Pore-lining residues of MEC-4 and MEC-10 channel subunits tune the Caenorhabditis elegans degenerin channel’s response to shear stress

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Abstract

The Caenorhabditis elegans MEC-4/MEC-10 channel mediates the worm’s response to gentle body touch and is activated by laminar shear stress (LSS) when expressed in Xenopus oocytes. Substitutions at multiples sites within the second transmembrane helix (TM2) of MEC-4 or MEC-10 abolish the gentle touch response in worms, but the roles of these residues in mechanosensing are unclear. The present study therefore examined the role of specific MEC-4 and MEC-10 TM2 residues in the channel’s response to LSS. We found that introducing mutations within the TM2 of MEC-4 or MEC-10 not only altered channel activity, but also affected the channel’s response to LSS. This response was enhanced by Cys substitutions at selected MEC-4 sites (Phe-715, Gly-716, Gln-718, and Leu-719) between the degenerin and the putative amiloride-binding sites in this subunit. In contrast, the LSS response was largely blunted in MEC-10 variants bearing single Cys substitutions in the regions preceding and following the amiloride-binding site (Gly-677 to Leu-681), as well as with four naturally occurring MEC-10 touch-deficient mutations that introduced charged residues into the TM2 domain. An enhanced response to LSS was observed with a MEC-10 mutation in the putative selectivity filter. Overall, MEC-4 or MEC-10 mutants that altered the channel’s LSS response are primarily clustered between the degenerin site and the selectivity filter, a region that likely forms the narrowest portion of the channel pore. Our results suggest that pore-lining residues of MEC-4 and MEC-10 have important yet different roles in tuning the channel’s response to mechanical forces.

Mechanically-gated ion channels play key roles in mechanotransduction, a process that translates physical forces into biological signals (1). Members of the epithelial Na+ channel (ENaC)/degenerin family function as mechanosensors in epithelial and endothelial cells, as well as in sensory neurons (2-9). In Caenorhabditis elegans (C. elegans), gentle body touch is sensed via a proposed multi-protein mechanotransduction apparatus where the extracellular mantle, degenerin channel and cytoskeleton components are tethered together within the worm’s touch receptor neurons (8-10). At the core of this mechanotransduction apparatus is a mechanically-gated ion channel formed by MEC-4 and MEC-10 subunits, members of the ENaC/degenerin family (8,9). Recent studies suggest that C. elegans degenerin channels could adapt either a homomeric (three MEC-4) or heteromeric (two MEC-4 and one MEC-10) stoichiometry (11,12). MEC-4 and MEC-10 are homologous proteins that share extensive sequence (54% identity) and structural similarity (8), yet have distinct roles in mechanotransduction: MEC-4 is both required and sufficient to generate mechanosensitive ion channels (11,13,14), whereas MEC-10 is required for full sensitivity to mechanical stimuli (14-16).
Proteins of the ENaC/degenerin family share a common topology: short cytoplasmic amino and carboxyl termini and two transmembrane helixes connected by a large, highly organized extracellular domain (17,18). Several lines of evidence (19-22) suggest that the channel’s gate resides in the outer part of the pore, in the vicinity of the “degenerin” site. The introduction of bulky residues to this site in ENaC subunits, MEC-4 and acid sensing ion channel (ASIC) subunits result in a dramatic increase in channel open probability (Po). Strikingly, a large number of touch-deficient alleles have mutations within the pore-lining region of MEC-4 (16 alleles at 13 sites) and MEC-10 (4 out of 5 alleles), highlighting the importance of the channel’s gate in sensing mechanical stimuli (23). Additionally, pore-lining residues within the second transmembrane helix (TM2) of ENaC subunits are important for modulating channel gating in response to flow-mediated laminar shear stress (LSS) (24-26). However, the role of the pore-lining residues of MEC-4 or MEC-10 in mechanosensation has not been systemically examined in worms.

The present study used the LSS response to examine the role of TM2 domains of the two pore-forming subunits, MEC-4 and MEC-10 in channel gating by a mechanical force. We found that the LSS response was reversible in oocytes expressing both MEC-10 and MEC-4d (A713T, a gain-of-function mutation at the MEC-4 degenerin site). The LSS response was largely diminished by touch-deficient mec-10 alleles that introduce charged residues into the channel’s pore, as well as by Cys substitution at five sites within TM2 of MEC-10. In contrast, we observed an enhanced LSS response with five MEC-4 mutants, as well as one MEC-10 mutant. In addition, MEC-4 or MEC-10 mutants that altered the LSS response are primarily clustered between the degenerin site and the selectivity filter, a region that forms the narrowest portion of the channel pore in the related channel, ASIC1 (17,27). Together, our data support the hypothesis that pore-lining residues within the TM2 domain of MEC-4 or MEC-10 have important yet different roles in accommodating conformational rearrangements within the channel pore during channel gating in response to mechanical forces.

**Results**

**Touch-deficient mutations of MEC-10—*C. elegans* MEC-4/MEC-10 channels are activated by flow-mediated LSS in *Xenopus* oocytes and MEC-10 is required for a robust LSS response (14). In oocytes expressing MEC-4 A713T (MEC-4d) with MEC-10, MEC-2 and MEC-6, the LSS response is reversible as the currents returned to the baseline when the vertical flow was stopped (Fig. 1a). Worms bearing mec-10 alleles that introduce charged residues within the TM2 of MEC-10 (see Fig. 1b, G679R (u20), L679R (u390), G680E (u332) and G684R (e1715)) are defective in their response to gentle body touch (16). We therefore examined whether these touch-deficient mutations of MEC-10 also blunt the channel’s LSS response when expressed in *Xenopus* oocytes. An individual MEC-10 mutant or wild-type (WT) MEC-10 was co-expressed with MEC-4 A713T (MEC-4d), as well as MEC-6 and MEC-2. Whole cell Na+ currents were measured just prior to the initiation of LSS (Ibasal) and at 40 s post initiation of LSS (ILSS). The applied shear stress (0.12 dynes/cm²) is comparable to the external force required to evoke mechanoreceptor currents in worm’s touch receptor neurons (13). In response to LSS of 0.12 dynes/cm², ILSS/Ibasal was 2.0 ± 0.4 in oocytes expressing WT MEC-10 (Fig. 1d). The LSS response was significantly diminished by each of the four MEC-10 mutations (Fig. 1). In addition, time constants of the LSS response (tau) were also reduced by each MEC-10 mutant (Fig. 1d), suggesting a faster but diminished channel activation by LSS relative to the WT channel.

The blunted response and faster kinetics (Fig. 1) recapitulate the LSS response of channels lacking MEC-10 (i.e. MEC-4d homomeric channels) (14). This observation raises the question whether the individual MEC-10 mutants are, like WT MEC-10, present in the functional channel complex. It has been shown that the association of MEC-10 with MEC-4d in the channel complex reduces channel activity and enhances the channel’s sensitivity to pore blockers (28). We therefore examined the inhibitory effect of benzamil on
channels containing WT or mutant MEC-10. As shown in Fig. 2, co-expression of WT MEC-10 with MEC-4d resulted in a left shift in the benzamil dose-response curve, indicating enhanced sensitivity to benzamil. We then used the difference in benzamil sensitivity as a readout to determine whether MEC-10 mutants with blunted LSS responses were present within functional channel complexes. The dose-response curves for G676R and G680E fell between those for MEC-4d alone (no MEC-10) and MEC-4d with MEC-10 (WT MEC-10) curves (Fig. 2). The dose-response curves for L679R and G684R were similar to that of channels with MEC-10 (Fig. 2). Our data showing that these touch-deficient MEC-10 mutants were able to confer higher benzamil sensitivity as compared to MEC-4d alone channels (no MEC-10) suggest that the mutant MEC-10 subunits are a part of the functional complex. In addition, co-expression of these MEC-10 mutants reduced baseline whole cell Na+ currents, when compared to either homomeric MEC-4d channels (no MEC-10) or channels with WT MEC-10 (Table 1). Taken together, our results suggest that these touch-deficient MEC-10 mutants are components of channel complexes that are deficient in their response to LSS.

**Pore-lining residues of MEC-10**— As the LSS response was largely lost with naturally occurring MEC-10 touch-deficient mutations located within the channel’s pore (Fig. 1), we decided to systematically investigate the role of the pore-forming region of MEC-10 in the channel’s response to LSS. We introduced Cys residues at individual sites within MEC-10 TM2, from the degenerin site (Ala675) through the putative selectivity filter to Val687 (Fig. 3a). We chose to introduce Cys substitutions to allow us to perform additional studies with Cys reactive reagents, if warranted. Each mutant MEC-10 subunit was co-expressed with MEC-4d, MEC-2, and MEC-6 in *Xenopus* oocytes, and the LSS response was examined 4 to 7 days after cRNA injection. As shown in Fig. 3, the magnitude of channel activation was significantly reduced in five mutants (G677C, H678C, L679C, G680C and L681C). These reduced LSS responses were characterized by faster kinetics (smaller tau values). Strikingly, LSS of 0.12 dynes/cm² elicited a 7.1 ± 2.8-fold increase of whole cell Na+ currents in oocytes expressing MEC-10 S683C, compared to the 2.9 ± 1.8-fold increase of whole cell Na+ currents in WT MEC-10 (Fig. 3c). This greater LSS response was characterized by slower kinetics, as was the LSS response of the V685C mutant (Fig. 3c). Our results suggest that key sites within the pore-segment of MEC-10 accommodate conformational changes during channel gating in response to LSS.

As shown in Fig. 3, the LSS response was blunted by mutations at a stretch of residues (Gly677 to Leu681) adjacent to the putative amiloride binding site (Gly680) of MEC-10. It is possible that the blunted LSS response resulted from the absence of mutant MEC-10 in the channel complex. To test this possibility, we probed whether these MEC-10 mutants sensitized the channel to external cadmium (Cd²⁺). Multiple endogenous Cys residues are present within the pore-lining segments of MEC-4 and MEC-10, which are potentially accessible to Cd²⁺. Increased concentrations of Cd²⁺ were applied to oocytes expressing MEC-4d or MEC-4d/MEC-10 channels via basal perfusion (Fig. 4a). We found that the presence of WT MEC-10 in channel complexes increased the channel’s sensitivity to Cd²⁺ (Fig. 4a). For example, 0.1 mM Cd²⁺ reduced whole cell Na+ currents by 26.1 ± 11.6% in oocytes co-expressing WT MEC-10 and MEC-4d (Fig. 4b). This inhibition was completely reversible (Fig. 4a). In contrast, perfusion with 0.1 mM Cd²⁺ had minimal effect on whole cell currents of homomeric MEC-4d channels (no MEC-10, 4.4 ± 7.7% inhibition), suggesting that MEC-10 is required for Cd²⁺ inhibition. The difference in Cd²⁺ sensitivity provided us with an approach to test if certain MEC-10 mutants were present in the channel complex. Indeed, we observed that 0.1 mM Cd²⁺ inhibited the currents of oocytes expressing each of the LSS-deficient MEC-10 mutants (G677C, H678C, L679C, G680C and L681C) to a similar or greater magnitude than that seen with oocytes expressing WT MEC-10 (Fig. 4b). These data suggest that these MEC-10 mutants were present in channel complexes that failed to respond to shear stress. In addition, Cd²⁺ also inhibited LSS-activated...
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currents in oocytes expressing MEC-4d/MEC-10 channels (19.3 ± 4.8 %, Fig. 4c and 4d).

**MEC-4 degenerin site**—LSS activates ENaC by increasing channel P_{o} (29,30). Accordingly, this response was diminished in ENaCs that were “locked open”, e.g. bearing a Lys substitution at the degenerin site of the β subunit (βS518K), or modification of an introduced Cys near the degenerin site of the α subunit (αS580C) (29).

There are three gain-of-function mec-4 alleles, e1611 (MEC-4 A713V), u231 (MEC-4 A713T) and u56 (MEC-4 A713D) that cause degeneration of touch receptor neurons and loss of touch sensitivity in worms by increase channel Po, leading to excess Ca^{2+} and/or Na^{+} influx (9,31). We therefore examined the LSS response of each gain-of-function MEC-4 variants (A713D, A713T and A713V), as well as A713C. As shown in Figure 5, the magnitude of the LSS response was not altered by A713C, A713T, or A713V, but nearly abolished by the A713D mutant, as compared to WT MEC-4. These results are consistent with previous reports that channels with an A713D mutation have the highest channel P_{o} (~ 0.76) at baseline among MEC-4 degenerin site mutants (22), and suggest that the blunted LSS response seen in this mutant (A713D) is due to a high intrinsic P_{o}.

**Pore-lining residues of MEC-4**—We also screened the TM2 of MEC-4 (residues Asn^{710} to Thr^{729}) for sites that are important for the LSS response. We generated each Cys mutant in the MEC-4d background as oocytes expressing WT MEC-4 exhibited very low currents even after prolonged incubation (14). Each MEC-4d mutant was co-expressed with WT MEC-10, MEC-2, and MEC-6 in Xenopus oocytes. Five MEC-4d mutations increased the magnitude of channel activation (I_{LSS} / I_{basal}): F715C, G716C, Q718C, L719C and L728C (Fig. 6c). Partially overlapping with this group, five MEC-4d mutations increased the rate of the LSS response: N710C, Q718C, L719C, I725C, and L728C (Fig. 6d). The most striking changes were observed with Q718C, L719C and L728C where the magnitude of the LSS response was nearly doubled (Fig. 6c). These results contrast with the effect of mutating the sites equivalent to Gln^{718} and Leu^{719} in MEC-10 (His^{678} and Leu^{679}), which blunted the LSS response (Fig. 3). Na^{+} currents were not detected in channels bearing Cys substitutions at Gly^{720}, Trp^{722}, Gly^{724}, Ser^{726} or Thr^{729} of MEC-4d, even after prolonged incubation (Table 2). We therefore examined whether MEC-4d mutations near the putative selectivity filter affect the channel’s permeability to Na^{+}. MEC-4d/MEC-10 channels expressed in Xenopus oocytes are selective to Na^{+}, but also permeable to K^{+} (Table 3). We did not detect benzamil-sensitive currents in oocytes expressing MEC-4d G720C, W722C, G724C or S726C when Li^{+} or K^{+} (100 mM) was the major carrying ion in the buffer. Only modest changes in cation selectivity were found for channels with the I725C or L728C mutant (Table 3).

**Discussion**

Worms with mutations at key sites within the TM2 of MEC-4 or MEC-10 exhibit an abnormal gentle touch response (8,13,16,23), indicating that the pore-lining residues of both subunits have key roles in mechanosensation. In the present study, we examined the role of TM2 residues of MEC-4 and MEC-10 in the channel’s LSS response. We found that both subunits contain key sites within the pore-forming region of MEC-4 or MEC-10 clearly differed. For example, introducing Cys mutations at the corresponding sites within TM2 of MEC-4 (Gln^{718} and Leu^{719}) and MEC-10 (His^{678} and Leu^{679}) had opposite effects on the channel’s response to LSS (Figs. 1, 3 and 6). When Cys mutants were placed between the degenerin site and putative amiloride binding site of MEC-4 or MEC-10, we found that the LSS response was enhanced by four MEC-4d mutants but dampened by five MEC-10 mutations (Figs. 1, 3 and 6). These results are consistent with different physiological roles of MEC-4 and MEC-10 in the worm’s touch response. MEC-4 is expressed exclusively in the six touch receptor neurons whereas MEC-10 has been detected in other sensory neurons, and thus also implicated in the worm’s harsh touch response (8,15). Functional ENaC is also a heterotrimeric channel that adopts an asymmetric organization within its pore and pore-lining residues from individual...
subunits contributed differently to the channel’s activation by LSS (25,26,32,33). It is possible that TM2 residues of MEC-4 or MEC-10 do not align symmetrically within the pore of the C. elegans degenerin channel. In addition, we found the LSS response was largely blunted in MEC-4 A713D, a degenerin mutation with a high Po at baseline (Fig. 5). It was previously reported that the A713D mutant has higher channel Po than other MEC-4 degenerin site mutants (the baseline Po is 0.76 for A713D, and 0.36 for A713T). It is reasonable to postulate that the intermediate Po of A713T (~0.36) allows the channel to open in response to LSS, whereas the blunted LSS response seen in A713D is due to a high intrinsic Po (~0.76, (22)).

In the resolved open state structures of ASIC1, there is a break in the α helix near the putative selectivity filter (GXS belt) within TM2. This feature may facilitate extensive conformational changes during gating (27). It is not clear whether the TM2 of MEC-4 or MEC-10 is a continuous helix or adopts an analogous helical break as TM2 of ASIC1 does. We generated a homology model of the MEC-4d/MEC-10 channel pore based on the structure of ASIC1 in the open state (Fig. 7). When we mapped the location of the functionally important residues identified in the present study, we found them in close proximity to the helical break in our model, clustering between the degenerin site (DEG in Fig. 7a) and the selectivity filter. For example, we observed a dramatic change in the LSS response (both magnitude and kinetics) when a Cys was introduced at MEC-10 Ser 683, one residue before the selectivity filter GXS motif (Fig. 3). Meanwhile, the rate of the channel’s LSS response was altered by mutating each site of the MEC-10 GXS motif (Gly684, Val685 and Ser686) as well as MEC-4d Ile725, the equivalent site of MEC-10 Val685 (Figs. 3, 6 and 7). We previously identified key residues for the channel’s LSS response in the TM1 of MEC-10 (residues 130-132 and 134-137, (14)). Interestingly, these residues are predicted to neighbor the residues in the TM2 of MEC-10 that also blunt the channel’s LSS response (Fig. 7b). Overall, we noticed that most mutants that altered the LSS response are clustered in a region that is predicted to form the narrowest portion of the channel pore, based on the resolved ASIC1 structure (Fig. 7). Together, our data support the hypothesis that the region preceding and including the GXS belt within MEC-4 and MEC-10 undergoes conformational changes during channel gating in response to LSS (27).

MEC-2 and MEC-6 were included in our experiments to enhance the functional expression of WT or mutant MEC-4d/MEC-10 channels in oocytes. Neither MEC-2 nor MEC-6 contributes to the channel pore (11). Although we have previously shown that neither MEC-2 nor MEC-6 are required for the channel’s response to LSS for WT MEC-4d/MEC-10 channels (14), we do not know whether the presence of MEC-2 or MEC-6 influences our results or the proposed channel pore model.

Several Cys mutations generated in this study (MEC-4d G720C, W722C, G724C or S726C) failed to generate functional channels in oocytes (Table 2), limiting our ability to test the role of MEC-4 TM2 in channel gating in response to LSS. Consistent with our observations, mutations introduced at Trp722 Gly724 or Ser726 in MEC-4 cause a gentle touch defect in worms (23,34). It is not known whether these mutations affect the structure of the channel pore that influences channel conductance, ion selectivity, mechanical gating or pore structure. As they are localized near the selectivity filter, we attempted to test if these Cys mutants alter the channel’s cation selectivity. However, our results suggest that these mutant channels do not carry Na+, Li+ or K+ currents. We suspect that these MEC-4d mutations reduce channel conductance, as ENaC subunits with specific mutations within the selectivity filter exhibited significantly reduced whole cell currents and channel conductance (35,36).

In summary, we systemically examined the contributions of MEC-4 TM2 and MEC-10 TM2 in the channel’s response to a mechanical force. Together, our results suggest that the pore-forming segments between the degenerin site and the putative selectivity filter of both MEC-4 and MEC-10 have important, but differing roles in accommodating conformational changes during channel gating in response to LSS. Future studies
are needed to identify other sites and structural features within MEC-4 and MEC-10 that are involved in sensing mechanical stimuli, and whether similar sites or regions within other mechanosensitive members of the ENaC/Degenerin family have roles in mechanosensing.

**Experimental procedures**

**Site-directed Mutagenesis and Xenopus Oocyte Expression**—Single mutations were generated with the QuickChange kit (Agilent Technologies, Santa Clara, CA), using WT MEC-10 or MEC-4, or MEC-4 A713T (MEC-4d) as templates. Standard DNA sequencing was performed to confirm the desired mutations. cRNAs of WT or mutant subunits was synthesized and purified using T7 mMESSAGE mMACHINE® kit (Ambion Invitrogen). Defolliculated stage V-VI oocytes of *Xenopus laevis* were injected with cRNA mixtures of MEC-6 (1 ng) and MEC-2 (5 ng), along with WT or mutants MEC-10 and MEC-4d cRNA (5 ng) as indicated. MEC-2 and MEC-6 cRNAs were co-injected to enhance the functional expression of WT or mutant channels in oocytes. We presume that each component of the cRNA mixture was expressed at similar levels. Following microinjection, oocytes were initially maintained at 18°C in modified Barth's saline (MBS: 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 15 mM HEPES, 0.3 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, pH adjusted to 7.4) supplemented with 10 µg/ml sodium penicillin, 10 µg/ml streptomycin sulfate and 100 µg/ml gentamicin sulfate. On the following day, surviving oocytes were transferred into fresh MBS solution supplemented with 50 µM benzamil prior to functional assays. Oocyte survival following cRNA injection improves when a channel blocker (benzamil) is present in the bath solution (37). Following microinjection, oocytes were initially maintained at 18°C in modified Barth's saline (MBS: 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 15 mM HEPES, 0.3 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, pH adjusted to 7.4) supplemented with 10 µg/ml sodium penicillin, 10 µg/ml streptomycin sulfate and 100 µg/ml gentamicin sulfate. On the following day, surviving oocytes were transferred into fresh MBS solution supplemented with 50 µM benzamil prior to functional assays. Oocyte survival following cRNA injection improves when a channel blocker (benzamil) is present in the bath solution (37). Oocytes were incubated at 18°C for additional 4 to 7 days for optimal channel expression. Excess benzamil was removed by rinsing oocytes in benzamil-free MBS before starting each recording (14). All chemicals were purchased from Sigma-Aldrich unless stated otherwise. The protocol for harvesting oocytes from *Xenopus laevis* was approved by the University of Pittsburgh’s Institutional Animal Care and Use Committee.

**LSS Response**—Oocytes were placed in a recording chamber (20-mm diameter and 6-mm deep) that was constantly perfused with the NaCl-110 solution (110 mM NaCl, 2 mM KCl, 1.54 mM CaCl₂, and 10 mM HEPES, pH adjusted to 7.4 with NaOH) at a rate of 3.5 ml/min. Ca²⁺ is included in the bath solution, as we found that lowering or eliminating bath Ca²⁺ results in a large leak current. Oocytes were clamped at -60 mV while whole cell Na⁺ currents were continuously recorded. 0.12 dynes/cm² of shear stress was applied by perfusion (1.5 ml/min) through a vertical pipette submerged near the top of the oocyte (38). At the end of each experiment, 20 µM benzamil (50 µM for channels with reduced sensitivity to benzamil) was added to the bath perfusion to determine leak currents. Three measurements were taken: (i) the current measured just prior to the initiation of LSS (Ibasal), (ii) the current measured 40 s following the initiation of LSS (ILSS), (iii) and the current measured after the application of benzamil. Whole cell currents were corrected for the benzamil-insensitive component when determining Ibasal and ILSS. The magnitude of the LSS response was expressed as a ratio between the LSS stimulated current (ILSS) and the basal current (Ibasal). Time constants for channel activation (τ) were determined by fitting the first 40 s of current increases following the initiation of LSS with an exponential equation as previously described (38,39). To examine whether the LSS response is reversible, oocytes expressing MEC-4d/10/2/6 were exposed to four sequential LSS stimulations. Each LSS stimulation lasted 120 s, alternating with 240 s of basal bath perfusion. Benzamil was added via the basal perfusion at the end of each experiment to determine the leak current.

**Benzamil Dose-Response Relationship**—Whole cell Na⁺ currents were continuously recorded while oocytes were clamped at -60 mV. For the first 60 s, basal currents (I₀) in the absence of benzamil were measure by perfusing oocytes were with NaCl-110. A benzamil stock solution (0.1 M in DMSO) was diluted to a series of concentrations (10⁻⁹, 10⁻⁸, 10⁻⁷, 10⁻⁶, 10⁻⁵, and 10⁻⁴ M) with NaCl-110 and delivered to oocytes in sequence. Oocytes were perfused with each
Degenerin TM2 modify channel gating concentration of benzamil for 20 s to obtain stabilized currents (I). Normalized Na⁺ currents (I/Io values) were plotted as a function of benzamil concentrations (M), and dose-response curves were generated by fitting data to the following sigmoidal equation (14).

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y = \frac{1}{1 + 10^{(\log IC_{50} - \log X)}}
\]

where \(X\) is the concentration of benzamil (M). The IC_{50} is defined as the concentration of benzamil that inhibits 50% of the whole cell Na⁺ current (\(y\)). Dose-response curves were compared by analyzing the best-fit logIC_{50} values with the extra sum-of-squares F test.

Cation permeability– Different cation solutions (100 mM, Li⁺, Na⁺, K⁺) were prepared with according chloride salts (36,40). These solutions also contained 0.82 mM MgCl₂, 0.41 mM CaCl₂, 10 mM HEPES. In addition, 5 mM BaCl₂ and 10 mM TEA-Cl were included in bath solutions to block endogenous K⁺ currents. The present study did not test whether the addition of Mg²⁺, Ca²⁺, Ba²⁺ and TEA alter channel permeation or selectivity. The pH was adjusted to 7.4 with a hydroxide of the main permeating cation (Li⁺, Na⁺ or K⁺) in the solution. Whole cell currents were continuously measured while switching the perfusion buffer to each predominant cation. At the end of each experiment, 20 μM benzamil was added to bath solution containing each cation to determine leak currents. Benzamil-sensitive currents recorded at -60 mV were used to calculate the ratios of Li⁺ current (\(I_{Li⁺}\)) and K⁺ current (\(I_{K⁺}\)) relative to Na⁺ current (\(I_{Na⁺}\)).

Data and Statistical Analyses– Experiments were repeated with a minimum of two batches of oocytes obtained from different frogs. Electrophysiology data was analyzed using Clampfit (Molecular Devices, Sunnyvale CA) and OriginPro (OriginLab Corporation, Northampton, MA). Data were expressed as the mean ± standard deviation (S.D.) throughout. Prism (GraphPad Software, La Jolla, CA) was used for graphing and statistical analysis. Scatter-dot plots were used to show every data point along with a horizontal bar for the mean. Statistical comparisons were obtained from analysis of variance (ANOVA) followed by a Bonferroni test. A \(p\) value of less than 0.05 was considered statistically different.

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Conflict of Interests
The authors declare no conflicts of interests.

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Footnotes
The abbreviations used are: ANOVA, analysis of variance; ASIC, acid sensing ion channel; ENaC, epithelial sodium channel; *I*_basal, current measured just prior to the initiation of LSS; *I*_LSS, current measured 40 s following the initiation of LSS; LSS, laminar shear stress; MBS, modified Barth’s solution; MEC, mechanosensory abnormality; *P*_o, open probability; TM2, the second transmembrane domain; WT, wild-type.
Table 1. Select MEC-10 mutations alter the channel activity

|                | $I_{\text{Na}}$, $\mu$A | S.D. | n   | $P$  |
|----------------|--------------------------|------|-----|------|
| WT MEC-10      | -2.52                    | 2.49 | 92  |      |
| no MEC-10      | -4.28                    | 2.92 | 84  | *    |
| G676R          | -0.72                    | 0.30 | 14  | **** |
| L679R          | -0.51                    | 0.19 | 15  | **** |
| G680R          | -0.59                    | 0.18 | 15  | **** |
| G684E          | -0.43                    | 0.14 | 15  | **** |
| A673C          | -4.08                    | 2.81 | 15  | Ns   |
| D674C          | -1.08                    | 1.14 | 10  | **   |
| F675C          | -2.11                    | 1.43 | 10  | Ns   |
| G676C          | -2.14                    | 1.56 | 11  | Ns   |
| G677C          | -0.80                    | 0.71 | 9   | **   |
| H678C          | -0.60                    | 0.33 | 13  | **** |
| L679C          | -0.96                    | 0.96 | 11  | **   |
| G680C          | -0.52                    | 0.15 | 10  | ***  |
| L681C          | -1.58                    | 1.15 | 15  | *    |
| W682C          | -1.61                    | 0.74 | 15  | *    |
| S683C          | -1.40                    | 1.69 | 15  | **   |
| G684C          | -0.30                    | 0.16 | 15  | **** |
| V685C          | -1.52                    | 0.57 | 10  | Ns   |
| S686C          | -0.36                    | 0.28 | 12  | **** |
| V687C          | -1.86                    | 1.04 | 10  | Ns   |

$Xenopus$ oocytes were injected with cRNA mixtures of MEC-4 A713T (MEC-4d), MEC-2 and MEC-6 in the presence of WT MEC-10 or individual MEC-10 mutants. Basal currents (prior to the initiation of LSS stimulation) were measured at -60 mV by perfusing oocytes with NaCl-110 TEV solution. At the end of each recording, 20 μM benzamil (50 μM for channels without MEC-10) was added to the bath to determine leak currents. $n$ indicates the number of oocytes assessed for each group. Statistical comparisons of whole cell Na⁺ currents measured in oocytes expressing WT MEC-10 versus mutant channels were determined with one-way ANOVA followed by a Bonferroni test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns, not significant).
Table 2. Mutations of the pore-lining residues of MEC-4 alter the channel activity

|         | $I_{Na^+}$, μA | S.D. | n  | P       |
|---------|----------------|------|----|---------|
| A713 (WT) | -0.12          | 0.08 | 12 | ****    |
| A713C     | -0.48          | 0.85 | 17 | ****    |
| A713D     | -0.83          | 0.96 | 18 | ****    |
| A713V     | -0.59          | 0.26 | 18 | ****    |
| A713T (MEC-4d) | -4.31     | 2.90 | 90 |         |
| A713T N710C | -1.13         | 0.51 | 20 | ****    |
| A713T L711C | -4.19          | 1.96 | 22 | Ns      |
| A713T L712C | -2.76          | 1.39 | 22 | **      |
| A713T D714C | -0.60          | 0.39 | 17 | ****    |
| A713T F715C | -1.51          | 1.19 | 16 | ****    |
| A713T G716C | -1.66          | 1.41 | 11 | ****    |
| A713T G717C | -0.42          | 0.18 | 19 | ****    |
| A713T Q718C | -2.91          | 3.07 | 16 | Ns      |
| A713T L719C | -5.62          | 3.40 | 16 | Ns      |
| A713T G720C | -0.03          | 0.02 | 12 | ****    |
| A713T L721C | -1.77          | 1.38 | 16 | ****    |
| A713T W722C | -0.05          | 0.08 | 11 | ****    |
| A713T G724C | -0.02          | 0.02 | 12 | ****    |
| A713T I725C | -2.31          | 1.64 | 20 | ***      |
| A713T S726C | -0.02          | 0.02 | 12 | ****    |
| A713T F727C | -0.54          | 0.46 | 12 | ****    |
| A713T L728C | -2.26          | 1.28 | 12 | **       |
| A713T T729C | 0.00           | 0.01 | 11 | ****    |

*Degenerin TM2 modify channel gating*

Xenopus oocytes were injected with cRNA mixtures of WT MEC-10, MEC-2 and MEC-6 in the presence of indicated MEC-4 constructs. Whole cell basal currents (prior to the initiation of LSS stimulation) were measured at -60 mV by perfusing oocytes with NaCl-110 TEV solution. At the end of each recording, 20 μM benzamil was added to the bath to determine the leak current. n indicates the number of measurements for each group. Significant changes in whole cell Na$^+$ currents when compared to MEC-4 A713T (MEC-4d) were noted as (*$*$ *p < 0.01; *** *p < 0.001; **** *p < 0.0001; ns, not significant, determined with one-way ANOVA followed by a Bonferroni test).
**Table 3. Ion Selectivity of MEC-4 Cys mutants near the selectivity filter**

| Mutant          | $I_{Li}^+ / I_{Na}^+$ | $I_{K}^+ / I_{Na}^+$ | n  |
|-----------------|-----------------------|----------------------|----|
| A713T           | 0.75 ± 0.15           | 0.16 ± 0.08          | 17 |
| A713T L721C     | 0.67 ± 0.20           | 0.18 ± 0.06          | 24 |
| A713T I725C     | 0.68 ± 0.24           | 0.24 ± 0.06*         | 22 |
| A713T F727C     | 0.72 ± 0.14           | 0.16 ± 0.07          | 30 |
| A713T L728C     | 0.59 ± 0.24*          | 0.10 ± 0.02*         | 18 |

Benzamil sensitive whole cell currents were measured for the MEC-4 A713T (MEC-4d) channel or individual Cys mutant channels by perfusing the oocytes with TEV solutions containing 100mM Na⁺, Li⁺, or K⁺ as major carrying cations (see Methods). The average whole cell Na⁺ current of oocytes expressing MEC-4 A713T was -5.1 ± 4.4 μA. Experiments were repeated in four batches of oocytes. n indicates the number of oocytes measured for each group. Statistical significance was determined with one-way ANOVA followed by a Bonferroni test (* p < 0.05).
Figure 1. The LSS response is reversible and is largely diminished by touch-deficient MEC-10 mutations. (a) Oocytes expressing the *C. elegans* MEC-4d/MEC-10 channel were exposed to repetitive stimulation by LSS (0.12 dynes/cm², grey bars). Benzamil was added to the bath perfusion at the end of each recording to determine the leak current. (b) Sequence alignment of the pore-lining residues within MEC-4 and MEC-10. The degenerin site, proposed amiloride binding site and selective filter region are noted. MEC-10 sites where mutations cause touch-deficient phenotype in worms are underlined. (c) Representative traces of oocytes expressing MEC-10 touch-deficient mutants. The LSS response (0.12 dynes/cm², 2 mins) was examined in oocytes expressing MEC-4d/MEC-2/MEC-6 (no MEC-10), with WT MEC-10 or MEC-10 touch-deficient mutants (as indicated). Dash lines indicate the zero of the Y axis. (d) The LSS response of oocytes expressing MEC-10 touch-deficient mutants. The magnitude ($I_{LSS}/I_{basal}$, top) of the LSS response was determined as a ratio between the peak response of the whole cell current following the initiation of LSS ($I_{LSS}$) and the basal whole cell current prior to initiation of LSS ($I_{basal}$). The time constants of channel activation by LSS (tau, bottom) were determined by fitting the current increase within the first 40s following LSS stimulation as described in the Experimental Procedures. The experiment was repeated in two batches of oocytes for the mutant channels and the number of recordings (n) for each group was indicated. Significant changes when compared to WT MEC-10 (**** $p < 0.0001$) or homomeric MEC-4d channels (†††† $p < 0.0001$) were analyzed with one-way ANOVA followed by a Bonferroni test.
Figure 2. The channel’s sensitivity to benzamil is altered by select MEC-10 mutations. Benzamil dose-response was measured in oocytes expressing MEC-4d homomeric channel (no MEC-10) or channels with either WT MEC-10 or or individual MEC-10 mutants. (a) Oocytes were sequentially perfused with TEV buffer or TEV buffer containing each concentration of benzamil (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, \text{and} 10^{-4} \text{M}) while whole cell Na^+ currents were measured at a holding potential of -60 mV. (b-f) Benzamil dose-response curves. Whole cell Na^+ currents measured in the presence of increasing concentrations of benzamil (\(I\)) were normalized to the basal current in the absence of benzamil (\(I_0\)) to determine relative currents (\(I/I_0\)). Dose-response curves were generated by plotting \(I/I_0\) values as a function of benzamil concentrations (M) and fitting data to a sigmoidal equation (\(y=1/(1+10^{(\log IC_{50}-\log X)})\), where \(X\) is the concentration of benzamil. The IC_{50} was defined as the concentration of benzamil that inhibits 50% of the whole cell Na^+ current (\(y\)). Each data point represents the mean value from 6-10 measurements from at least two batches of oocytes. Error bars represent S.D. in each panel. Comparisons between the dose-response curves were analyzed with the extra sum-of-squares F test of the best-fit logIC_{50} values.
Figure 3. Mutations at the MEC-10 pore-lining region alter the channel’s LSS response. (a) Sequence alignment of the pore-lining residues within MEC-4 and MEC-10. The degenerin site, proposed amiloride binding site and selective filter region are noted. The LSS response was examined in oocytes injected with cRNA mixtures of MEC-4d, MEC-2 and MEC-6 in the presence of MEC-10 or individual MEC-10 Cys mutant, underlined. (b) Representative traces of MEC-10 mutants with altered LSS response. Oocytes were exposed to flow-mediated LSS of 0.12 dynes/cm² for 2 mins (grey bars). Dash lines indicate where the zero of the Y axis. (c) The relative LSS response (top) was estimated by normalizing the magnitude of the LSS response ($I_{LSS}/I_{basal}$) of each mutant channel to that of WT MEC-10 from the same batch of oocytes. The time constants of channel activation by LSS (tau, bottom) were determined as described in the Experimental Procedures. The dash line indicates the average values of the magnitude ($I_{LSS}/I_{basal}$) and kinetics (tau) of LSS response in oocytes expressing homomeric MEC-4d channels (no MEC-10: $I_{LSS}/I_{basal} = 1.6 \pm 0.5$; tau = $4.6 \pm 1.7$ s). The experiment was repeated in a minimal of two batches of oocytes for the mutant channels with the number of recordings (n) indicated. Significant changes when compared to WT MEC-10 (** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$) were analyzed with one-way ANOVA followed by a Bonferroni test.
Figure 4. Cadmium inhibits degenerin channels with functional MEC-10. Oocytes were injected with cRNA mixtures of MEC-4d, MEC-2, MEC-6, WT MEC-10 or individual MEC-10 construct as indicated. MEC-4d homomeric channels (no MEC-10) were examined in each experiment as a control. (a). Oocytes were exposed to increasing concentrations (0.1 mM, 1 mM and 5 mM) of Cd^{2+} dissolved in NaCl-110 TEV buffer for 40 s, followed by 40 s of washout with NaCl-110. At the end of each recording, benzamil was added to the bath to determine the leak currents. Tracings are representative of 3 to 4 recordings. (b) The percentage of Cd^{2+} (0.1 mM) inhibition on whole cell Na^{+} currents were determined for each mutant channel, as well as channels containing either WT MEC-10 or no MEC-10. Experiments were repeated in three batches of oocytes for each group with the number of recordings (n) indicated. Significant changes when compared to WT MEC-10 (**p < 0.001 and ****p < 0.0001) or homomeric MEC-4d channels (††††p < 0.0001) were analyzed with one-way ANOVA followed by a Bonferroni test. (c) The LSS was examined in oocytes expressing MEC-4d/MEC-10 channels and Cd^{2+} was applied through the vertical peptide to examine its inhibitory effect on whole cell Na^{+} current in the presence of LSS. (d) The percentages of Cd^{2+} inhibition on LSS-activated currents were quantified. Experiments were repeated in two batches of oocytes (n = 8). Statistical significance was analyzed with one-sample Student’s t-test (****p < 0.0001).
Degenerin TM2 modify channel gating

Figure 5. MEC-4 A713D mutation abolishes the LSS response. (a) The LSS response was examined in oocytes injected with cRNA mixtures of MEC-2, MEC-6 and MEC-10 with either WT MEC-4 or individual MEC-4 degenerin mutants. Oocytes were exposed to flow-mediated LSS of 0.12 dynes/cm² (grey bars). Dash lines indicate where the zero of the Y axis. (b) The magnitude ($I_{LSS}/I_{basal}$) and (c) the time constants (tau) the LSS response were determined for each group as described in the Experimental Procedures. The experiment was repeated in a minimum of three batches of oocytes and the number of recordings (n) for each group is indicated. Statistical comparisons were made against WT MEC-4 with one-way ANOVA followed by a Bonferroni test (**** p < 0.0001).
Figure 6. Select pore mutations of MEC-4 alter the channel's response to LSS. (a) Sequence alignment of the pore-lining residues within MEC-4 and MEC-10. The degenerin site, proposed amiloride binding site and selective filter region are noted. The LSS response was examined in oocytes injected with cRNA mixtures of MEC-2 and MEC-6, MEC-10 in the presence of MEC-4d or individual MEC-4d Cys mutant, underlined. (b) Representative traces of MEC-4d mutants with altered LSS response. Oocytes were exposed to flow-mediated LSS of 0.12 dynes/cm$^2$ for 2 mins (grey bars). Dash lines indicate where the zero of the Y axis. (c) The relative LSS response was estimated by normalizing the magnitude of the LSS response ($I_{LSS}/I_{basal}$) of each mutant channel to that of the MEC-4d group from the same batch of oocytes. (d) The time constants of channel activation by LSS (tau) were determined as described in the Experimental Procedures. The dash lines in panels (c) and (d) indicate the average values of the magnitude ($I_{LSS}/I_{basal} = 2.4 \pm 0.6$) and time constant ($tau = 10.6 \pm 3.2$ s) of the LSS response in oocytes expressing MEC-4d. nd, not determined. Statistical significance when compared to the MEC-4d group was determined with one-way ANOVA followed by a Bonferroni test (\textbf** $p < 0.01$ and \textbf**** $p < 0.0001$).
Degenerin TM2 modify channel gating

Figure 7. A model of the MEC-4/MEC-10 channel pore. (a) Alignments of the TM1 and TM2 segments of the chicken ASIC1 and *C. elegans* MEC-4 and MEC-10 (GenBank® numbers NP_001035557, NP_510712 and NP_509438). Conserved residues are highlighted in the grey background. The degenerin site (DEG), putative amiloride binding site and selectivity filter region are noted. (b) The bottom and side views of the channel pore formed by two MEC-4 (Cyan) and one MEC-10 (Grey). The model is generated based on the resolved structure of chicken ASIC1 (PDB # 4NTW). In both panels, MEC-4 and MEC-10 TM2 sites where mutations altered the channel's LSS response are highlighted in Blue (blunted LSS response) or Red (enhanced LSS response). Two key regions within MEC-10 TM1 that are required for a robust LSS response are also highlighted in Blue.
Pore-lining residues of MEC-4 and MEC-10 channel subunits tune the *Caenorhabditis elegans* degenerin channel's response to shear stress

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