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The Nematode Degenerin UNC-105 Forms Ion Channels that Are Activated by Degeneration- or Hypercontraction-Causing Mutations

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Summary

Nematode degenerins have been implicated in touch sensitivity and other forms of mechanosensation. Certain mutations in several degenerin genes cause the swelling, vacuolation, and death of neurons, and other mutations in the muscle degenerin gene *unc-105* cause hypercontraction. Here, we confirm that *unc-105* encodes an ion channel and show that it is constitutively active when mutated. These mutations disrupt different regions of the channel and have different effects on its gating. The UNC-105 channels are permeable to small monovalent cations but show voltage-dependent block by Ca^{2+} and Mg^{2+}. Amiloride also produces voltage-dependent block, consistent with a single binding site 65% into the electric field. Mammalian cells expressing the mutant channels accumulate membranous whorls and multicompartment vacuoles, hallmarks of degenerin-induced cell death across species.

Introduction

Mechanosensitive channels, which open or close when a mechanical force is applied to them, may constitute the basis for the senses of touch, hearing, and balance. They have also been detected in many nonsensory cells, where they may participate in volume regulation or response to mechanical stress. Despite their wide distribution, there is only one cloned channel known to be primarily activated by mechanical stimuli: the bacterial MscL (Sukharev et al., 1994). This channel senses stretch directly applied through the lipid bilayer, whereas many eukaryotic mechanosensitive channels seem to require attachments to intracellular or extracellular structures that convey tension to them. Moreover, no MscL homologs have been detected in eukaryotes.

The nematode degenerin proteins of *C. elegans* have been implicated in several forms of mechanotransduction: MEC-4 and MEC-10 are necessary for the detection of touch by the six touch receptor neurons in which they are expressed (Chalfie and Sulston, 1981; Driscoll and Chalfie, 1991; Huang and Chalfie, 1994), UNC-8 has been proposed as a proprioceptor of motoneurons needed for proper locomotion (Tavernarakis et al., 1997), and UNC-105 is believed to mediate stretch sensitivity in muscle (Liu et al., 1996). No degenerin genes have been identified in genomes other than those of nematodes, but their sequence is similar to that of several amiloride-sensitive ion channels (Figure 1b). These include the epithelial sodium channels (ENaCs), primarily expressed in vertebrate epithelial tissues (Canessa et al., 1993, 1994; Lingueglia et al., 1993; Waldmann et al., 1995a); the brain sodium channels (BNaCs) and the related DRASIC, expressed in mammalian neurons and activated by extracellular pH (Price et al., 1996; Waldmann et al., 1996, 1997a, 1997b; Bassilana et al., 1997; García-Añoveros et al., 1997; Lingueglia et al., 1997); a FMRFamide-peptide–activated channel from the mollusk *Helix aspersa* (FANaC; Lingueglia et al., 1995); and a newly discovered branch from *Drosophila* of unknown function (Adams et al., 1998). The degenerins are thus thought to be ion channels and are to date the only putative channels cloned from eukaryotes that are strongly implicated in mechanosensation (García-Añoveros and Corey, 1997).

Certain gain-of-function mutations in the degenerin genes *deg-1* (Chalfie and Wolinsky, 1990; García-Añoveros et al., 1995), *mec-4* (Driscoll and Chalfie, 1991), *mec-10* (Huang and Chalfie, 1994), and *unc-8* (Shreffler et al., 1995; Tavernarakis et al., 1997) cause the degeneration of some or all of the neurons in which they are expressed: the cells accumulate whorls (concentric spheres: of membrane) and vacuoles, swell to several times their original diameter, and often die (Hall et al., 1997). Some of these mutations affect a residue in the second hydrophobic region, the predicted pore-forming domain, and others disrupt an extracellular region. It has been proposed that these mutations activate the channels, most likely by interfering with their closing, and that the ensuing constitutive currents damage the cells (García-Añoveros et al., 1995). In fact, a similar mutation in the related mammalian channels BNaC1 (also called BNC1 and mDEG1) and BNaC2 (also called ASIC), and in the *Drosophila Ripped Pocket* (RPK), increases currents and kills cultured cells that express the channel (Waldmann et al., 1996; Adams et al., 1998; Bassilana et al., 1997; A. Jacobs, J. G.-A., D. P. C., and X. O. Breakefield, unpublished data). The degenerin gene *unc-105* is not expressed in neurons but in muscles of *C. elegans*, and the gain-of-function mutations found in this gene cause not muscle degeneration but hypercontraction (Park and Horvitz, 1986b; Liu et al., 1996). These mutations disrupt extracellular residues situated near the two predicted transmembrane domains of the protein, at sites different from the degeneration-causing mutations. It was proposed that constitutive channel activation might also cause the hypercontraction phenotype (Liu et al., 1996).

Most of the residues disrupted by these degeneration- and hypercontraction-causing mutations in degenerin genes are not conserved in the related ion channels that have been studied electrophysiologically (ENaCs, BNaCs, and FANaC). Therefore, these residues are likely to be involved in a form of regulation unique to the

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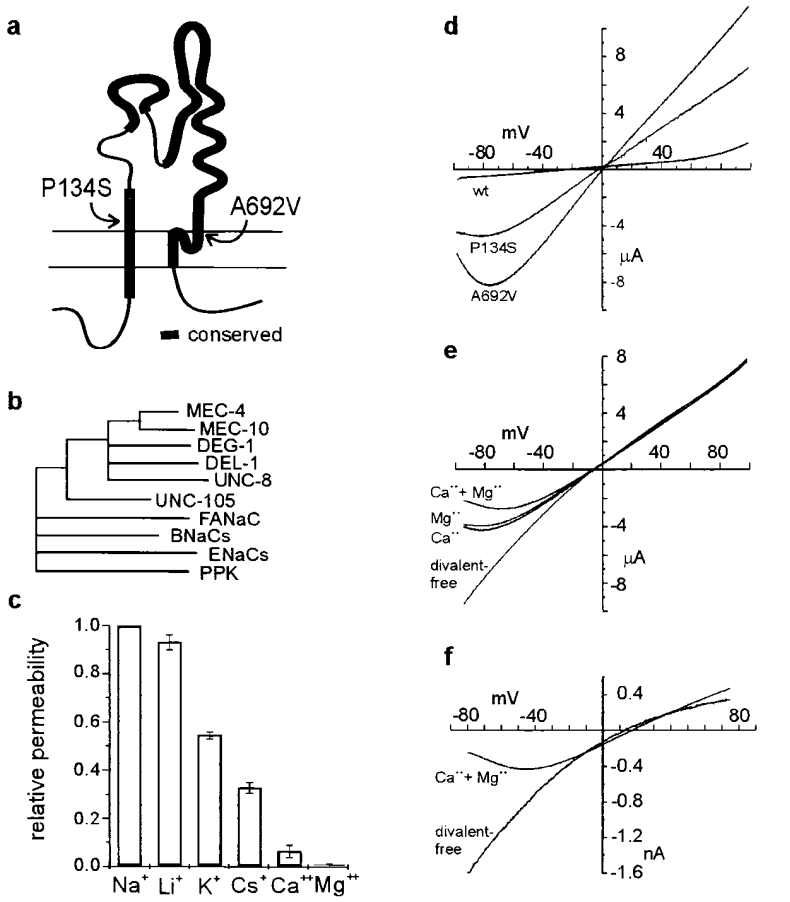

Figure 1. Channels Formed by Mutated UNC-105

(a) Schematic representation of UNC-105 showing the regions conserved with other degenerins and the amino acid substitutions resulting from two gain-of-function mutations.

(b) Phylogenetic tree of the degenerin family and other branches of the DEG/ENaC superfamily, obtained with the PAUP program (Swofford, 1993).

(c) Selectivity of UNC-105(A692V) channels to cations, determined by comparing the reversal potential in an external solution of 116 mM NaCl, 7.5 mM HEPES, and 0.375 mM EDTA with the reversal potentials in similar external solutions of LiCl, KCl, and CsCl, or by comparing the total magnitude of the inward current at −100 mV (for Ca^{2+} and Mg^{2+} with respect to Na^{+}).

(d) Whole-cell currents of oocytes expressing wild-type (wt) or mutant UNC-105 (A692V or P134S). The small currents detected in oocytes expressing UNC-105(wt) were probably just leak, since they are not blocked by amiloride. The external solution was frog saline, which contains 1.8 mM Ca^{2+}.

(e) Block by Ca^{2+} and Mg^{2+} of oocyte whole-cell currents. The bath solutions contained 0.5 mM EDTA (divalent-free), 1.8 mM $CaCl_2$ (Ca^{2+}), 1.8 mM $MgCl_2$ and 0.5 mM EGTA (Mg^{2+}), or 1.8 mM $CaCl_2$ and $MgCl_2$ (Ca^{2+} + Mg^{2+}). Current in the presence of Ca^{2+} was fitted with the equation $I = I_{0Ca}[1/(1 + [Ca]/[K_{D0mV} \exp(V_m \delta 2F/RT)])]$, where I_{0Ca} is the current in the absence of calcium, K_{D0mV} is the half-blocking concentration of Ca^{2+} at 0 mV, δ is the proportion of the field sensed at the binding site, and F, R, and T have their usual thermodynamic meanings. The same analysis was done for current in the presence of Mg^{2+}. The resulting fits superimpose the traces and thus are not shown.

(f) Whole-cell currents of HEK-293 cells expressing UNC-105(A692V), in the absence (divalent-free) or presence (Ca^{2+} + Mg^{2+}) of 2 mM $CaCl_2$ and 1 mM $MgCl_2$ in the bath solution (150 mM NaCl and 10 mM HEPES [pH 7.4]). The pipette solution contained 130 mM CsCl, 10 mM NaCl, 10 mM HEPES, 5 mM EGTA, and 2 mM $MgCl_2$ (pH 7.4). Because Cs^{+} permeates one-third as well as Na^{+} (c), currents are smaller at positive than at negative potentials.

degenerins, i.e., their suspected mechanical gating, and cannot be characterized by studying the related ion channel families. Attempts to form channels by heterologous expression of other degenerins have failed, perhaps because an essential component of the channel, or an essential modification, is missing in these heterologous systems. In fact, the products of *mec-4* and *mec-10* (Huang and Chalfie, 1994), and of *unc-8* and *del-1* (Tavernarakis et al., 1997), are thought to form heteromultimers. In addition, the degenerations caused by most degenerin genes (*deg-1*, *mec-4*, *mec-10*, and *unc-8*) require another gene, *mec-6*, suggesting that its product MEC-6 is needed to form an active channel (Chalfie and Wolinsky, 1990; García-Añoveros et al., 1995; Shreffler et al., 1995). Additional genetic and molecular analyses further suggest that degenerins are part of a macromolecular complex with cytoskeletal and extracellular matrix attachments that would be necessary for opening the channel (García-Añoveros et al., 1995; García-Añoveros and Corey, 1996). Thus, even if the channel was formed it might be closed and remain undetected.

In order to study gating and permeation of a putative mechanosensitive ion channel, we have expressed the *unc-105* gene from *C. elegans* in two heterologous expression systems: *Xenopus* oocytes and human embryonic kidney cells (HEK-293). A phylogeny analysis had indicated that UNC-105 was closer than others to a primordial degenerin, suggesting that it may function as a homomultimer (Corey and García-Añoveros, 1996; Figure 1b). In addition, an extensive genetic study did not show a need for *mec-6* in the function of *unc-105* (Park and Horvitz, 1986a, 1986b). To activate these channels, we introduced gain-of-function mutations predicted to cause constitutive activation. One of these (P134S) was originally identified in *unc-105* as causing muscle hypercontraction (Park and Horvitz, 1986a; Liu et al., 1996). The other mutation (A692V) was originally identified in *deg-1* and *mec-4* as causing neuronal degeneration (Chalfie and Wolinsky, 1990; Driscoll and Chalfie, 1991; García-Añoveros et al., 1995; Shreffler et al., 1995). The mutated UNC-105 did produce constitutive currents, allowing us to characterize the channel properties of degenerins, and did cause degeneration

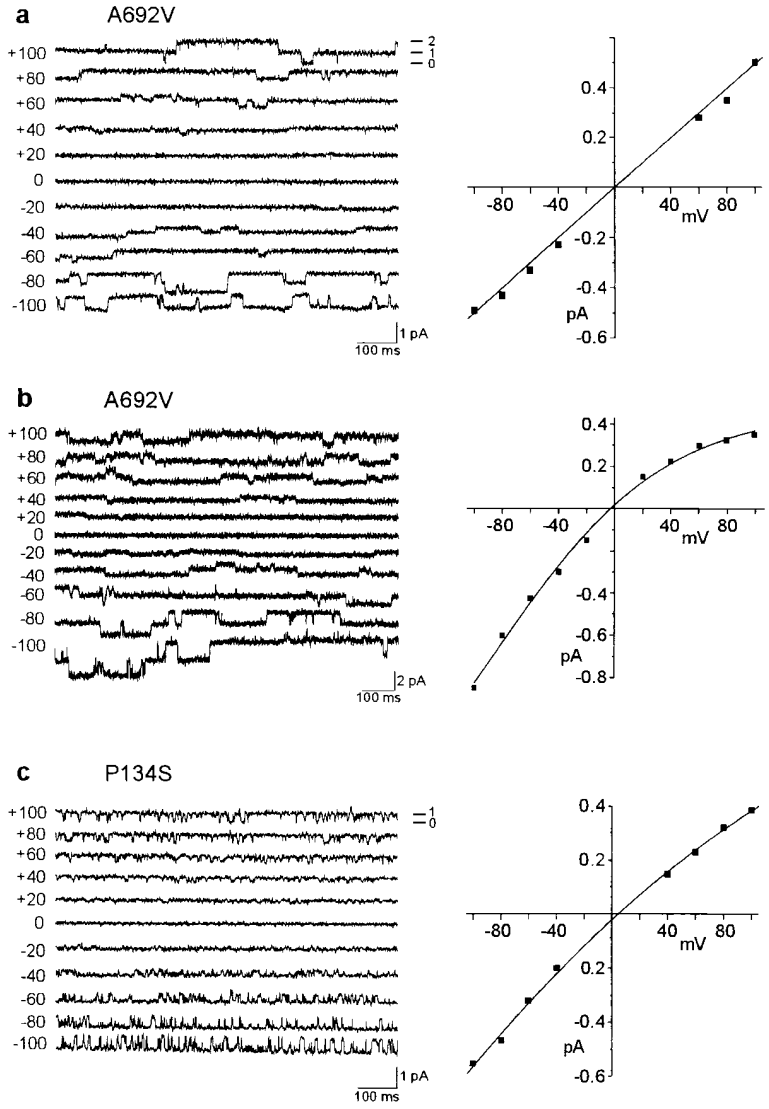

Figure 2. Single Channel Records of Mutated UNC-105

Inside-out (a, left) and cell attached (b and c, left) single channel recordings and corresponding current–voltage relationship (right) of UNC-105(A692V) (a and b) or UNC-105(P134S) (c) expressed in *Xenopus* oocytes. Pipette and bath solutions were frog saline with no divalent cations.

of expressing oocyte and HEK cells. The results provide an explanation for both the degeneration and the hypercontraction phenotypes.

Results

UNC-105 Mutants Form Hyperactive Channels

The mechanically gated channels of vertebrate hair cells are thought to have an open probability of $<0.1\%$ in the absence of applied tension (Assad and Corey, 1992), suggesting that a channel expressed without mechanical connection might normally be closed. Expression of wild-type UNC-105 in cultured HEK-293 cells or in *Xenopus* oocytes resulted in no detectable currents above background, as expected for channels that must be properly activated (i.e., by mechanical stimuli) in order to open. To detect currents near background level, we applied up to 1 mM amiloride. Amiloride blocks the related ENaC, FANaC, and BNaC channels but caused no decrease in conductance in either oocytes or HEK cells expressing wild-type UNC-105. However, constitutive currents were observed in HEK cells and oocytes expressing UNC-105(A692V) or UNC-105(P134S) (Figures 1d and 1f). The fact that UNC-105 formed channels in two different heterologous cell types without cotransfection of other potential subunits suggests that UNC-105 channels can assemble as homomultimers.

Conductances were only slightly selective for Na^+ and Li^+ over K^+ and Cs^+ (1.0 : 0.93 $\pm$ 0.03 : 0.54 $\pm$ 0.02 : 0.32 $\pm$ 0.02; Figure 1c). By contrast, all nondegenerin ion channels of the DEG/ENaC superfamily that have been analyzed are several times more selective for Na^+ and Li^+ over K^+ (Canessa et al., 1993, 1994; Voilley et al., 1994; Ismailov et al., 1995; Lingueglia et al., 1995; Waldmann et al., 1995a, 1997a, 1997b; Price et al., 1996). UNC-105 channels are not significantly permeable to Ca^{2+} and Mg^{2+} (Figure 1c). The fraction of the current carried by Ca^{2+} and Mg^{2+} at physiological concentrations would be several hundred-fold lower than that of Na^+. Instead of permeating, both divalent cations

blocked these channels in a voltage-dependent manner (Figures 1e and 1f). At physiological concentrations (1.8 mM Ca^{2+} in frog saline), the block was only partial, but increased with hyperpolarization. A simple fit to the data (not shown; see legend of Figure 1e) suggests that Ca^{2+} and Mg^{2+}, both of which block with a K_D of 25 mM at 0 mV, bind to the same site within the pore, and that this site senses 40% of the membrane field.

To identify the individual channel currents that form the basis of these constitutive whole-cell currents, we obtained cell-attached and inside-out patch recordings (Figure 2). Membrane patches from oocytes injected with either UNC-105(A692V) (n = 8) or UNC-105(P134S) (n = 4) cRNA, but not from water- or noninjected control oocytes (n = 10), contained channels characterized by a single channel conductance of 4.6 ± 1.6 pS (measured at hyperpolarized potentials). The current–voltage relation for channels with either mutation was approximately linear in divalent-free medium on inside-out patches. However, on cell-attached patches the outward currents were smaller than the inward currents, since the main cation of the cytosol, K^+, permeates half as well as Na^+ (Figure 1c).

Cells injected with UNC-105(A692V) cRNA also expressed channels with different conductances. Individual channels appeared to have single, fixed conductances, that ranged from 2–30 pS, but all had similar kinetics (n = 15). Examples of channels with 5 and 16 pS conductances are shown in Figures 2a and 2b. Because kinetics were similar, because amiloride blocked 80%–99% of the whole-cell current of oocytes expressing UNC-105(A692V), and because these constitutively active channels were never detected in control oocytes, these channels are likely to be alternate conductance forms of UNC-105, rather than endogenous channels activated or unmasked by the sequelae of this constitutive current.

Both the UNC-105(A692V) and the UNC-105(P134S) channels were open most of the time (P_{open} = 0.50–0.99). However, their gating kinetics differed: UNC-105(A692V) channels had very long open and closed times of tens to hundreds of milliseconds (τ_o = 164 ± 11 ms, τ_c = 20 ± 3 ms; Figure 2), whereas UNC-105(P134S) channels opened and closed within a few milliseconds (τ_o = 10.5 ± 0.4 ms, τ_c = 2 ± 0.08 ms; Figure 2). Because both channels fluctuated constantly between open and closed states, we consider them to be constitutively active, rather than constitutively open. Thus, constitutive activity of the mutated UNC-105 channels forms the basis for the currents observed in whole cells. When tested physiologically with the patch clamp in the voltage-clamp mode, injected cells were found to be depolarized, with a resting potential near 0 mV, as expected from their large nonselective conductance.

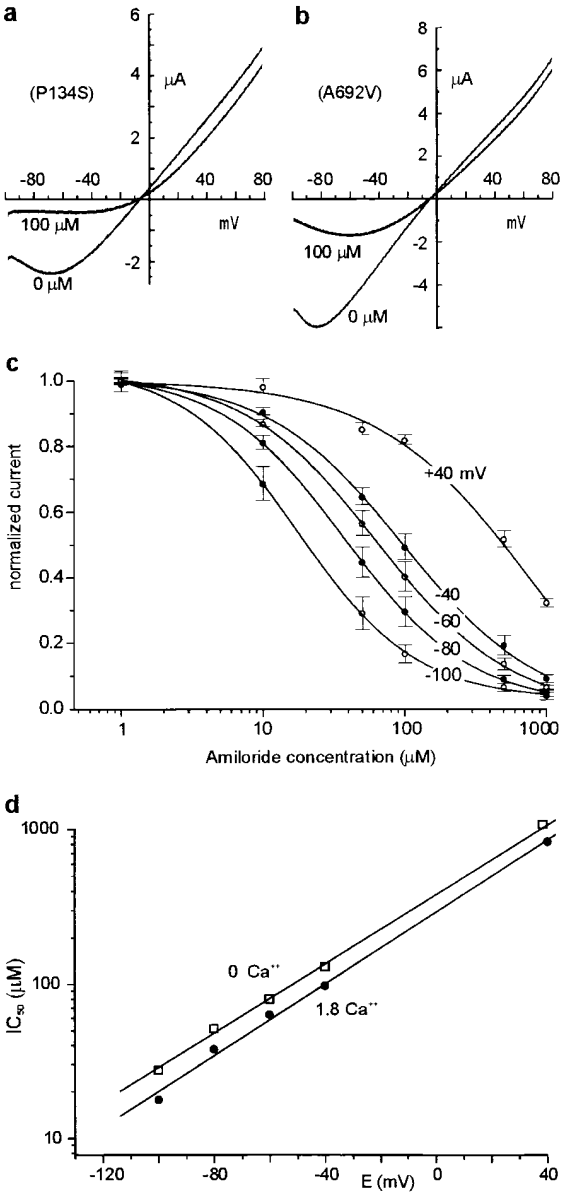

Figure 3. Amiloride Block of UNC-105 Channels

(a) Current–voltage relations of *Xenopus* oocytes expressing UNC-105(P134S) in the presence or absence of 100 μM amiloride.

(b) Oocytes expressing UNC-105(A692V).

(c) Dose-response curves at various membrane potentials (mean current ± SEM) for oocytes expressing UNC-105(A692V).

(d) Voltage dependence of half-blocking concentrations for oocytes expressing UNC-105(A692V). Amiloride blocks slightly better in the presence of 1.8 mM Ca^{2+} than in its total absence. In both cases, the electrical distance of the amiloride site in the pore (δ), calculated by fitting the data to the equation $IC_{50}(E) = IC_{50}(0\ mV) \times e^{z\delta EF/RT}$, was similar (0.65 and 0.68, respectively).

Amiloride Binds Deeply into the Pore and Blocks the Channel

Amiloride blocked UNC-105 channels in a voltage-dependent manner: at −60 mV the IC_{50} is 80 ± 10 μM for UNC-105(A692V) (n = 7; Figures 3c and 3d). All other branches of this superfamily of ion channels have slightly or significantly higher affinity for amiloride at the same voltage (IC_{50} = 0.1–60 μM at −70 or −60 mV; Canessa et al., 1993, 1994; Lingueglia et al., 1993; Voilley et al., 1994; Price et al., 1996; Waldmann et al., 1996, 1997a, 1997b; Lingueglia et al., 1997). The voltage sensitivity of the block by amiloride, a monovalent cation, is less pronounced than the voltage sensitivity of the block

by divalent cations, as expected for blockers that are driven into the pore by the electric field (Figures 1e, 1f, and 3). We determined dose-response relations of amiloride block both in the presence of Ca^{2+}, and, to avoid any potential competition among blockers, in the absence of Ca^{2+} and Mg^{2+}. Hill coefficients were calculated at the various voltages at which an entire dose-response curve could be recreated (i.e., −100, −80, and −60 mV). In all cases, the Hill coefficient was close to 1 (0.92–1.05 for UNC-105[A692V] in the presence of Ca^{2+} and Mg^{2+}, 0.88–1.02 for UNC-105[A692V] in the absence of Ca^{2+} and Mg^{2+}, and 0.89–1.2 for UNC-105[P134S] in the absence of Ca^{2+} and Mg^{2+}), consistent with one binding site per channel (Figure 3c).

Fitting the IC_{50} at different voltages using a Woodhull (1973) model of voltage-dependent block, we calculate that amiloride penetrates two-thirds of the way into the electric field of the UNC-105 pore ($\delta = 0.65$ and 0.68; Figure 3d). Amiloride block of the ENaC is also thought to occur by binding into the pore. However, the affinity of ENaC for amiloride decreases 100-fold when six extracellular residues are deleted (Ismailov et al., 1997b). These six extracellular residues are absent from the degenerins, and this may account for their low sensitivity to amiloride.

Ca^{2+} did not reduce the effectiveness of the amiloride block, as would be expected if both blockers competed for the same site. In fact, Ca^{2+}, which only penetrates 40% of the electrical distance of the pore, induced a small and voltage-independent enhancement of the amiloride block (Figure 3d). Perhaps Ca^{2+} momentarily traps amiloride in its blocking site, augmenting its blocking effect. The amiloride site is considerably deeper in the pore of UNC-105 than in that of ENaC ($\delta = 0.13$–0.15; Palmer, 1985; McNicholas and Canessa, 1997). Both the depth and the different ionic selectivities described above suggest a structural difference between the pores of ENaCs and degenerins. However, in both cases the data are consistent with one charged (i.e., protonated) molecule of amiloride binding in the pore of the channel to block it.

The UNC-105(P134S) channels were about 5-fold more sensitive to amiloride (IC_{50} at −80 mV = 7.7 ± 1.0 μM, n = 5) than the UNC-105(A692V) channels (IC_{50} at −80 mV = 52 ± 3 μM, n = 11). A mutation in BNaC1 equivalent to A692V apparently decreases the sensitivity of the channel to amiloride (compare the results of Waldmann et al., 1996, with those of Price et al., 1996). Therefore, one of the two *unc-105* mutations, probably A692V, affects amiloride's access to or stability within the pore. Mutations near the equivalent residue of the ENaCs also alter its sensitivity to amiloride (Schild et al., 1997). Indeed, residue 692 is at or near the predicted pore-forming domain of the UNC-105 protein (García-Añoveros et al., 1995; Waldmann et al., 1995b; Schild et al., 1997).

Hypercontraction- and Degeneration-Causing Mutant Channels Cause Nonapoptotic, Vacuolated Degeneration of Cultured Cells

Is the open channel phenotype of mutated UNC-105 sufficient to explain degenerations induced by the A692V mutation in other degenerins? We expressed both mutant forms of UNC-105 in HEK cells along with an in vivo marker, either green fluorescent protein (GFP) or CD8. GFP was detected by fluorescence microscopy; CD8 was detected by binding of beads coated with anti-CD8 antibodies. Expression of either mutant channel resulted in the death of those cells expressing the channel. Dying HEK-293 cells did not have the hallmarks of appoptotic cell death, such as shrunken nuclei, clumped chromatin (as determined with the nuclear dye H 33258), or fragmented DNA (as determined by the TUNEL assay; Gavrieli et al., 1992; data not shown, but see the Experimental Procedures). Instead, transfected cells appeared round, having lost most or all of their extensions and attachments to the substrate. Like the degenerating neurons described in the nematode (Hall et al., 1997), they contained vacuoles. However, these cells did not appear to be swollen (Figure 4).

Transmission electron microscopy confirmed the presence of vacuoles and revealed additional pathologic features (Figure 5). Vacuoles were very large and contained smaller vacuoles inside (Figures 5E and 5D). The walls of these vacuoles were considerably thicker than a single lipid bilayer, as if formed by several layers of membrane. Often these vacuoles occupied most of the cytoplasmic space and pushed the nucleus aside, deforming it (Figures 5C and 5E). In 5 out of 11 dying cells, we detected 1–3 whorls, comprised of 6–60 concentric layers of membrane, and in one case we noticed that the membranes of one large whorl connected to the membrane of adjacent vacuoles (Figures 5E–5G). Because we observed only one section per degenerating cell, the fraction of degenerating cells with whorls may be larger than half. Interestingly, similar whorls have also been described with transmission electron microscopy in nematode neurons at the early stages of degeneration (Hall et al., 1997). Among 18 control cells transfected solely with the CD8-encoding marker plasmid, we detected only one small whorl. Some degenerating cells also had electron-lucent cytoplasm and abnormal mitochondria. These mitochondria, normally elongated with clearly defined cristae (Figure 5B), were uniformly spherical with darkened and condensed matrix distributed against the outer membrane, leaving an electron-lucent center (Figures 5H–5J). Mildly abnormal mitochondria have been observed in late stages of degeneration in nematode neurons (Hall et al., 1997) but not to the extent observed here. Perhaps severely abnormal mitochondria correspond to even later stages of degeneration than observed in the nematode. Alternatively, the different mitochondrial phenotype may reflect a difference between nematode neurons and cultured human kidney cells. Otherwise, degenerating HEK cells expressing mutant UNC-105 channels appeared ultrastructurally similar to degenerating nematode neurons expressing other mutant degenerins.

Discussion

UNC-105 May Form Homomultimeric Channels

Channels of the DEG/ENaC superfamilly appear to function as multimers. The ENaC branch comprises homologous α, β, and γ subunits, although the stoichiometry

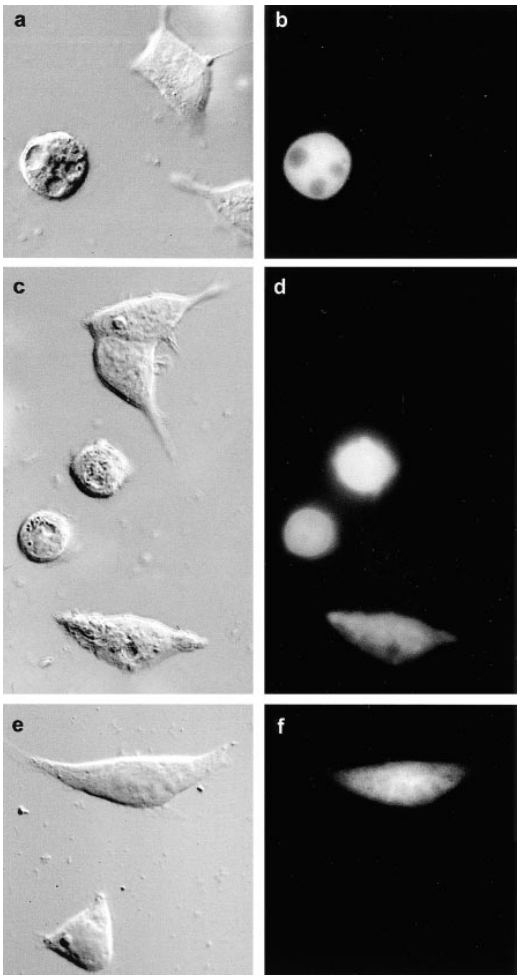

Figure 4. Morphology of Degenerating HEK Cells

(a–d) Cultured HEK-293 cells expressing UNC-105(P134S).
(e and f) Control cells expressing wild-type UNC-105.
Transfected cells are identified with GFP labeling (b, d, and f) and observed under differential interference contrast (DIC) (a, c, and e). Untransfected cells or cells expressing UNC-105 appear normal, whereas cells expressing UNC-105(P134S) often appear rounded, contain vacuoles, and have a rough surface. One of the transfected cells (c and d) is not yet round but already contains a small vacuole and a ragged surface. The same morphology is attained by cells expressing UNC-105(A692V).

is undetermined (Canessa et al., 1994). *trans*-dominant suppression of a dominant degeneration-causing mutation of *deg-1* by another *deg-1* allele suggested that several *deg-1* products assembled in one macromolecular structure (García-Añoveros et al., 1995; Shreffler et al., 1995). Similar observations were made for *mec-4* and *mec-10* (Hong and Driscoll, 1994; Huang and Chalfie, 1994). In some cases, genetic evidence and coexpression patterns suggest that several degenerins assemble into heteromultimers (MEC-4 and MEC-10, Huang and Chalfie, 1994; UNC-8 and DEL-1, Tavernarakis et al., 1997). In contrast, the degenerin UNC-105 appears able to form channels without other exogenous proteins. It is possible but unlikely that UNC-105 forms heteromultimers with endogenous proteins in oocytes and HEK cells; thus, the UNC-105 channel studied here is most likely a multimer of a single protein isoform (although the native channel in the nematode could be a heteromultimer). Degenerins other than UNC-105 require MEC-6 for function. Perhaps UNC-105, which phylogenetically appears to be the least evolved degenerin, is more like the primordial degenerin, which probably formed homomultimeric channels. In any case, the molecular simplicity of the UNC-105 channel makes it an ideal choice for the study of degenerins in general, since this protein still contains all of the suspected regulatory regions common to the degenerins.

An Electrochemical Explanation for the Hypercontraction and Degeneration Phenotypes

UNC-105 channels can be constitutively activated by certain gain-of-function mutations. One of these (P134S) causes muscular hypercontraction in the nematode. Oocytes expressing the mutant channel have depolarized resting potentials of $\sim$0 mV. Such constant and large depolarization, when in muscle, could lead to hypercontraction via an increase in Ca^{2+} influx through other voltage-activated conductances.

The other mutation (A692V) was originally identified in *deg-1* and *mec-4* as the cause of neuronal degeneration, a form of nonapoptotic cell death characterized both by the accumulation of membrane whorls and vacuoles and by the swelling of the whole cell. In cultured cells, both the hypercontracting (P134S) and the degenerating (A692V) mutant UNC-105 channels caused the accumulation of whorls and vacuoles and nonapoptotic cell death. However, these cells did not appear to swell, perhaps revealing a difference in volume regulatory abilities between nematode neurons and mammalian-cultured epithelial cells. The mitochondria in the degenerating nematode neurons rarely appeared damaged, but were severely deformed in dying HEK cells, reflecting another difference in the way these two cell types respond to a qualitatively similar insult. However, the anatomy of degenerating neurons in the intact nematode and cultured HEK cells is very similar and may be considered a signature of degenerin-induced cell death.

Mutation of a pore residue in a *C. elegans* acetylcholine receptor causes cell death with a morphology that is indistinguishable (by light microscopy) from degenerin-induced cell death. This cell death is probably also caused by hyperactive channels, since cholinergic antagonists prevent it (Treinin and Chalfie, 1995). In addition, degenerin-induced cell death has morphological similarities to that of dying, vacuolated neurons and glia of the *weaver* mouse (Rakic and Sidman, 1973a, 1973b), in which a mutation disrupts the pore of an inward rectifying K^+ channel (Patil et al., 1995) and makes it nonselective with respect to K^+ and Na^+ (Kofugi et al., 1996; Navarro et al., 1996; Slesinger et al., 1996). Thus, constitutive Na^+ currents appear to cause a morphologically distinct form of cell death.

What is the cause of cell death? The small influx of Ca^{2+} through the mutant UNC-105 channels is unlikely to cause degeneration on its own, because the similarly mutated BNaC1 channel is impermeable to Ca^{2+} and

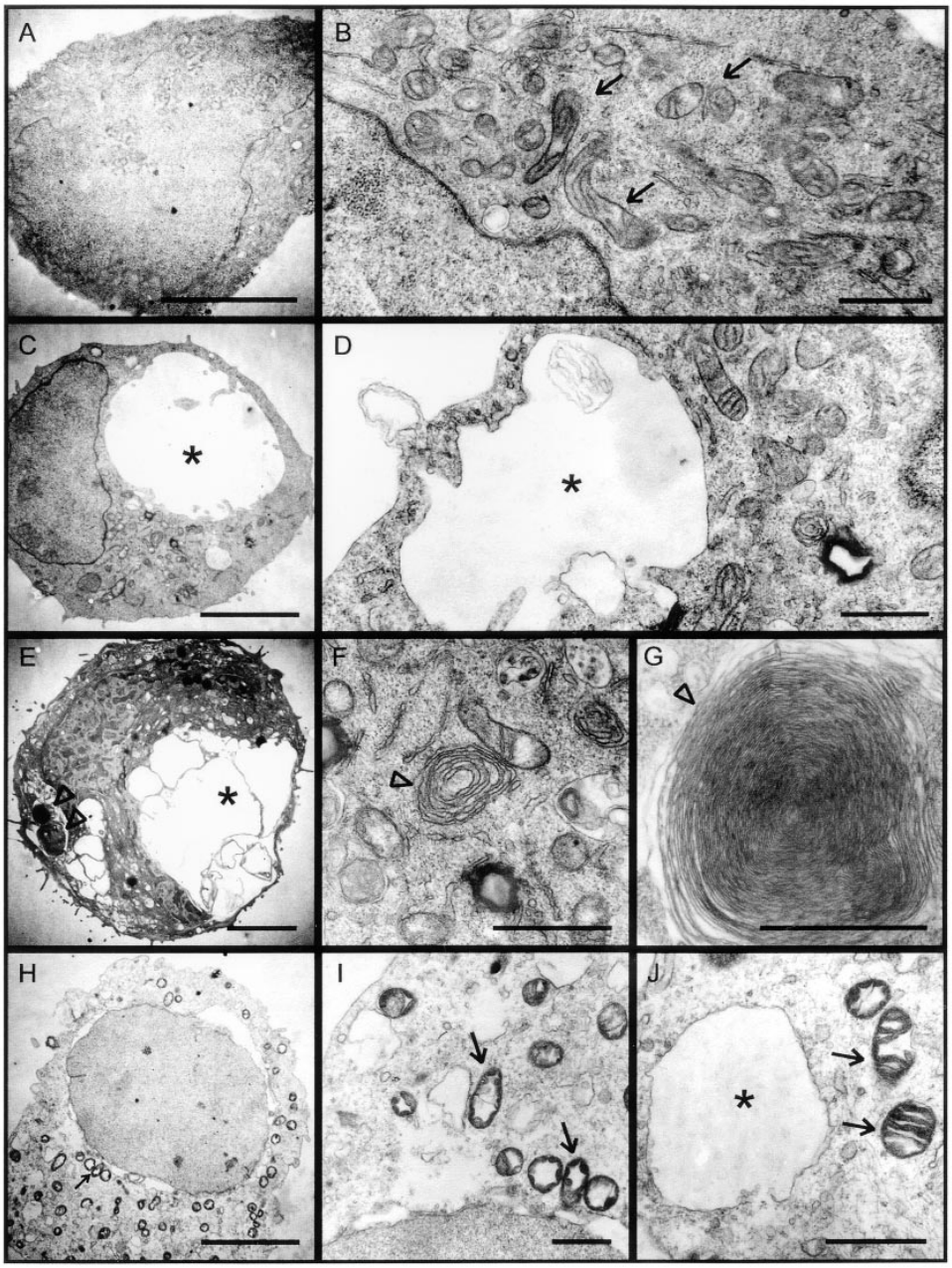

Figure 5. Ultrastructure of Degenerating HEK-293 Cultured Cells Expressing UNC-105(A692V)

(A and B) Control cells that do not express UNC-105(A692V) have no vacuoles, and their mitochondria appear normal.

(C–J) Degenerating cells, which express UNC-105(A692V), often contain large vacuoles that displace the nucleus (C) or even squeeze it (E). Vacuolated cells may also contain whorls of various sizes (E–G). The mitochondria and cytosol of cells with vacuoles and whorls appear normal (D and F). In contrast, other degenerating cells contain smaller vacuoles, but their cytoplasm is clear and their mitochondria are damaged (H–J). Mitochondria are indicated by arrows, whorls by open arrowheads, and vacuoles by asterisks. Scale bars, 5 μm (A, C, E, and H) or 1 μm (B, D, F, G, I, and J).

still causes degeneration (Waldmann et al., 1996). The Na^+ influx may be lethal by altering the osmotic balance of the cell or by exhausting the cell by overloading its Na^+/K^+ ATPases. Alternatively, a reduction of the Na^+ gradient would diminish the activity of the Na^+/H^+ exchanger (a mutation of which causes neuronal death in mice; Cox et al., 1997) or of the Na^+/Ca^{2+} exchanger (leading to an accumulation of Ca^{2+}, which is toxic to cells; Limbrick et al., 1995).

All of these potential mechanisms of degeneration predict that blockade of the channel would prevent cell death. However, attempts to prevent degenerations by blocking the UNC-105 channels with amiloride were unsuccessful. The IC_{50} of amiloride for these channels is

high and gets much higher with depolarization because of the voltage sensitivity. For instance, if a cell is depolarized by Na^+ influx to −20 mV, it would require a lethal dose of more than 700 μM amiloride to block 95% of the current.

It is unclear why any of the aforementioned phenomena would lead to the formation of whorls and vacuoles. One possible explanation given the morphology of dying cells is that, in an attempt to reduce leak, the cell internalizes plasma membranes, and numerous channels are incorporated into intracellular whorls and vacuoles. Once the constitutively active channels are in the intracellular compartments, cationic influx may swell them, perhaps turning whorls into vacuoles. Indeed, the vacuole walls were in some cases many times thicker than a single bilayer.

The damaged mitochondria observed in a few apparently terminally degenerating cells with electron-lucent cytoplasms may be due to the dilution of the cytosol, which would in turn dilute and swell the intermembrane space of the mitochondria (since the outer mitochondrial membrane is considerably more water-permeable than the inner mitochondrial membrane) and compress the mitochondrial matrix. Mitochondria might also be damaged by the excessive demand for ATP needed to pump Na^+ from the cytosol.

Several Mechanosensitive Channels Are Unrelated to the Degenerins

It seems likely that MEC-4 and MEC-10 form mechanically gated channels in nematode touch receptor neurons, and, by extension, degenerins in other nematode tissues may be mechanosensitive. Like the degenerin channels, some vertebrate mechanosensitive channels are also blocked by amiloride, notably the mechanotransducer of hair cells (with an IC_{50} = 50 μM, quite similar to that of UNC-105 channels) and the stretch-activated channel of *Xenopus* oocytes (IC_{50} = 500 μM). These and other considerations have led to suggestions that these channels may also be degenerins or the related ENaCs (Hackney and Furness, 1995).

Both pharmacologic and molecular sequence evidence make this unlikely: first, two molecules of amiloride are needed to block the hair cell and oocyte channels, and amiloride appears not to bind into the electric field of the pore. Rather, the voltage sensitivity of this block has been explained as a voltage-dependent conformational change of the channel that allows for a voltage-independent binding of amiloride, at sites outside of the electric field of the pore (Lane et al., 1991; Rüsch et al., 1994), quite different from the simple block observed for UNC-105 in which one molecule appears to bind deep in the pore.

Second, no degenerins have been detected in organisms other than nematodes. Sequences characteristic of nematode degenerins have not appeared in genomic, protein, or expressed sequence databases, except those of *C. elegans*. In the genomes of several bacteria and of the yeast *Saccharomyces cerevisiae*, which have been completely sequenced, there are no members of the DEG/ENaC superfamily. The stretch-activated channels that have been observed in these species (Martinac et al., 1987; Gustin et al., 1988; Sukharev et al., 1993; Berrier et al., 1996) must be composed of nondegenerin proteins, creating a precedent for several gene families of mechanically gated channels.

It remains unclear whether nondegenerin members of the DEG/ENaC superfamily may form mechanically gated channels in other species. So far, the BNaCs are proton gated, the FANaC is gated by peptides, and the ENaCs form constitutively active channels with no known form of gating: none seem to be mechanically gated. The open probability of the ENaC channel reconstituted in lipid bilayers could apparently be increased by stretch (Awayda et al., 1995; Ismailov et al., 1996, 1997a), and there is a report that expression of α-ENaC in LM(TK) fibroblasts confers upon them mechanical sensitivity (Kizer et al., 1997). However, thorough attempts to recreate this mechanical sensitivity by expressing ENaCs in *Xenopus* oocytes have not succeeded (Awayda and Subramanyam, 1998), and there is no in vivo evidence that ENaC is stretch modulated in the epithelia where it is expressed. A recently discovered new branch of the DEG/ENaC superfamily in *Drosophila* comprises two proteins: PPK and RPK. PPK localizes to the dendrites of some sensory neurons, and thus it has been suggested that it may form a mechanically gated channel (Adams et al., 1998). Therefore, members of the DEG/ENaC family might mediate mechanosensitivity in other eukaryotes; however, they appear not to include the hair cell or oocyte stretch-activated channels.

Degeneration- and Hypercontraction-Causing Mutations Interfere with Gating and Leave Channels Open Most of the Time

Degenerins containing certain gain-of-function mutations cause cellular degeneration or, if the expressing cells are muscles, hypercontraction. It was proposed that these mutations in some way deregulate the activity of a channel, for example by disabling a gate, rendering it constitutively active (García-Añoveros et al., 1995). We have found that both mutations indeed affect gating, and in different ways. Both mutations cause P_{open} to be nearly 1.0, and thus reduce the relative free energy of the open state. But UNC-105(P134S) channels open and close more readily than UNC-105(A692V) channels, and thus these two mutations have different effects on the activation energy barrier.

The A692V mutation subtitutes a residue at or near the second transmembrane domain, the suspected pore of the channel, and therefore could easily affect its gating properties. The P134S mutation disrupts a residue situated past the first predicted transmembrane domain, in the extracellular loop of the protein. In fact, most of the gain-of-function mutations identified in degenerins disrupt extracellular residues, and they all probably affect gating. The effects of P134S on gating support the suggestion that degenerins are regulated by their extracellular portion (García-Añoveros et al., 1995). Since proline 134 and the majority of the other mutated residues are not conserved in other related channels of the DEG/ENaC superfamily, the mode of gating of degenerin channels might be unique.

Experimental Procedures

Expression of *unc-105* in HEK-293 Cells and Oocytes

The mutations P134S and A692V were induced with BioRad's MutaGene kit into the plasmid pJL256, which contains the full-length cDNA of *unc-105* (Liu et al., 1996). A 3151 bp EcoRI fragment of cDNA that contains the complete coding sequence of *unc-105*, intact or with the mutations P134S or A692V, was subcloned into GW1-CMV (British Biotechnology, Oxford; Choi et al., 1991) for expression in HEK-293 cells (American Type Culture Collection, Rockville, MD) or into pGEM-HE (Liman et al., 1992) for in vitro transcription. HEK-293 cells were electroporated with 3 μg of plasmid πH3-CD8 (which expresses CD8 as a cotransfection marker) alone (as control) or together with 25 μg of plasmids GWunc-105, GWunc-105(P134S), or GWunc-105(A692V). Alternatively, 3 μg of plasmid pEGFP-N1 (Clontech) was used as a cotransfection marker. Transfected cells were detected with beads coated with anti-CD8 antibodies (Jurman et al., 1994) or epifluorescence using a GFP filter set, respectively. No electrophysiological or viability differences were detected between the CD8 and GFP markers, and both sets of data have been pooled for this study. Transfected HEK-293 cells were incubated for 1–2 days at 37°C in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% fetal calf serum and 0.1% gentamicin (Gibco).

Xenopus laevis were anesthetized with ice and 1 g/l of methanesulfonate (Sigma, St. Louis, MO), and oocytes were removed by laparotomy. Oocytes were defolliculated in 0.2 mg/ml of collagenase (Gibco, Grand Island, NY) dissolved in nominally zero Ca^{2+} solution (OR-2) containing 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES (pH 7.6) for 2–2.5 hr at room temperature with gentle agitation. Stage V–VI oocytes were injected with 12.5 ng of cRNA in a final volume of 50 nl. Injected oocytes were incubated for 1–2 days before experiments at 18°C in ND-96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES [pH 7.6]) supplemented with 2.5 mM pyruvic acid (Sigma) and 50 μg/ml gentamicin (Gibco). In cases where Na^+ loading was a possible problem, oocytes were grown in a modified ND-96 (ND-10) containing 10 mM NaCl, 88 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES (pH 7.6).

Electrophysiology

Whole-cell oocyte currents were obtained using a two-electrode voltage clamp (OC-725C Oocyte Clamp, Warner Instrument, Hamden, CT). Electrodes were fabricated from borosilicate microcapillary glass (VWR Scientific) and pulled to resistances of 0.5–2.0 MΩ when filled with 3 M KCl. Voltage ramps were done from −100 mV to +80 mV at 360 mV/s. Currents were digitized using a Digidata 1200 Series interface (Axon Instruments) and stored on a personal computer. Injected oocytes typically had very low input resistances due to the relatively nonselective, constitutive cationic currents caused by the A692V and P134S mutations. Thus, the voltage, current, and bath electrodes were sanded down to bare silver and rechlorided in bleach for 20 min after each recording to prevent electrode drift and uncorrected junction potentials during an experiment. A gravity fed perfusion system was used to effect bath exchanges, and complete solution exchanges were achieved within 2 min. A 10 mM stock solution of amiloride (Research Biochemicals International, Natick, MA) was made in water and diluted into solutions prior to each experiment.

Oocytes used for single channel recordings were transferred to a hyperosmolar solution and the vitelline membrane removed using fine forceps. Oocytes were then transferred to a recording chamber filled with frog saline solution containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, and 10 mM HEPES (pH 7.2). Cell-attached and inside-out patch pipettes were fabricated from borosilicate microcapillary glass (VWR Scientific), coated with Sylgard (Dow Corning, Midland, MI), and fire polished to resistances of 3–5 MΩ when filled with frog saline. Recordings were done with an Axopatch 200B (Axon Instruments, Foster City, CA) patch-clamp amplifier in voltage-clamp mode. Currents were low-pass filtered at 500–2000 Hz with an 8-pole bessel filter and digitized at 4–5 kHz with the Digidata 1200 Series interface (Axon Instruments). Data were acquired using Clampex and Fetchex software and analyzed using the Clampfit (pCLAMP6, Axon Instruments) and Origin programs (Microcal Software, Northampton, MA).

Single channel current levels and P_{open} were calculated from histograms. Relative permeabilities for monovalent cations were calculated from the reversal potentials in different monoionic external solutions. Relative permeabilities for divalent cations with respect to Na^+ were calculated from the magnitude of the inward current at −80 mV in different monoionic external solutions.

Apoptosis Detection

HEK-293 cells were tested 24 hr after electroporation for DNA fragmentation with the In Situ Cell Death Detection Kit (Boehringer Mannheim) according to the manufacturer's instructions, and chromatin clumping or changes in nuclear shape were detected using the nuclear dye H 33258 (Calbiochem). Results were evaluated and quantitated using fluorescence microscopy.

Electron Microscopy of Cells Expressing Mutated UNC-105

Transfected HEK-293 cells grown on glass coverslips were incubated in 0.5 μg/ml of anti-CD8 antibody (Dako Corporation, Carpinteria, CA) in culture medium at 37°C for 30 min, followed by several washes in culture medium. The cells were then incubated for 30 min at 37°C in culture medium with a 1:50 dilution of goat anti-mouse secondary antibody conjugated to 10 nm gold particles (Nanoprobes, Stony Brook, NY) for detection of transfected cells. The cells were washed free of culture medium and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1.5 hr, followed by multiple rinses in PBS. These and all subsequent steps were performed at room temperature. The antibody reaction was stabilized with 2.5% glutaraldehyde in PBS for 1.5 hr, followed by multiple rinses in PBS. The cells were postfixed with 2% osmium tetroxide (OsO_4) in 1.5% potassium ferrocyanide for 1 hr and rinsed with 100 mM sodium cacodylate buffer, followed by en bloc staining for 1 hr in 2% uranyl acetate in maleate buffer (pH 6.0). The cells were rinsed in maleate buffer (pH 5.2), followed by dehydration in an ethanol series. The cells were rinsed briefly in 100% propylene oxide, followed by overnight incubation in increasing concentrations of epon/araldite (EMbed 812; Electron Microscopy Sciences, Fort Washington, PA). A beem capsule topped off with 100% epon/araldite was inverted over the coverslip and cured for 48 hr at 60°C. The glass coverslip was removed from the epon stub by multiple immersions in liquid nitrogen with rapid thawing and finally by gentle twisting of the epon stub relative to the glass coverslip. The epon stub was trimmed and thin sections were generated using a Reichert Ultramicrotome E. Thin sections (silver-gold) were collected onto formvar-coated slotted grids, poststained with uranyl acetate and Reynold's lead citrate, and viewed with a JEOL 100CX electron microscope.

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