Stomatin and Sensory Neuron Mechanotransduction

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INTRODUCTION

Almost all sensory neurons of the dorsal root ganglion (DRG) are specialized to detect mechanical stimuli impinging on their receptor endings in the periphery (Lewin and Moshourab 2004). There is still little information available on the molecules that enable vertebrate sensory neurons to transduce mechanical stimuli into action potentials. The process of mechanotransduction involves the transduction of a mechanical stimulus into a unidirectional electrical impulse that can be discriminated by the central nervous system (CNS). This process is achieved by the activation of specific ion channels in the plasma membrane of sensory neurons (Lewin and Chalfie 2002). The degree of homology between stomatin and MEC-2 is striking especially within the core domain where the amino acid residues are 65% identical. Recently, we have shown that another protein closely related to stomatin, called stomatin like protein-3 (SLP3) is essential for mechanoreceptor function in the mouse (Wetzel et al. 2007). In the worm, MEC-2 is thought to function as an essential subunit of a mechanosensitive channel complex that also includes MEC-4/MEC-10 proteins, members of the Deg/ENaC superfamily (Chelur et al. 2002; Ernstorn and Chalfie 2002; Goodman et al. 2002). Consistent with these findings, it was recently shown that both SLP3 and stomatin can interact with and modulate the physiological properties of acid sensitive ion channels (ASICs), also members of the Deg/ENaC superfamily (Price et al. 2004; Wetzel et al. 2007). Furthermore, stomatin and SLP3 are found in almost all DRG neurons in mice (Mannsfeldt et al. 1999; Wetzel et al. 2007).

METHODS

Electrophysiology

The skin nerve preparation was used essentially as previously described (Koltzenburg et al. 1997; Wetzel et al. 2007). Mice were killed using CO2 inhalation, and the saphenous nerve together with the skin of the hind limb was dissected free and placed in an organ bath. The skin was placed with the corium side up, and the nerve was placed in an adjacent chamber for fiber teasing and single-unit recording. Single units were isolated with a mechanical search stimulus applied with a glass rod and classified by conduction velocity and adaptation properties to suprathreshold stimuli. In some experiments, a series of increasing displacement stimuli were applied manually with a calibrated micrometer. In other experiments, a computer-controlled nanomotor (Kleindiek, Reutlingen) was used to apply controlled displacement stimuli of known amplitude and velocity. Standardized displacement stimuli of 10-s duration were applied to the receptive field at regular intervals (interstimulus period: 30 s). The signal driving the movement of the linear motor and raw electrophysiological data were collected with a Powerlab 4.0 system (AD instruments), and spikes were discriminated off-line with the spike histogram extension of the software. Mechanoreceptors were tested with an ascending series of displacement stimuli in which the ramp had a constant velocity of 1,435 μm/s. The same neurons were further tested with an intercalated...
series of stimuli with a constant displacement of 96 μm but with increasing velocities from 26 to 2,945 μm/s. The experimenter was blinded to the genotype of the animal throughout data collection and analysis.

**Patch-clamp recordings**

Whole cell electrophysiological recordings were made using fire-polished glass electrodes of 3–5 MΩ resistance pulled from borosilicate glass (Hilgenberg, Malsfeld, Germany) on a laser micropipette puller (P-2000, Sutter Instrument, Novato, CA). The recording chamber (volume of 500 μl) containing a coverglass with adherent neurons was continuously superfused (2–3 ml/min) with extracellular solution containing (in mM) 154 NaCl, 5.6 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 8 glucose. pH was adjusted to 7.4 with NaOH and osmolality was adjusted to 325 mosM with sucrose. The electrodes were filled with solution containing (in mM) 122 KCl, 10 Na⁺, 1 MgCl₂, 1 EGTA, and 10 HEPES. pH was adjusted to 7.3 with KOH and osmolality was adjusted to 290 mosM with sucrose. Neurons were visualized using phase contrast illumination at ×63 magnification on a Leica DMIRB inverted microscope. The diameter of each neuron was calculated from the mean of the longest and shortest diameters measured with a calibrated reticle. Only small-diameter neurons (<26 μm), which are predominantly C-fiber nociceptors, were used (Stucky et al. 2002). For heat tests, temperature in the recording bath was monitored using a miniature thermocouple (response time constant of 5 ms, Physitemp, Clifton, NJ) that was placed 1 mm from the recorded neuron. Heat ramp stimuli (24–49°C in 10 s) were applied by heating the extracellular solution immediately before it entered the bath. Bath temperature was maintained at room temperature (22–24°C) except during subsequent cell tests. For experiments with rosette neurons, the cultures were incubated overnight with 10 ng/ml human recombinant neurotrophin-4 (Prepro Tech). Recording solutions used for rosette neurons differed from those used to record I_{heat} (Hu and Lewin 2006). Extracellular solution contained (in mM) 140 NaCl, 1 MgCl₂, 2 CaCl₂, 4 KCl, 4 glucose, and 10 HEPES, pH 7.4 and electrodes were filled with solution containing (in mM) 110 KCl, 10 Na⁺, 1 MgCl₂, 1 EGTA, and 10 HEPES, pH 7.3. Note that the experiments in which recordings were made from rosette neurons were carried out separately from those in which I_{heat} was measured. The size of the rosette neurons recorded was significantly larger than the cells recorded to measure I_{heat}. Means are shown with SE.

**RESULTS**

We used an in vitro skin saphenous nerve preparation to examine in detail mechanoreceptor properties in mice with a null mutation of the stomatin gene. Stomatin mutant mice are viable and appear behaviorally normal (Zhu et al. 1999). We recorded from a large number of myelinated (n = 367) and unmyelinated (n = 157) sensory fibers with a mechanosensitive receptive field in the hairy skin of stomatin −/− mice or their wild-type littermates derived from heterozygote parents (Table 1). A total of 26 stomatin −/− and 27 stomatin +/- mice were used in the electrophysiological studies. Large myelinated fibers with conduction velocities >10 m/s in the saphenous nerve can be classified according to their adaptation properties as either rapidly or slowly adapting low-threshold mechanoreceptors (RAM and SAM, respectively). We initially used a manually applied series of step displacements to characterize the stimulus response properties of each mechanoreceptor type. We then used a computerized series of stimuli with a constant displacement of 96 μm but with increasing velocities from 26 to 2,945 μm/s. The experimenter was blinded to the genotype of the animal throughout data collection and analysis.

**TABLE 1. Proportions and some physiological properties of different primary afferent mechanoceptors**

<table>
<thead>
<tr>
<th>Receptor Type</th>
<th>Stomatin +/+</th>
<th>Stomatin −/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Total</td>
<td>CV m/s</td>
<td>vFT, mN</td>
</tr>
<tr>
<td>Aβ-Fibers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAM</td>
<td>35 (30/86)</td>
<td>16.0 ± 0.70</td>
</tr>
<tr>
<td>SAM</td>
<td>65 (56/86)</td>
<td>17.4 ± 0.58</td>
</tr>
<tr>
<td>Aβ-Fibers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>63.1 (46/73)</td>
<td>4.8 ± 0.38</td>
</tr>
<tr>
<td>C-Fibers</td>
<td>36.9 (27/73)</td>
<td>5.9 ± 0.21</td>
</tr>
<tr>
<td>C-M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-MH</td>
<td>63 (31/49)</td>
<td>0.51 ± 0.01</td>
</tr>
<tr>
<td>C-M</td>
<td>37 (18/49)</td>
<td>0.57 ± 0.02</td>
</tr>
</tbody>
</table>

The proportions and some physiological properties of different primary afferent mechanoreceptors recorded in stomatin +/+ and stomatin −/− mice. The proportions and some physiological properties of different primary afferent mechanoreceptors recorded in stomatin +/+ and stomatin −/− mice. Conduction velocity and median von Frey thresholds (with quartile ranges) for each mechanoreceptor type are shown. There were no statistically significant differences between any of the parameters measured in wild-type and stomatin mutant mice. RAM and SAM, respectively, rapidly or slowly adapting low-threshold mechanoreceptors; AM, A-fiber mechano receptor; C-M, C-mechanoreceptor; C-MH, C-mechanoheat; d-hair, Down hair receptor.
noxious heat stimulus (a transient heat pulse to $>$50°C) applied directly to the receptive field of single fibers to further sub-classify nociceptors into noxious heat-sensitive C-mechanoheat units (C-MH) (sometimes called polymodal nociceptors) and C-mechanonociceptors (C-M) that lack a response to noxious heat. The proportion of C-MH and C-M units as well as their median von Frey thresholds and mean conduction velocity was not different between stomatin $-/-$ and $+/+$ mice (Table 1). In another series of experiments we also measured heat-induced inward ($I_{\text{heat}}$) currents (Cesare and McNaughton 1996) in acutely isolated sensory neurons using the whole cell patch-clamp technique as previously described (Stucky et al. 2002). We found that the proportion of presumptive nociceptors, identified by their small size ($<$26 μm) and humped spike configuration, with a significant $I_{\text{heat}}$ was virtually identical to the control population. Thus of 22 small-diameter neurons recorded from stomatin $-/-$ mice ($n = 4$), 11 (50%) possessed a significant inward current ($>$100 pA) to application of a heat ramp. In an equivalent control population tested with noxious heat (47 cells), 21 (45%) were found to possess $I_{\text{heat}}$ and this is not significantly different from that found for stomatin $-/-$ neurons ($\chi^2$ test $P > 0.5$). Moreover the mean amplitude of $I_{\text{heat}}$ found in the stomatin $-/-$ neurons (552 ± 164 pA) was also not significantly different (unpaired $t$-test) from that found in control heat-responsive cells (357 ± 58 pA). We also noted the resting membrane potential of the small-diameter cells recorded and found that the mean resting membrane potential of stomatin $-/-$ mice ($-$1.0 mV; $n = 186$ single units), using a computer controlled mechanical stimulator. The relative incidence of mechanorreceptors of the RA or SA type but not other mechanosensitive sensory neurons within the Aβ-fiber range was not significantly different from that found in control wild-type neurons (50.7 ± 1.0 mV; $n = 70$, unpaired $t$-test, $P > 0.3$). The mean diameter of the cells recorded in wild-type (22.3 ± 2.5 μm) and stomatin $-/-$ (23.7 ± 0.5 μm) groups was not significantly different (unpaired $t$-test, $P > 0.10$).

We further characterized the deficit in $\alpha$-hair receptors in stomatin $-/-$ mice by making additional recordings from mechanoreceptors in stomatin $-/-$ and wild-type controls ($n = 186$ single units), using a computer controlled mechanical stimulator. The relative incidence of mechanoreceptor types found in this experiment was essentially normal in stomatin mutant mice and corroborated the results from the initial dataset shown in Fig. 1 (data not shown). Using a computer-

![Diagram](image-url)
controlled mechanical stimulator, the receptive field of each D-hair receptor was subjected to a series of increasing ramp and hold displacement stimuli, starting with the smallest effective stimulus. For stimulus strengths of 48 and 96 µm, the receptor was in also subjected to a series of ramp stimuli of varying velocity from 26 to 2,945 µm/s to quantify receptor sensitivity to moving stimuli. The use of the computer-controlled stimulus allowed us to calculate the exact firing rate during probe movement for each applied stimulus as the duration of the ramp in relation to spike firing could be precisely measured. In this case, a rapidly adapting response, typical of D-hair or RAM fibers was characterized by cessation of firing at the end of the ramp (allowing for small delay for conduction time). This analysis revealed that the deficit in the stimulus response properties of D-hair receptors to displacement and velocity stimuli was substantial in stomatin-deficient mice compared with wild-type controls (Fig. 2, A–C). It is clear that D-hair receptors encode the velocity of skin displacement much better than they encode displacement amplitude. Thus by using different stimulus velocities (constant amplitude), a steep relationship between stimulus strength and firing rate was observed (compare Fig. 2, B and C). We found that the coding properties of D-hair receptors in stomatin −/− mice is preserved, as the slope of the stimulus response is not changed, but the mean firing rate is dramatically reduced (−50% reduction) at all stimulus strengths compared with control neurons (Fig. 2). We also measured mechanical latency (Dubreuil et al. 2004; Shin et al. 2003) at each stimulus strength which is the time between onset of mechanical probe movement and the first spike (corrected for conduction delay). This parameter was not substantially altered in stomatin −/− mice at most stimulus strengths (Fig. 2D). Because mechanical latency is directly related to threshold, this finding suggests that absence of stomatin does not increase the absolute mechanical threshold for activation of D-hair receptors. In agreement with our earlier experiments using a manually applied stimulus, there was no impairment in the ability of RAM and SAM fibers to encode displacement or velocity stimuli in stomatin-deficient mice (Fig. 2, E and F).

**FIG. 2.** Coding properties of D-hair receptors in stomatin−/− mice. A: typical example traces from D-hair receptor neurons from wild-type and stomatin −/− mice in response to a 96-µm ramp displacement (velocity: 1.4 mm/s). B: mean firing rate during the movement of the probe onto the skin is shown for increasing displacements. The displacement velocity remained constant so that the firing rate increases only slightly with increasing amplitude. Note that the mean firing rate of D-hair receptors from stomatin −/− mice is lower than in controls, a difference that was significantly different [repeated-measures ANOVA analysis F(4,92) = 8.37, P < 0.001]. C: same neurons were also tested with varying velocities of ramp with amplitudes of 96 µm. Here the mean rates of firing for those neurons with a response to the ramp are plotted against ramp velocity (n is indicated in brackets above each data point). Note here that for both wild-type and stomatin −/− neurons there is a steep relationship between firing rates and probe velocity. The mean rates of firing were again reduced by −50% in stomatin mutant mice compared with controls [repeated-measures ANOVA F(4,72) = 3.89, P < 0.01]. D: mean mechanical latency for each displacement amplitude is plotted. Interestingly, mechanical latency is low for all displacement amplitudes, and the neurons from stomatin −/− mice show little tendency to have longer mechanical latencies. The difference between the 2 sets of mechanical latencies from control and stomatin −/− mice did not reach statistical significance with a repeated-measures ANOVA analysis [F(4,88) = 1.6, P > 0.15]. E and F: we observed no changes in mechanoreceptive properties in RAM fibers tested with varying velocities of ramp movement (E) or in SAM fibers stimulated with an increasing series of displacement stimuli (F).
The experiments described thus far were carried out using a mechanical search stimulus to identify mechanically sensitive single units. It is possible that, as in SLP3 mutant mice, a substantial proportion of sensory axons may not form a mechanosensitive receptive field in the skin of stomatin-deficient mice (Wetzel et al. 2007). To address this question, we used an electrical search stimulus to initially isolate single cutaneous fibers irrespective of modality and then determined the proportion of these that respond to mechanical stimuli. Using this experimental approach, it is consistently found that there is a low proportion (5–10%) of fibers for which a mechanosensitive receptive field cannot be found (Kress et al. 1992; Wetzel et al. 2007). We found no significant increase in the proportion of nonmechanosensitive afferents with Aβ-fibers (control: 5.3%, 7/132 tested; stomatin −/−: 7.7%, 6/78 tested), or Aδ-fibers (control: 5.0%, 7/141 tested; stomatin: −/−: 7.8%, 4/51 tested), or C-fibers (control: 7.5%, 5/66 tested; stomatin: −/−: 12.5%, 2/16 tested). χ² test P > 0.2 in all cases.

The reduced mechanosensitivity of δ-hair receptors could be due to a primary deficit in the conversion of the mechanical stimulus into a receptor potential. Alternatively, the absence of stomatin might influence the electrical excitability of the δ-hair receptor membrane. We made new recordings from a subpopulation of medium to large neurons in culture (mean diameter stomatin +/+: 33.6 ± 1.0 μm n = 8; stomatin −/−: 33.0 ± 1.7 μm, n = 6) that have a unique “rosette”-like morphology (Fig. 3). Rosette neurons have characteristics of δ-hair receptors such as expression of very large T-type calcium channel currents and a dependence on neurotrophin-4 (Dubreuil et al. 2004; Shin et al. 2003). All the rosette cells recorded from both wild-type and stomatin −/− mice had very narrow action potentials (AP half-width in stomatin −/− cells 0.89 ± 0.15 ms compared with 0.66 ± 0.06 in stomatin +/+ cells, n = 6–8). Neither the resting membrane potential nor the current required to evoke an action potential in these neurons was different between the two genotypes (Fig. 3). These data indicate that lack of stomatin does not affect the electrical excitability of these neurons or their ability to generate action potentials.

**DISCUSSION**

In this study, we have shown that the absence of the MEC-2-like protein stomatin results in an impairment of the transduction of mechanical stimuli by δ-hair receptors (Figs. 1 and 2). The physiological properties of all other types of cutaneous sensory neurons, including nociceptive C-fibers were not significantly affected in stomatin −/− mice despite the fact that stomatin is present in all sensory neurons (Mansfeldt et al. 1999).

A molecular model of mechanotransduction by body touch sensory neurons in *C. elegans* incorporates two Deg/ENaC channel subunits, MEC-4 and MEC-10, together with two presumptive accessory proteins, MEC-2 and MEC-6 (Chelur et al. 2002; Goodman et al. 2002). Recent physiological recordings made from sensory neurons in *C. elegans* mutants indicates that the MEC-2 protein is not only required for touch evoked behavior but is also absolutely necessary for mechanosensory responses of sensory neurons (O’Hagan et al. 2005; Suzuki et al. 2003). Stomatins and its close relative SLP3 are, on the basis of their amino acid sequences, the closest vertebrate proteins to the worm MEC-2 protein (Wetzel et al. 2007). The SLP3 protein is absolutely required for mechanosensitivity of many Aβ and Aδ fibers in the saphenous nerve (Wetzel et al. 2007). Here we show that stomatin is, unlike SLP3, not absolutely required for sensory neuron mechanosensitivity (Wetzel et al. 2007). However, we did observe a robust decrease in the mechanosensitivity of one type of rapidly adapting receptor the δ-hair receptor. These neurons displayed substantially reduced firing rates (>50% reduction) to both displacement stimuli and moving stimuli. Our data indicate that the absolute mechanical threshold for activation of δ-hair receptors was not elevated in stomatin mutants as assessed with von Frey hairs and by measuring the mechanical latency for the first spike at different stimulus strengths (Fig. 2). The loss in sensitivity of δ-hair receptors was not accompanied by any shift in the coding properties as the slope of the stimulus response functions were unaltered in stomatin −/− mice (Fig. 2). The phenotype is consistent with a requirement for stomatin in normal transduction of mechanical stimuli and might be explained by a reduction in the magnitude of the receptor current in the absence of stomatin. Although it is possible to measure mechanosensitive currents in cultivated neurons (Hu and Lewin 2006), it is not possible to measure the receptor potential of single identified δ-hair receptors in vivo (Hu et al. 2006). Thus it is difficult to exclude the possibility that stomatin is required for maintenance of normal electrical excitability.
of d-hair receptor endings. However, in identified “rosette” sensory neurons, which have many characteristics of d-hair receptors (Dubreuil et al. 2004; Shin et al. 2003), we found no evidence for changes in membrane excitability in sensory neurons taken from stomatin−/− mutant mice. This finding is in contrast to what we have observed in isolated neurons taken from SLP3 mutant mice that have a small but statistically significant higher resting membrane potential than wild-type neurons (Wetzelaer et al. 2007). It is striking that the deficits we observe in mechanoreception in stomatin mutant mice is so much milder than seen in the SLP3 mutant (Wetzelaer et al. 2007), and there is a possibility that these two highly related proteins substitute for each other functionally. Future experiments using stomatin/SLP3 double mutant mice should resolve this question.

An important point to note is that the phenotype that we have described here is the first direct evidence for any in vivo cellular function of stomatin and further suggests that stomatin may directly regulate ion channels in sensory cells. The hypothesis that stomatin might regulate cation channels in the erythrocyte membrane was prompted some time ago by the observation that lack of stomatin protein in erythrocytes, taken from patients with an inherited stomatocytosis, was correlated with a plasma membrane leaky to monovalent cations (Stewart et al. 1992, 1993). However, no primary genetic lesion in the stomatin gene has been identified in patients and families with hereditary stomatocytosis (Argent et al. 2004; Fricke et al. 2004; Innes et al. 1999), and the lack of stomatin in stomatocytosis is therefore thought to be secondary. Indeed, deletion of the stomatin gene itself in mice does not lead directly to leaky erythrocyte membranes (Zhu et al. 1999).

We conclude that the loss of stomatin leads to a specific loss of mechanoreceptor function in vivo. This protein may therefore participate directly in the detection of mechanical stimuli by subsets of vertebrate mechanoreceptors. Our data provide the first evidence that stomatin is a physiological regulator of ion channels in vivo.

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