19(n582) IV*, JD303 *egl-19(n582)IV*; *gar-3(lg1201) V*, MT6129 *egl-19(n2368sd) IV*, JD301 *egl-19(n2368sd) IV*; *gar-3(lg1201) V*, JT73 *itr-1(sa73) IV*, JD265 *gpb-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 DA837 (Davis *et al.* 1995); 100 mm arecoline in M9 buffer (Lewis and Fleming 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 Fleming 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* for 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 PC-501A amplifier (Warner Instruments, Hamden, CT), a Digidata 1322A acquisition system (Axon Instruments, Union City, CA), and Axoscope 8.1 software (Axon Instruments). All recordings were filtered with a low-pass 5-Hz filter and a high-pass 20-Hz filter. Traces shown in figures underwent an additional low-pass filter of 350 Hz using Clampfit 8.1 software (Axon Instruments). For analysis of pump duration, traces from at least five different worms were examined (no more than 15 pumps were examined from each individual worm). For experiments involving *avr-15*, only traces in which worms pumped 40 or more times per minute were analyzed (in the wild-type background, this restriction was unnecessary because no worms pumped <40 times/minute). Measurements were taken from the point at which measured current began rising steeply (beginning of the E-phase spike) to the point at which the measured current returned to zero after an R1 or R2 spike.

Identification of *gar-3* mutant alleles: The *gar-3(vu78)* allele was isolated in a screen for mutations that suppress the arecoline sensitivity of *gpb-2(ad541)* mutant worms. Briefly, *gpb-2 (ad541)* hermaphrodites were mutagenized with EMS (Anderson 1995) and their F_2 progeny were transferred to plates seeded with *E. coli* DA837 and containing 5 mm arecoline. Surviving worms were selected and the mutations conferring

survival were mapped using TC1 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 *lg1201* allele of *gar-3* was isolated from a deletion library by EleGene. The deletion breakpoints are 5′-AAAATGCAAAACAATAA GTA//TTATGCATCGATTATTTAAT-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′-ggt aca cag ctc ctt tcc aat caa-3′; gfp-gar-3F, 5′-cag aca cac tta ttc cag atg ttg gcc aaa gga ccc aaa g-3′; gfp-gar-3R, 5′-ctt tgg gtc ctt tgg cca aca tct gga ata agt gtg tct g-3′; and gfp-R, 5′-caa acc caa acc ttc ttc cga tc-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 (Improvision, 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′-gtg agg tta cta atg cga aca ttg c-3′; gar-3-43F, 5′-cgg cag aca cac tta ttc cag atg-3′; and gar-3-5124R, 5′-gtc aac aat gaa tga gca atg att 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 CB1072 *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 $G_o\alpha$ and $G_q\alpha$ 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 mm 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 excitation (E) 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 $G_o\alpha$ and $G_q\alpha$ 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.

GAR-3 is an important target of arecoline. Two different mutant alleles of *gar-3* suppress the arecoline sensi-

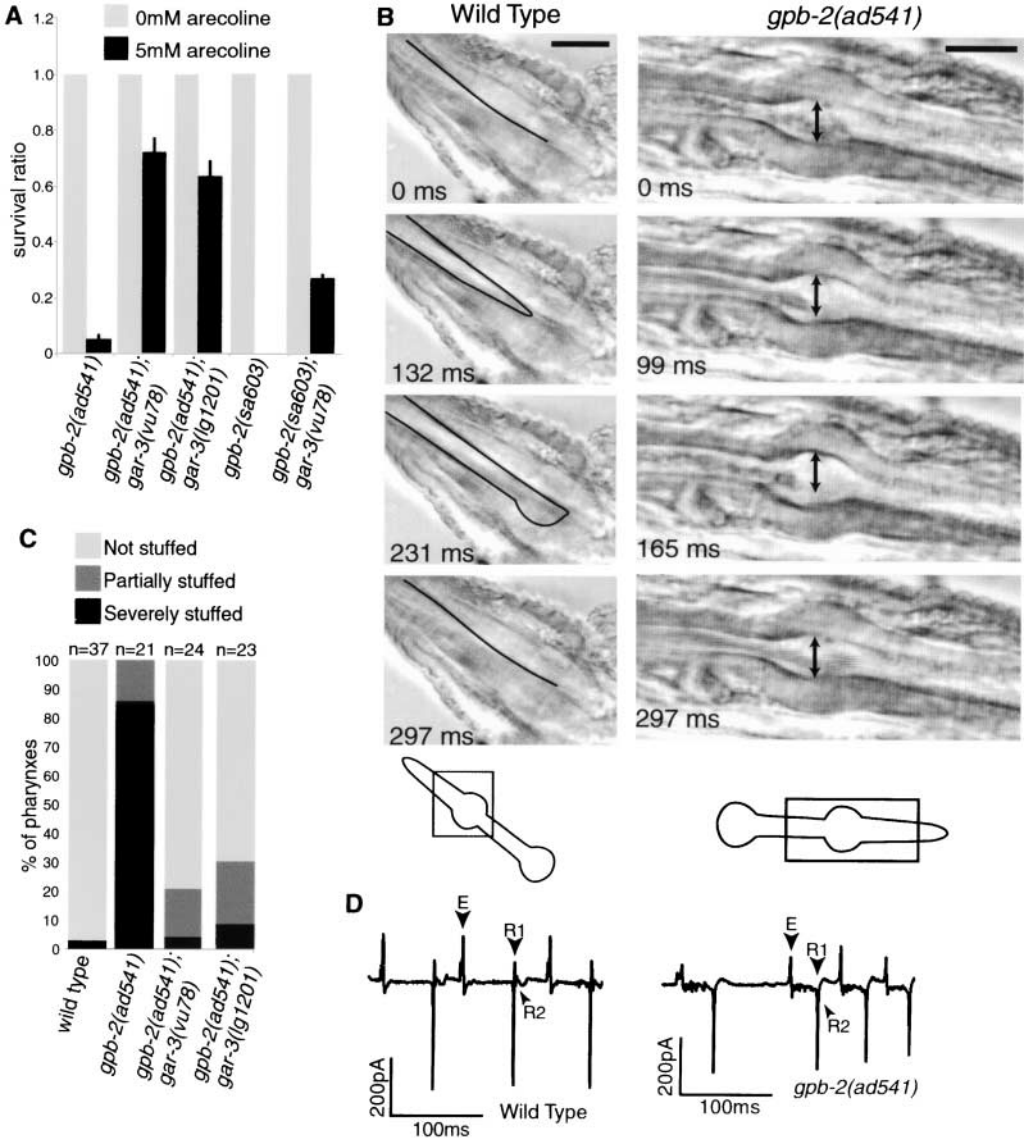

FIGURE 1.—*gpb-2* mutant pharynxes are hypersensitive to arecoline. (A) Ratio of worms that survive to adulthood in the presence (solid) or absence (shaded) of 5 mM arecoline. *gpb-2* mutants are prevented from growing by 5 mM arecoline, but two *gar-3* mutations restore growth. See MATERIALS AND METHODS 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.

tivity 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 2A). *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)* 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-

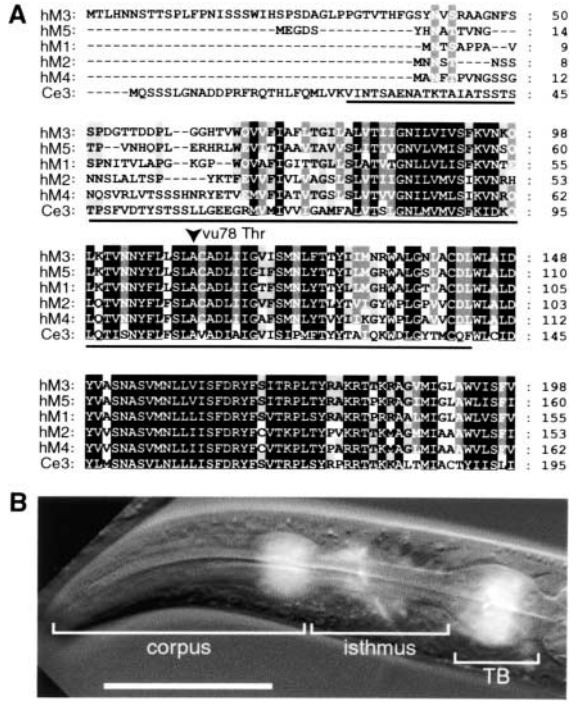

FIGURE 2.—*gar-3* encodes a muscarinic acetylcholine receptor expressed in neurons and in the pharyngeal muscle. (A) Alignment of the N-terminal portion of the GAR-3 amino acid sequence with human muscarinic receptors (the GAR-3 sequence data are available in the GenBank databases under accession no. 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 (obscured by muscle staining). Bar, 50 μm.

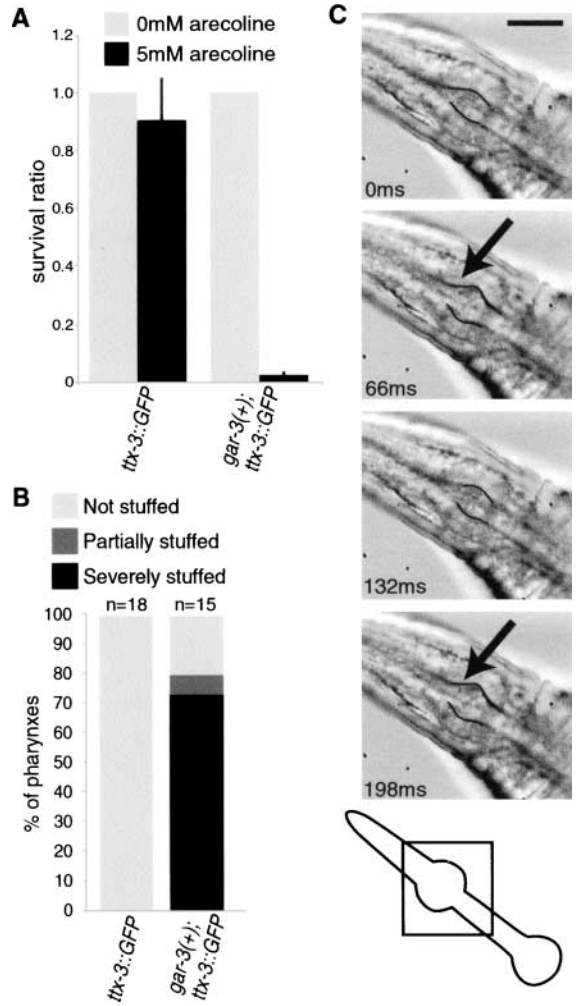

FIGURE 3.—Overexpression of *gar-3* causes arecoline hypersensitivity. (A) Ratio of worms that survive to adulthood in the presence (solid) or absence (shaded) of 5 mM arecoline. Transgenic worms carry extrachromosomal arrays containing either a GFP marker alone (*ttx-3*::GFP) or the marker plus extra copies of wild-type *gar-3* [*gar-3(+); ttx-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.

cle contraction. 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.

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-

TABLE 1

Loss of *gar-3* function alters pharyngeal pumping rate and action potential duration

Genotype	Pump rate per minute	Brief?	Action potential segment intervals (msec)		
			Total length	E to R1	R1 to R2
Wild type	200.9 ± 2.7	No	204.0 ± 4.3	134.4 ± 7.1	69.6 ± 3.7
gar-3(lg1201)	231.3 ± 2.3	Yes	171.8 ± 3.7	131.6 ± 4.2	40.1 ± 2.9
gar-3(vu78)	227.7 ± 2.0	Yes			
eat-2(ad465)	49.5 ± 2.1	No	277.8 ± 3.0	165.0 ± 4.5	112.0 ± 4.3
eat-2(ad465); gar-3(lg1201)	69.9 ± 2.5	Yes	214.4 ± 2.2	178.5 ± 2.9	35.9 ± 1.6
avr-15(ad1051)	133.1 ± 5.0	No	246.0 ± 8.8	216.6 ± 8.6	29.3 ± 1.2
avr-15(ad1051); gar-3(lg1201)	187.6 ± 4.5	No	244.8 ± 4.4	226.8 ± 4.8	18.0 ± 1.6
egl-19(n2368sd)	151.1 ± 4.6	No (extended)			
egl-19(n2368sd); gar-3(lg1201)	188.8 ± 5.1	Yes (relative to *n2368*)			
egl-19(n582)	134.2 ± 3.0	No			
egl-19(n582); gar-3(lg1201)	178.9 ± 2.5	Yes			

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.

tions 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 (Niacaris 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 α-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

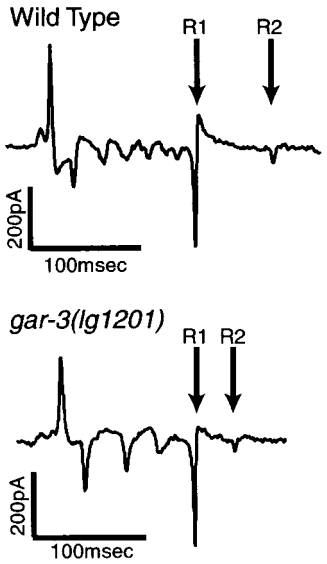

FIGURE 4.—Loss of *gar-3* function hastens terminal bulb repolarization. Representative EPGs from wild-type and *gar-3 (lg1201)* worms. The interval between the E-phase spike (pharyngeal muscle depolarization) and the R1 spike (corpus repolarization) is approximately the same in both traces, but the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

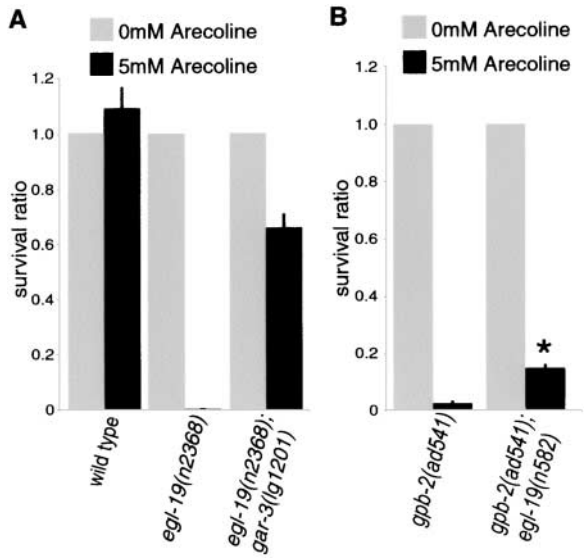

FIGURE 5.—Effect of calcium channel activity on arecoline sensitivity. (A) Ratio of worms that survive to adulthood in the presence (solid) or absence (shaded) of 5 mM arecoline. *egl-19(n2368sd)* mutants carry a gain-of-function mutation in the *egl-19* calcium channel gene and are unable to grow in 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.

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 IP_3, 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 G_o 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-

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 $G_q\alpha$ 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 $G_q\alpha$ proteins is to stimulate the production of IP_3 and DAG by phospholipase Cβ (Berridge and Irvine 1984). In mammals, IP_3 is crucial for smooth muscle contraction (Berridge 1993). IP_3 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 IP_3-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 *gpb-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 *gpb-2* function enhances the ovulation defects exhibited by *itr-1* mutants (Clandinin *et al.* 1998; Dal Santo *et al.* 1999). Mutation of *itr-1* did not decrease the arecoline sensitivity of *gpb-2(ad541)* mutants in our survival assay (a total of ~40 adult worms grew from 600 *gpb-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 *gpb-2; itr-1* mutant larvae makes interpretation of this result difficult. Therefore, the role of IP_3-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(lg1201)* 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 *avr-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,

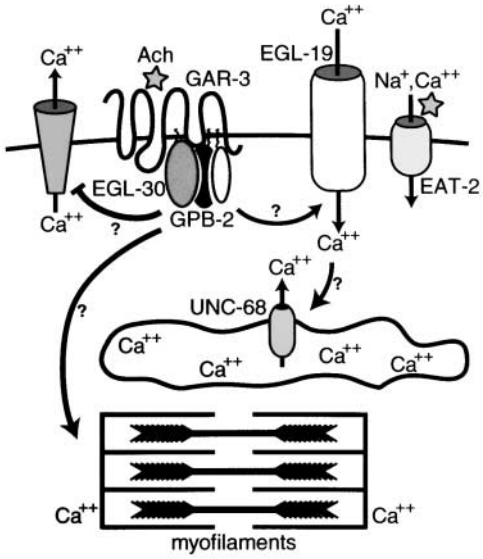

FIGURE 6.—Model of GAR-3 signaling in pharyngeal muscle. Acetylcholine (star) binds both muscarinic (GAR-3) and nicotinic (EAT-2) receptors. Acetylcholine binding to EAT-2 allows cation influx that depolarizes the muscle membrane and activates voltage-gated calcium channels (EGL-19). Increased intracellular calcium may trigger additional calcium release from intracellular stores or may directly trigger contraction. Signaling through GAR-3 seems to influence intracellular calcium handling at several points. GAR-3 signaling may directly modulate the activity of EGL-19, may reduce the effectiveness of calcium efflux, and may alter the relationship of calcium to myofilaments.

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 $G_q\alpha$ 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 $G_q\alpha$ proteins often act through IP_3-mediated calcium release in vertebrate systems, we have not observed any role for the IP_3 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 defecation, 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.

We thank Joseph Dent for critical reading of the manuscript. We also thank the *Caenorhabditis* Genetics Center for providing some of the strains used in this work and EleGene for providing the *gar-3 (lg1201)* strain. This work was supported by National Institutes of Health research grant HL46154.

LITERATURE CITED

Adelstein, R. S., M. A. Conti and M. D. Pato, 1980 Regulation of myosin light chain kinase by reversible phosphorylation and calcium-calmodulin. Ann. NY Acad. Sci. **356:** 142–150.

Anderson, P., 1995 Mutagenesis. Methods Cell Biol. **48:** 31–58.

Avery, L., 1993 The genetics of feeding in *Caenorhabditis elegans.* Genetics **133:** 897–917.

Avery, L., and H. R. Horvitz, 1989 Pharyngeal pumping continues after laser killing of the pharyngeal nervous system of C. elegans. Neuron **3:** 473–485.

Avery, L., and H. R. Horvitz, 1990 Effects of starvation and neuroactive drugs on feeding in Caenorhabditis elegans. J. Exp. Zool. **253:** 263–270.

Avery, L., D. Raizen and S. Lockery, 1995 Electrophysiological methods. Methods Cell Biol. **48:** 251–269.

Benham, C. D., T. B. Bolton and R. J. Lang, 1985 Acetylcholine activates an inward current in single mammalian smooth muscle cells. Nature **316:** 345–347.

Bernheim, L., A. Mathie and B. Hille, 1992 Characterization of muscarinic receptor subtypes inhibiting Ca2+ current and M current in rat sympathetic neurons. Proc. Natl. Acad. Sci. USA **89:** 9544–9548.

Berridge, M. J., 1993 Inositol trisphosphate and calcium signalling. Nature **361:** 315–325.

Berridge, M. J., and R. F. Irvine, 1984 Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature **312:** 315–321.

Bers, D. M., 2002 Cardiac excitation-contraction coupling. Nature **415:** 198–205.

Boyer, H. W., and D. Roulland-Dussoix, 1969 A complementation analysis of the restriction and modification of DNA in Escherichia coli. J. Mol. Biol. **41:** 459–472.

Brundage, L., L. Avery, A. Katz, U. J. Kim, J. E. Mendel *et al.*, 1996 Mutations in a C. elegans Gqalpha gene disrupt movement, egg laying, and viability. Neuron **16:** 999–1009.

Caulfield, M. P., and N. J. Birdsall, 1998 International union of pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol. Rev. **50:** 279–290.

Chase, D. L., G. A. Patikoglou and M. R. Koelle, 2001 Two RGS proteins that inhibit Galpha(o) and Galpha(q) signaling in C. elegans neurons require a Gbeta(5)-like subunit for function. Curr. Biol. **11:** 222–231.

Clandinin, T. R., J. A. DeModena and P. W. Sternberg, 1998 Inositol trisphosphate mediates a RAS-independent response to LET-23 receptor tyrosine kinase activation in C. elegans. Cell **92:** 523–533.

Dal Santo, P., M. A. Logan, A. D. Chisholm and E. M. Jorgensen, 1999 The inositol trisphosphate receptor regulates a 50-second behavioral rhythm in C. elegans. Cell **98:** 757–767.

Davis, M. W., D. Somerville, R. Y. Lee, S. Lockery, L. Avery *et al.*, 1995 Mutations in the Caenorhabditis elegans Na,K-ATPase alpha-subunit gene, eat-6, disrupt excitable cell function. J. Neurosci. **15:** 8408–8418.

Dent, J. A., M. W. Davis and L. Avery, 1997 avr-15 encodes a chloride channel subunit that mediates inhibitory glutamatergic neurotransmission and ivermectin sensitivity in Caenorhabditis elegans. EMBO J. **16:** 5867–5879.

Doncaster, C. C., 1962 Nematode feeding mechanisms. I. Observations on *Rhabditis* and *Pelodera.* Nematologica **8:** 313–320.

Eckert, R., and J. E. Chad, 1984 Inactivation of Ca channels. Prog. Biophys. Mol. Biol. **44:** 215–267.

Gomeza, J., H. Shannon, E. Kostenis, C. Felder, L. Zhang *et al.*, 1999 Pronounced pharmacologic deficits in M2 muscarinic acetylcholine receptor knockout mice. Proc. Natl. Acad. Sci. USA **96:** 1692–1697.

Gomeza, J., L. Zhang, E. Kostenis, C. C. Felder, F. P. Bymaster *et al.*, 2001 Generation and pharmacological analysis of M2 and M4 muscarinic receptor knockout mice. Life Sci. **68:** 2457–2466.

Hamilton, S. E., S. N. Hardouin, S. G. Anagnostaras, G. G. Murphy, K. N. Richmond *et al.*, 2001 Alteration of cardiovascular and neuronal function in M1 knockout mice. Life Sci. **68:** 2489–2493.

Hobert, O., I. Mori, Y. Yamashita, H. Honda, Y. Ohshima *et al.*, 1997 Regulation of interneuron function in the C. elegans thermoregulatory pathway by the ttx-3 LIM homeobox gene. Neuron **19:** 345–357.

Hwang, J. M., D. J. Chang, U. S. Kim, Y. S. Lee, Y. S. Park *et al.*, 1999 Cloning and functional characterization of a Caenorhabditis elegans muscarinic acetylcholine receptor. Receptors Channels **6:** 415–424.

Kiriazis, H., and E. G. Kranias, 2000 Genetically engineered models with alterations in cardiac membrane calcium-handling proteins. Annu. Rev. Physiol. **62:** 321–351.

Lackner, M. R., S. J. Nurrish and J. M. Kaplan, 1999 Facilitation of synaptic transmission by EGL-30 Gqalpha and EGL-8 PLCbeta: DAG binding to UNC-13 is required to stimulate acetylcholine release. Neuron **24:** 335–346.

Lee, H. K., O. Bayguinov and K. M. Sanders, 1993 Role of nonselective cation current in muscarinic responses of canine colonic muscle. Am. J. Physiol. **265:** C1463–C1471.

Lee, R. Y., L. Lobel, M. Hengartner, H. R. Horvitz and L. Avery, 1997 Mutations in the alpha1 subunit of an L-type voltage-activated Ca2+ channel cause myotonia in Caenorhabditis elegans. EMBO J. **16:** 6066–6076.

Lewis, J. A., and J. T. Fleming, 1995 Basic culture methods. Methods Cell Biol. **48:** 3–29.

Maryon, E. B., B. Saari and P. Anderson, 1998 Muscle-specific functions of ryanodine receptor channels in Caenorhabditis elegans. J. Cell Sci. **111:** 2885–2895.

Matsui, M., D. Motomura, H. Karasawa, T. Fujikawa, J. Jiang *et al.*, 2000 Multiple functional defects in peripheral autonomic organs in mice lacking muscarinic acetylcholine receptor gene for the M3 subtype. Proc. Natl. Acad. Sci. USA **97:** 9579–9584.

McKay, J., D. M. Raizen, A. Gottschalk, W. R. Schafer and L. Avery, 2004 *eat-2* and *eat-18* are required for nicotinic neurotransmission in the *Caenorhabditis elegans* pharynx. Genetics **166:** 161–169.

Mello, C., and A. Fire, 1995 DNA transformation. Methods Cell Biol. **48:** 451–482.

Meza, U., R. Bannister, K. Melliti and B. Adams, 1999 Biphasic, opposing modulation of cloned neuronal alpha1E Ca channels by distinct signaling pathways coupled to M2 muscarinic acetylcholine receptors. J. Neurosci. **19:** 6806–6817.

Miller, K. G., M. D. Emerson and J. B. Rand, 1999 Goalpha and diacylglycerol kinase negatively regulate the Gqalpha pathway in C. elegans. Neuron **24:** 323–333.

Niacaris, T., and L. Avery, 2003 Serotonin regulates repolarization of the *C. elegans* pharyngeal muscle. J. Exp. Biol. **206:** 223–231.

Raizen, D. M., and L. Avery, 1994 Electrical activity and behavior in the pharynx of Caenorhabditis elegans. Neuron **12:** 483–495.

Raizen, D. M., R. Y. Lee and L. Avery, 1995 Interacting genes required for pharyngeal excitation by motor neuron MC in *Caenorhabditis elegans.* Genetics **141:** 1365–1382.

Rand, J. B., and R. L. Russell, 1984 Choline acetyltransferase-deficient mutants of the nematode *Caenorhabditis elegans*. Genetics **106:** 227–248.

Robatzek, M., T. Niacaris, K. Steger, L. Avery and J. H. Thomas, 2001 eat-11 encodes GPB-2, a Gbeta(5) ortholog that interacts with G(o)alpha and G(q)alpha to regulate C. elegans behavior. Curr. Biol. **11:** 288–293.

Seymour, M. K., K. A. Wright and C. C. Doncaster, 1983 The action of the anterior feeding apparatus of *Caenorhabditis elegans* (Nematoda: Rhabditida). J. Zool. **201:** 527–539.

Sulston, J. E., and J. Hodgkin, 1988 Methods, pp. 587–606 in *The Nematode C. elegans*, edited by W. Wood. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Valenzuela, D., X. Han, U. Mende, C. Fankhauser, H. Mashimo *et al.*, 1997 G alpha(o) is necessary for muscarinic regulation of Ca2+ channels in mouse heart. Proc. Natl. Acad. Sci. USA **94:** 1727–1732.

van der Linden, A. M., F. Simmer, E. Cuppen and R. H. Plasterk, 2001 The G-protein β-subunit GPB-2 in *Caenorhabditis elegans* regulates the $G_o\alpha$-$G_q\alpha$ signaling network through interactions with the regulator of G-protein signaling proteins EGL-10 and EAT-16. Genetics **158:** 221–235.

Walker, D. S., N. J. Gower, S. Ly, G. L. Bradley and H. A. Baylis, 2002 Regulated disruption of inositol 1,4,5-trisphosphate signaling in Caenorhabditis elegans reveals new functions in feeding and embryogenesis. Mol. Biol. Cell **13:** 1329–1337.

Williams, B. D., 1995 Genetic mapping with polymorphic sequence-tagged sites. Methods Cell Biol. **48:** 81–96.

Yamada, M., K. G. Lamping, A. Duttaroy, W. Zhang, Y. Cui *et al.*, 2001 Cholinergic dilation of cerebral blood vessels is abolished in M(5) muscarinic acetylcholine receptor knockout mice. Proc. Natl. Acad. Sci. USA **98:** 14096–14101.

Communicating editor: J. Tamkun