Critical Residues of the Caenorhabditis elegans unc-2 Voltage-Gated Calcium Channel That Affect Behavioral and Physiological Properties

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The Caenorhabditis elegans unc-2 gene encodes a voltage-gated calcium channel α1 subunit structurally related to mammalian dihydropyridine-insensitive high-threshold channels. In the present paper we describe the characterization of seven alleles of unc-2. Using an unc-2 promoter-tagged green fluorescent protein construct, we show that unc-2 is primarily expressed in motor neurons, several subsets of sensory neurons, and the HSN and VC neurons that control egg laying. Examination of behavioral phenotypes, including defecation, thrashing, and sensitivities to aldicarb and nicotine suggests that UNC-2 acts presynaptically to mediate both cholinergic and subsets of sensory neurons, and the HSN and VC neurons that control egg laying. Examination of behavioral phenotypes, including the voltage dependence of activation, steady-state inactivation, as well as channel kinetics. Overall, our findings suggest that UNC-2 plays a pivotal role in mediating a number of physiological processes in the nematode and also defines a number of critical residues important for calcium channel function in vivo.

Key words: calcium channel; mutation; behavior; electrophysiology; presynaptic; C. elegans

Introduction

Voltage-gated calcium (Ca) channels play a central role in a number of biological processes, including neurotransmitter release, excitation–contraction coupling, regulation of gene expression, and neuronal migration. In addition, compounds that directly affect either Ca channels or proteins that modulate their activity are used to treat a number of cardiovascular and neurological pathologies (Miller, 2001). Understanding the nature of Ca channel functional diversity will provide insight into both normal calcium signaling mechanisms, as well as contributing to our understanding of the roles that these molecules play in various pathological conditions.

High-threshold Ca channels are multisubunit complexes consisting of four to five subunits designated α1, α2δ, β, and γ (for review, see Catterall, 2000). The α1 subunit forms the voltage-sensor and channel proper, whereas the remaining proteins interact with the α1 subunit to modulate channel activity. In mammals, five pharmacologically distinct types of Ca channels have been described (designated L-, N-, P/Q-, T-, and R-types) and that are encoded by 10 distinct α1 subunit genes (Stea et al., 1995b; Catterall, 2000). Genetic and molecular studies have revealed the presence of three α1 subunit genes in the nematode Caenorhabditis elegans: unc-2 (Schafer and Kenyon, 1995), egl-19 (Lee et al., 1997), and cca-1 (for review, see Bargmann 1998) and which appear to be ancestral to the mammalian N/P/Q-, L-, and T-type channels, respectively. Genes encoding other Ca channel subunits have also been identified in the C. elegans genome: unc-36 and T24F1.6 encode α1β subunit homologues and ccb-1 (T28F2.5) and W10C8.1 both encode putative β subunits (Bargmann, 1998). Schafer and Kenyon (1995) first noted that the slow, uncoordinated movement and constitutive egg-laying defects displayed by unc-2 mutants resembled the affects of adding exogenous serotonin to wild-type worms. In addition, unlike wild-type animals, unc-2 worms were unable to adapt to either serotonin or dopamine (Schafer and Kenyon, 1995). Subsequently, it was shown that mutations in the unc-36 gene exhibited similar mutant phenotypes to that of unc-2, suggesting that these two genes act together in a Ca-dependent pathway to modulate responses.
to serotonin (Schafer and Kenyon, 1995; Schafer et al., 1996). Genetic mosaic analysis and in situ hybridization experiments have shown that UNC-2/UNC-36 both act in the HSN and/or VCs neurons known to control egg laying in *C. elegans* (Schafer and Kenyon, 1995; Schafer et al., 1996). Mutations in unc-2 have also been shown to affect migration of specific neuronal cells. For example, the sensory neuron AVM and the interneuron SDQR undergo aberrant postembryonic migrations in unc-2 mutants (Tam et al., 2000). *unc-2* also plays an integral role in a Ca-dependent signal transduction pathway that controls the asymmetric expression of the *str-2* odorant receptor gene in the AWC sister sensory neurons (Troemel et al., 1999). In both processes, mutations in *unc-2* and *unc-36* show similar phenotypes, implying that these two gene products act in the same functional complex. Mutations in genes encoding factors that are known to regulate Ca channel αs-subunits, such as CaM kinase II (unc-43) (Reiner et al., 1999; Rongo and Kaplan, 1999), syntaxin (unc-64) (Saíee et al., 1998), and G-proteins (gou-1: Lochrie et al., 1991; gpb-1: Brundage et al., 1996; Zwaal et al., 1996), have also been identified. Interestingly, genetic analysis of the Ca signaling pathways involving UNC-2 that affect cell migration and *str-2* odorant receptor gene expression have demonstrated that one of the downstream effectors is unc-43/CaM kinase II (Troemel et al., 1999; Tam et al., 2000). Taken together, these findings suggest that both potential modulators of Ca channel αs-subunits and the αβ-subunits themselves function in a variety of complex physiological processes. Consequently, the coordinated analysis of these genes in *C. elegans* using genetic, behavioral, and electrophysiological methodologies should add to our understanding of Ca channel physiological functions in vivo.

Here we describe a comprehensive characterization of the *unc-2* gene and the isolation of six new alleles of *unc-2* as well as the canonical allele, e55. We also present evidence that *unc-2* acts as a presynaptic Ca channel to mediate both acetylcholine and voltage-dependent and kinetic properties. Consequently, the coordinated analysis of these genes in *C. elegans* using genetic, behavioral, and electrophysiological methodologies should add to our understanding of Ca channel physiological functions in vivo.

Materials and Methods

*Nematode strains and growth conditions.* Nematodes were grown on nematode growth media (NGM) plates streaked with *Escherichia coli* (OP50 strain), as described (Brenner, 1974). Strains in this work include the wild-type strain N2; *unc-2(e55)*, CB55; *unc-2(ra605)* TS79, *unc-2(ra606)* TS80; *unc-2(ra609)* TS82; *unc-2(ra610)*, TS83; *unc-2(ra612)*, TS85; *unc-2(ra614)*, TS118; *unc-2(e55)* dpy-1(e27), DM2601; *unc-25(e265)*, CB265; *unc-36(e251)*, CB251; *lin-13(e765)*; and val-9, TS67. The identification of the *ra605* and *ra610* mutations were previously reported in Tam et al. (2000).

Isolation of new *unc-2* alleles. A non-complementation screen was used to isolate new alleles of *unc-2*. Briefly, N2 males were mutagenized with 50 μM ethyl methane sulfonate and crossed to dpy-3(e27) *unc-2(e55)* hermaphrodites. Twenty mating plates, each with six males and three hermaphrodites, were set up, and their progeny were screened for the presence of Unc non-Dpy progeny. Eleven independent Unc non-Dpy animals were identified and picked to new plates. From each plate, single Unc animals were transferred to new plates, and their progeny were scored for the presence of Dpy Uncs. Animals that failed to segregate Dpy Unc progeny were presumed to be homozygous for the new *unc-2* mutation. Strains were outcrossed at least three times before phenotypic analysis.

Phenotypic analyses of *unc-2* mutants. Resistance of wild-type and mutant worms to aldicarb was performed as described (Miller et al., 1996). Briefly, 20 animals were placed on plates containing 0.5 μg aldicarb and assayed for paralysis at 10 min intervals for 3 hr. Animals, except for five individuals, were removed from the plates and then scored progeny over 1, 2, and 3 weeks for the production of viable progeny. To test for sensitivity to nicotine, plates were flooded with 1% nicotine solution.

To examine defects in movement, young adult hermaphrodites were transferred to a microtiter well containing 60 μl of M9 buffer. After a 2 min recovery period, thrashes were counted for 2 min (Miller et al., 1996). A thrash was defined as a change in direction of bending at the mid-body. Ten animals from each strain were examined. Defects in defection were determined by examining young adult hermaphrodites with a dissecting microscope for the presence or absence of an expulsion event after each posterior body muscle contraction (PBoC) of the defection cycle (Thomas, 1990; Miller et al., 1996). Ten animals from each strain were observed for 10 consecutive cycles.

cDNA *unc* Overlapping cDNAs encoding the *unc-2* gene were isolated from several sources. These included RT-PCR of total RNA prepared from a mixed population of worms and both PCR amplification and screening of a cDNA library (AACR-RB2; kindly provided by Robert Barstead, Oklahoma Medical Research Foundation, OK). The entire predicted *unc-2* open reading frame (ORF) is encoded by five overlapping cDNA clones, namely punc2.1, which included the putative initiator methionine codon; cDNA 82–43; cDNA10; cDNA1, and yk131b1 which contained the predicted *unc-2* poly(A) site (AATATA) and the 3’ untranslated region (3’ UTR) (Fig. 1A). The cDNA clone yk131b1 was generously provided by Yuji Kohara (National Institute of Genetics, Japan). The composite sequence of the overlapping cDNA clones was submitted to GenBank (accession number AY264781). Existing GenBank entries for *unc-2* differ considerably from the sequence reported in this work. An explanation for this discrepancy is that previous GenBank submissions have been based on annotated predictions using the program GeneFinder rather than on actual physical comparison of cDNA and genomic sequences. Differences between the cDNA sequence described in this work and that from Tam et al. (2000) is attributable to the discovery of additional 5’ sequences and a nucleotide sequencing change in the 3’ end of the gene that altered the reading frame of the predicted gene product (see Results).

Identification of *unc-2* lesions. To identify the nucleotide changes in the *unc-2* mutants, two approaches were used. The first involved the use of an RNase protection assay performed according to the procedure outlined in the Mismatch Detect II kit (Ambion). In the second approach genomic DNA encompassing the *unc-2* gene from the corresponding homozygous mutant animals was amplified, and the DNA sequence was determined directly.

*unc-2*: green fluorescent protein fusion. Long-range PCR was used to amplify the 5’ region of the *unc-2* gene and the predicted first three exons (Fig. 1A). Two fragments were amplified from genomic DNA. One included ~4 kb of 5’ sequence upstream from the first exon, along with a 6.8 kb region encompassing exons one and two. The second long-range PCR product was 4.1 kb in length, overlapping with the first product by 694 bp and ending within exon three. The second product was cloned into the vector pGEM-T Easy. A green fluorescent protein (GFP) cassette isolated from the expression vector pPD95.70 (kindly provided by A. Fire) was then cloned in-frame with exon 3, generating the construct punc24.70. The 10.8 kb long-range PCR product was injected into the gonads of adult lin-15(n765ts) hermaphrodites along with punc24.70 and PHM23, which contains a wild-type copy of the lin-15 gene. Transgenic animals were rescued for the lin-15 multivulval phenotype, and stable lines were established. The array from one of these lines was integrated, and GFP expression was examined in this strain, called TS67. Cell identification was based on the position and characteristic morphology of GFP-positive cell nuclei using fluorescence and Nomarski differential interference microscopy.

Construction of rat brain αs mutant. *unc-2* mutations corresponding to e55 and ra612 were introduced into the rat brain αs/Ca.2.1 cDNA by site-directed mutagenesis. Details of the procedures used can be obtained from the authors on request. All mutations were made in the rat αs/Ca.2.1 clone pc3RBA1 (Starr et al., 1991). To generate the corresponding e55 mutation the αs/Ca.2.1 cDNA was truncated by the introduction of a stop codon after the R477 codon. The construct pαs55 encodes only the N terminus of the rat αs/Ca.2.1 subunit ending in the domain I-I.
linker. Plasmid pmsa18-612 contains the corresponding rat162 mutation in which the G1817 codon (GGC) was changed to arginine (CGT).

Electrophysiology and data analysis. Human embryonic kidney (HEK)-ttsa201 cells were transiently transfected by the Ca phosphate precipitation method with an equimolar ratio of cDNAs encoding either the wild-type rat brain Ca channel α1/Ca,2.1 or one of the plasmids carrying the engineered mutation plus the rat brain β1b and α2δ ancillary subunits. Coexpression of CD38 antigen was used to visually identify cells for electrophysiological experiments through the binding of anti-CD38 antibody-coated microspheres (Dynal, Great Neck, NY). Whole-cell inward currents were recorded 24–48 hr after transfection with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA). Recordings were filtered at 2 kHz and acquired using pClamp software, version 6.03 (Axon Instruments). The extracellular recording solution contained either 5 mM BaCl₂ or 5 mM CaCl₂ and 1 mM MgCl₂, 10 mM HEPES, 40 mM TEACl, 10 mM glucose, and 87.5 mM CsCl, pH 7.4. Pipettes of typical resistances ranging from 2 to 4 MOhm were filled with internal solution containing (in mM): 105 CsCl, 25 TEACl, 1 CaCl₂, 11 EGTA, and 10 HEPES, pH 7.2. The voltage dependence of activation was analyzed by step depolarizations from a holding potential of −120 mV to various test potentials ranging from −60 to +30 mV. Normalized current amplitudes were plotted as a function of membrane potential, and I–V curves were fitted according to equation: I = [Gmax(Vm − Vrev)]/[1 + exp((Vm − Vrev)/k)], where G is membrane conductance, Vrev is the reversal potential, Vrev is the midpoint, and k is the slope of the voltage dependence. To measure steady-state inactivation profiles, conditioning pre-pulses (15 sec) from −120 to 40 mV in 10 mV steps were applied, and the membrane was then stepped to the peak of the I–V curve. Currents were normalized to the maximal value obtained at the test pulse and plotted as a function of the prepulse potential. Data were fitted with Boltzmann equations (I/Imax = [1 + exp((V − Vrev)/k)]−1). All experiments were performed at room temperature. Recordings were analyzed using Clampfit 6.03 (Axon Instruments); figures and nonlinear regressions were done using the Origin software (version 6.0, Microcal, Northampton, MA). Data are presented as mean ± SEM. Significant differences were determined using Student’s t test with the significance set at p < 0.01.

Results

Isolation and characterization of the unc-2 gene

Previous analysis of the unc-2 gene suggested that it encoded the α₁ subunit of a non-L-type high voltage-activated (HVA) Ca channel (Schafer and Kenyon, 1995). To characterize the complete unc-2 gene structure and predicted coding region, a combination of cDNA cloning and RT-PCR were used. Comparison of the cDNA and nematode genomic sequences show that the unc-2 gene consists of at least 28 exons and 27 introns encompassing a genomic region used to generate the unc-2::GFP fusion construct (top). Also shown are the regions of the predicted gene product (Protein) that are encoded by the corresponding exons and the overlapping cDNAs or RT-PCR products used to determine the gene structure (cDNAs). B, Phylogenetic comparison of UNC-2 with other C. elegans and mammalian Ca channel α₁ subunits. The predicted amino acid sequences of representatives of each class of Ca channel α₁ subunit and the novel NCA ion channel family (Gene), including the amino acid polypeptide with a predicted molecular weight of 231 kDa and the overlapping cDNAs or RT-PCR products used to determine the gene structure (cDNAs).
specific amino acids of the L-type channels required to interact with these agents have been described (Moreno, 1999). Of the four specific residues in domain IV identified to confer DHP and phenyalkylamine binding in the L-type channels (Tang et al., 1999), UNC-2 does not match exactly at any position but instead possesses residues identical to those found only in the non-DHP-sensitive high voltage-activated Ca channel subfamily.

Isolation and characterization of novel unc-2 mutations

Using a non-complementation screen, six new EMS-induced alleles of unc-2 were isolated from ~20,000 F1 progeny examined. All alleles showed locomotor defects typical for unc-2 mutants, as described previously (Schafer and Kenyon, 1995; Schafer et al., 1996). However, differences in the mobility of mutant animals were noted. For example, the canonical unc-2 allel, e55, showed a severe movement deficit, as did ra605, ra606, ra609, and ra610. In contrast, the ra612 and ra614 alleles showed moderate to mild defects in movement. To understand the underlying differences between the mutants, the molecular lesions of several unc-2 alleles were determined (see Materials and Methods). The results show that five mutations, e55, ra605, ra606, ra609, and ra610 are all single base substitutions predicted to result in premature stop codons and, hence, truncated proteins (Fig. 2A). The e55 allele is a change of glutamine 111 to a stop in the domain I-II linker, the ra605 allele a change of glutamine 1288 to stop in domain IV S4, ra606 a change of glutamine 1039 to a stop in the domain III-IV loop, ra609 a change of tryptophan 179 to a stop in the IS3 transmembrane domain, and ra610 a change of arginine 1379 to stop in domain IV S5-S6. All of these mutations result in a severe uncoordinated phenotype, and together with their identification as premature stop codons in highly conserved structural regions of the channel are therefore likely to represent the unc-2 null phenotype (see below).

Two alleles, ra612 and ra614, were found to be missense mutations. The ra612 mutation altered glycine 1477 to an arginine in the C terminus in a region flanked by a conserved EF-hand and a downstream-IQ domain (Fig. 2B). The ra614 allele was found to possess an A to G mutation that alters a conserved tyrosine 1255 to a cysteine in the domain IV-S4/IV-S5 linker. Interestingly, the residues altered in both mutants are conserved in all vertebrate high voltage-activated Ca channels cloned to date including all splice variants of α1a/Ca,2.1 and α1b/Ca,2.2 (Stea et al., 1995b).

Phenotypic analysis of unc-2 alleles

In an attempt to further correlate the various unc-2 molecular lesions with locomotor activity, several phenotypic analyses were performed. Initially, e55, ra605, ra609, and ra612 were examined for their sensitivities to aldicarb and nicotine. unc-2 mutants have previously been shown to be resistant to the effects of the AChE inhibitor aldicarb, implicating the UNC-2 protein in cholinergic transmission (Miller et al., 1996). Within 1 h of being placed on NGM plates containing 0.5 mM aldicarb, wild-type animals became paralyzed, and the body wall muscles hypercontracted, causing eggs to be extruded from the uterus. In contrast, animals for the four unc-2 alleles tested were not noticeably affected after 1 h of exposure (Fig. 3A). After 1 week of aldicarb exposure, the wild-type animals had died without successfully reproducing. Again, in contrast to wild-type animals, the four unc-2 strains examined were hypercontracted by this point, but the original animals plated remained alive, and living progeny were also present. Differences among the four unc-2 alleles tested became apparent after 3 weeks of aldicarb exposure. Whereas all four strains produced some viable progeny, there were ~33–50% more animals on the e55, ra605, and ra609 plates than on the ra612 plates (Fig. 3B).

The addition of the acetylcholine agonist nicotine to C. elegans stimulates postsynaptic acetylcholine receptors causing contraction of the body wall muscles. Treatment of wild-type and the four unc-2 strains with nicotine caused both wild-type and mutant animals to hypercontract (data not shown). Because nicotine acts postsynaptically, these results suggest that mutations in unc-2 do not directly affect the postsynaptic transmission machinery. Taken together with the effects of aldicarb, the data also suggest that mutations in unc-2 most likely reduce ACh release at the neuromuscular junction, consistent with a role for UNC-2 as a presynaptic Ca channel that contributes to triggering neurotransmitter release.

For a more quantitative assessment of the neurotransmission defects in unc-2, we focused on the behaviors of locomotion and defecation. These behaviors are chiefly mediated by two different neurotransmitters, ACh and GABA (for review, see Rand and Nonet, 1997). As previously noted, unc-2 mutants are slow moving and resistant to the AChE inhibitor aldicarb, suggesting that cholinergic transmission is impaired (Miller et al., 1996). Using a thrashing assay in liquid media, we quantified the extent of the impairment in various unc-2 alleles. Thrashing was significantly reduced in e55, ra605, ra609, and ra612 mutants compared with wild-type animals. Figure 3C shows that thrashing of e55, ra605, and ra609 mutants was reduced to <3% of wild-type rates measured under similar conditions. Thrashing in ra612 mutants was reduced to 6.5% of thrashing rates compared with wild-type, a value that is significantly different from both wild-type animals and the unc-2 mutants examined (Fig. 3C).

The results of the thrashing assay provide further support for a defect in cholinergic transmission in unc-2 animals. To assess the effects of mutations in unc-2 on the release of another neu-
transmitter, we examined the expulsion step of the defecation process [enteric muscle contraction (EMC)]. EMC is mediated by GABA, and failure of EMC is indicative of a defect in GABA neurotransmission (McIntire et al., 1993a,b). As shown in Figure 3D, the expulsion failure rates of the unc-2 mutants were significantly different from that of wild-type. Wild-type animals failed to defecate in <2% of cycles, whereas ra612 mutants had an ~44% failure rate, and e55, ra605, and ra609 animals failed ~70% of the time. Interestingly, the failure rates of the latter unc-2 mutants were similar to those of unc-25(e265) animals, which are completely defective in GABA biosynthesis (Jin et al., 1999). Overall, the data are consistent with the notion that UNC-2 functions presynaptically to affect the release of the neurotransmitters ACh and GABA.

The unc-36 gene encodes a homolog of the mammalian Ca channel α1B subunit, and genetic analyses suggest that unc-2 and unc-36 may function as part of the same protein complex in vivo (Schafer and Kenyon, 1995; Schafer et al., 1996). A prediction of this hypothesis is that unc-36(null); unc-2(null) double mutants should have no worse a phenotype than either single mutant alone. In addition, it would be expected that unc-36; unc-2(ra612) double mutants should be more severe than unc-2(ra612) single mutants, but no worse than either unc-36 or unc-2 null mutants. To test this notion we constructed unc-36(e251); unc-2(ra605), and unc-36(e251); unc-2(ra612) double mutants. In all cases, the double mutants were indistinguishable from either the unc-36(e251) or unc-2(ra605) single mutants in their thrashing and defecation behaviors (Fig. 3CD). Taken together, the data are consistent with the hypothesis that UNC-36 and UNC-2 function as part of the same Ca channel complex in vivo.

unc-2 gene expression pattern

The results of the behavioral studies suggested that UNC-2 functions primarily in the nervous system and plays a prominent role in synaptic release. To confirm this hypothesis, we constructed an unc-2::GFP reporter construct to determine the expression pattern of the unc-2 gene in transformed animals. GFP fluorescence was found to be primarily localized within the nervous system (Fig. 4). Expression was first observed late in embryogenesis at ~450 min of development when most neurons have been generated, and continued throughout development to the adult stage. Of particular note, in contrast to previously reported in situ hybridization results (Schafer and Kenyon, 1995), GFP expression was observed only a subset of pharyngeal muscle cells but not in other types of muscle tissues. The unc-2 reporter gene was expressed in most motor neurons in the ventral nerve cord, nerve ring motoneurons—interneurons, and the touch cells (Fig. 4A). Postembryonic cell migration of the touch cell AVM and its sister, the interneuron SDQR, are often perturbed in unc-2 mutant animals (Tam et al., 2000). Examination of the unc-2::GFP transgenic animals showed expression of the reporter gene in these two cells (Fig. 4B), suggesting that UNC-2 activity is required within AVM and SDQR to control their migration. A number of neurons in the head and tail regions were also observed to express the unc-2::GFP reporter gene. Of particular note, neurons identified in the head included the olfactory sensory cells AWC (Fig. 4C). UNC-2 has been shown to function in a Ca-dependent pathway that controls the asymmetric expression of the str-2 olfactory receptor gene be-
expression of a single domain truncated product that could lead to the formation of functional voltage-gated channels with a structure similar to that for certain types of single domain voltage-gated potassium channels. To test this possibility, the corresponding e55 mutation (R477stop) was introduced into the rat brain α1A/Ca,2.1 (P/Q-type) cDNA and transfected into HEKtsa201 cells (together with rat brain β1b and α2δ subunits). As shown in Figure 5A, cells transfected with the plasmid did not result in detectable currents under conditions identical to that for cells transfected with wild-type rat brain α1A/Ca,2.1 cDNA. These results, together with the genetic analysis of e55, suggest that the nonsense mutation fails to produce functional voltage-gated Ca channels.

Of the three missense mutations examined, the ra612 allele with a lesion in the conserved C terminal domain, appeared to be the most interesting to pursue regarding functional analyses. The glycine residue at position 1477 is conserved among all high voltage-activated Ca channels and is located within a region flanked by an upstream Ca-binding EF-hand motif and a downstream IQ calmodulin binding motif. To test the effect of the G1477R mutation on channel physiological properties, we introduced the corresponding change (G1817R) into the α1A/P-Q-type channel and expressed the resulting construct in HEKtsa201 cells.

Currents recorded from HEK cells expressing wild-type α1A/Ca,2.1 (Fig. 5B) inactivated slowly during a test pulse to 0 mV ($\tau_{\text{inact}} = 200$ msec; $n = 5$). In contrast, α1A-ra612 currents decayed rapidly, with a biexponential time course ($\tau_1 = 14$ msec, $\tau_2 = 50$ msec; $n = 5$). At the end of a 200 msec test pulse, wild-type and mutant currents had decayed to 43 ± 4% ($n = 5$) and 94 ± 1% ($n = 5$) of the peak current, respectively. Comparison of the extent of inactivation measured as the magnitude of residual current as a function of membrane potential showed that the degree of inactivation of α1A-ra612 decreased monotonically with depolarization and reached a plateau at ~20% of the maximum current.

The current–voltage relationship for the α1A-ra612 channel was shifted ~10 mV more positive than that of the wild-type channel (Fig. 5C). In 5 mM Ba, currents through the wild-type α1A/Ca,2.1 channels first activated at approximately ~30 mV and peaked at approximately ~10 mV. In contrast, α1A-ra612 channels activated and peaked after depolarizations to ~20 and 0 mV, respectively. No significant differences were detected in the voltage dependence of activation ($K_v\alpha_{1A} = 3.97 ± 1.1$; $n = 5$; $K_v\alpha_{1A-ra612} = 4.03 ± 1.2$; $n = 13$).

Comparison of the steady-state inactivation properties of the wild-type α1A/Ca,2.1 and α1A-ra612 mutant channels demonstrated significant differences in the potential at which half of the current was inactivated ($V_{0.5\text{inact}}$) (Fig. 5D). Compared with α1A/Ca,2.1, the voltage dependence of inactivation of α1A-ra612 was shifted by ~20 mV to more hyperpolarized potentials ($V_{0.5\text{inact}}$ for: $\alpha_{1A}/Ca,2.1 = -52.5 ± 0.2$ mV; $n = 4$; $\alpha_{1A-ra612} = -73.6 ± 0.4$ mV; $n = 6$). Figure 5E shows that the current densities for the wild-type mutant ra612 mutant ra612 mutant channels were comparable using either Ba or Ca as the charge carriers ($n = 13$, wild-type in Ca; $n = 11$, mutant in Ca; $n = 8$, wild-type in Ba; $n = 13$, mutant in Ba). The fraction of noninactivating current was not affected by using Ca as the charge carrier, and kinetics of inactivation did not depend on the nature of the permeant ion (Fig. 6), suggesting that the inactivation mechanism produced by the G1447R mutation is unlikely caused by alteration of a typical Ca-dependent process.

**Discussion**

**Structural characterization of unc-2**

The initial description of the unc-2 gene structure and predicted UNC-2 protein were derived from the partial sequence from the nematode genome-sequencing project (Schafer and Kenyon,
The present study extends the initial unc-2 gene characterization and provides a more complete and detailed understanding of the intron/exon boundaries and the UNC-2 protein. The first two introns of unc-2 are quite large, spanning 5.4 and 4.9 kb, respectively. Although introns in C. elegans are mostly ~60 bp in size, a number of genes contain large introns near their 5′ ends. In some cases, these large introns have been shown to contain regulatory elements such as alternative promoters or transcriptional enhancers (Blumenthal and Steward, 1997). There does not appear to be any obvious relationship between the intron/exon structure of the unc-2 gene and the structural domains of the UNC-2 protein. For example, the putative transmembrane regions of domain I are encoded by five exons with no distinct boundaries that define any particular transmembrane region. By comparison, the majority of domain IV is encoded by only two exons. Furthermore, some exons encode portions of adjacent domains, such as with exon 17 that contains the last two transmembrane segments from domain III as well as the first two transmembrane segments of domain IV.

The predicted UNC-2 protein is structurally similar to other HVA Ca channel α1 subunits and is most closely related to the DHP-insensitive α1 subunit classes (N- and P/Q-type channels). Although the overall structure of UNC-2 is well conserved with its mammalian counterparts, the predicted intracellular domain II–III loop is considerably shorter and consists of only 97 residues, as compared with the ~500 residues encoded by the mammalian α1P/Q-type and α1N-type subunits. In this respect, UNC-2 is more similar in structure to the mammalian L-type and the Drosophila Dmca1A α1 subunits. In the mammalian P/Q-type and N-type channel domain II–III linkers, the Synprint site has shown to be required for the physical interaction of SNARE proteins involved in eliciting presynaptic neurotransmitter release (Sheng et al., 1994, 1996; Rettig et al., 1996, 1997). The physical binding of the SNARE protein syntaxin to the Synprint site results in modulation of channel steady-state inactivation properties (Bezprozvanny et al., 1995) and also a negative feedback between Ca channel function and Ca-dependent gene regulation (Sutton et al., 1999). The lack of a readily identifiable Synprint site in UNC-2 suggests that the mechanisms of neurotransmitter release and the control of Ca channel regulation in C. elegans may differ from those in vertebrates.

**UNC-2 contributes to both cholinergic and GABAergic neurotransmission**

The unc-2 mutant phenotype is primarily similar to that of mutants known to be defective in neurotransmission (Rand and Nonet, 1997). Furthermore, unc-2 animals are aldicarb-resistant (Miller et al., 1996), implicating UNC-2 specifically in cholinergic neurotransmission. The wild-type response of unc-2 animals to levamisole (Miller et al., 1996) and nicotine indicates that the aldicarb resistance is not caused by an abnormal response to ACh by the postsynaptic cell. Furthermore, expression of an unc-2 promoter::GFP fusion revealed fluorescence in the nervous system, but not the body wall, uterine, or vulval musculature. These
observations suggest that UNC-2 primarily functions in the nervous system to regulate neurotransmitter release.

In addition to aberrant locomotor behavior, unc-2 mutants are defective in EMC of the defecation motor program (Miller et al., 1996). The AVL and DVB neurons that stimulate the contraction of the enteric muscles are GABAergic. Thus, the unc-2 gene product appears to play a role in the release of GABA as well as ACh and may have a generalized role in neurotransmission in C. elegans.

The observed behavioral, pharmacological, and cellular expression studies suggest that UNC-2 functions in neurons in C. elegans in a manner analogous to that of the presynaptic N- and P/Q-type channels in vertebrate CNS. Null mutations in cha-1 and unc-17, which encode choline acetyltransferase and a synaptic vesicle ACh transporter, respectively, completely abrogate ACh release. These mutants are not viable, arresting at the L1 stage of development (Nonet et al., 1993). In contrast, the unc-2 null phenotype predicted by mutants e55, ra605, ra606, ra609, and ra610 are all significantly milder than that of cha-1 and unc-17, suggesting that even in the absence of unc-2, there is some residual release of ACh from motor neurons. This notion is substantiated by the observation that unc-2 animals become hypercontracted after prolonged exposure to aldicarb and suggests the release of ACh from at least some motor terminals.

Several mechanisms could account for residual neurotransmitter release in unc-2 animals. As shown in Figure 1 B, there are two other predicted Ca channel α1 subunits in C. elegans. One of these, the putative L-type channel encoded by egl-19, is thought to act predominantly in muscle, however it is also expressed in a subset of neurons (Lee et al., 1997), and thus could play a role in neurotransmission at some terminals. There are also two novel four domain Ca channel-like α1 subunits encoded by nca-1 and nca-2 (Fig. 1 B) (T. P. Snutch and K. Hamming, unpublished results), and a T-type-like Ca channel encoded by cca-1 that may play a role in regulating synaptic activity, although no mutations that affect neurotransmission have been mapped to these parts of the genome. The possibility of either overlapping or redundant roles in synaptic transmission could be examined in the future by the analysis of double and triple mutants of these genes. Alternatively, it is possible that the viability of unc-2 null mutants is due, at least in part, to the spontaneous release of ACh from the motor neurons. The presence of spontaneous miniature postsynaptic currents has been demonstrated in the absence of Ca (Richmond and Jorgensen, 1999), and it is possible that even in the absence of UNC-2 protein, sufficient ACh release occurs to maintain viability.

UNC-2 and UNC-36 likely function in the same channel complex

The unc-36 gene encodes a polypeptide that is highly similar to the Ca channel α1β subunits expressed in mammals (Lee et al., 1997). Several observations suggest that UNC-2 and UNC-36 function together in vivo. First, mutations in the unc-36 gene result in movement and egg-laying defects that are similar to those exhibited by unc-2(nll) mutants. Second, unc-2 and unc-36 mutants also exhibit very similar responses to the AChE inhibitor aldicarb (Nguyen et al., 1995) and also similar defects in adaptation to dopamine and serotonin (Schaefer and Kenyon, 1995; Schaefer et al., 1996). Third, mutations in these genes also exhibit similar genetic interactions; unc-36; egl-19 and unc-2; egl-19 double mutants are both essentially paralyzed and are indistinguishable in this respect (Schaefer et al., 1996). Finally, unc-36; unc-2 double mutants are indistinguishable from either single mutant in most behavioral assays, suggesting that these genes function in many of the same pathways (Schaefer et al., 1996).

We also find suggestive evidence that UNC-36 activity is required for the functional expression of the UNC-2 Ca channel complex. For example, the unc-2(ra612) allele reduces, but does not eliminate, unc-2 function. However, unc-36; unc-2(ra612) double mutant phenotype is identical to the unc-2(nll) phenotype. Because the absence of unc-36 (α1β subunit) function has the same effect as the absence of unc-2 (α1 subunit) function, the α1β subunit must be essential for the activity of the complex. This conclusion is consistent with results from a number of surrogate expression systems wherein co-expression of a cloned α1β subunit is necessary for the expression of detectable whole-cell Ca currents.

The ra612 mutation alters channel biophysical properties

The ra612 mutation alters a glycine residue that is located in the C terminal region of the protein, nine residues downstream of a Ca binding EF-hand motif and upstream of a calmodulin binding IQ motif, both implicated in regulating Ca channel function (de-Leon et al., 1995; Lee et al., 1999; DeMaria et al., 2001). All HVA Ca channels cloned to date contain a glycine residue at the homologous position, and it was of interest to determine whether the alteration in this region of the channel affected biophysical properties. Electrophysiological analysis of α1β/Ca2.1 channels harboring the ra612 (G1817R) mutation showed that compared with wild-type that a stronger depolarization is required to open the mutant channels which, once open inactivated more rapidly, thereby terminating Ca influx sooner. In addition, a hyperpolarizing shift in the steady-state inactivation curve indicated a significant reduction in the number of channels available for opening at a given membrane potential.

Although we are cautious about inferring the exact in vivo physiological implications of these results, they are consistent with the phenotypic observations that suggest that the ra612 mutation does not result in the complete elimination of channel function. For example, the apparent decrease in neuronal activity in unc-2(ra612) animals could be attributed to diminished Ca influx through the mutant channels. The increased rate of inactivation together with the changes in current–voltage properties would be predicted to reduce Ca influx resulting in decreased neurotransmitter release.

The mechanism underlying the increased rate of inactivation does not appear to be a direct alteration of Ca- or current-sensitive properties of the channel. Ion substitution experiments...
illustrate that the increase in the rate of inactivation of α1-A rat12 cannot be attributed to Ca-dependent modulation previously reported for L-type or N-type calcium channels (Cox and Dunlap, 1994; Ferreira et al., 1997). Potentially, the rat12 mutation might alter or occlude sites that interact with modulatory proteins that may or may not be Ca-activated. For example, a CaMKII–PKA consensus sequence is located near the altered glycine and phosphorylation of this site, which may serve to activate or facilitate the channel, might be inhibited by the rat12 alteration. This is just one possible model among others that will require additional investigation to determine the underlying mechanisms responsible for the altered biophysical properties of the mutant channel.

References


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