Speed and Temperature Dependences of Mechanotransduction in Afferent Fibers Recorded From the Mouse Saphenous Nerve

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Milenkovic N, Wetzel C, Moshourab R, Lewin GR. Speed and temperature dependences of mechanotransduction in afferent fibers recorded from the mouse saphenous nerve. J Neurophysiol 100: 2771–2783, 2008. First published September 24, 2008; doi:10.1152/jn.90799.2008. Here we have systematically characterized the stimulus response properties of mechanosensitive sensory fibers in the mouse saphenous nerve. We tested mechanoreceptors and nociceptors with defined displacement stimuli of varying amplitude and velocity. For each sensory afferent investigated we measured the mechanical latency, which is the delay between the onset of a ramp displacement and the first evoked spike, corrected for conduction delay. Mechanical latency plotted as a function of stimulus strength and velocity. For each sensory afferent investigated we measured the mechanical latency, which is the delay between the onset of a ramp displacement and the first evoked spike, corrected for conduction delay. Mechanical latency plotted as a function of stimulus strength and velocity.

INTRODUCTION

The receptive properties of rodent primary afferent neurons have been extensively studied using classical methodologies such as single-unit recording in vivo and in vitro (Lewin and Moshourab 2004). Using this methodology the response properties of single fibers can be probed with well-defined mechanical and thermal stimuli. The use of quantitative stimuli also allows the classification of sensory neurons into low-threshold mechanoreceptors or nociceptors. Many groups have adopted in vitro skin nerve preparations similar or identical to that first developed for rat by Reeh and modified for mouse by Koltzenburg and colleagues (Koltzenburg et al. 1997; Reeh 1986; Wenk et al. 2006). A considerable advantage of an in vitro skin nerve preparation is that it is a convenient tool for the quantitative study of primary afferents in the mouse, which has become a standard model organism to test the effects of targeted gene mutation on the development and function of sensory receptors (Bautista et al. 2007; Caterina et al. 2000; Price et al. 2000, 2001; Stucky et al. 2002a,b; Wetzel et al. 2007). In principle it is possible to make recordings from mouse sensory afferents in anesthetized preparations, although this is considerably more challenging technically (Boada and Woodbury 2007). Indeed comparison of data obtained from anesthetized preparations and in the in vitro preparation indicates that primary afferent properties are very comparable under these circumstances (Kress et al. 1992).

The cellular and molecular mechanisms by which mechanical stimuli are transduced into electrical impulses in the peripheral endings of dorsal root ganglion neurons (DRGs) remain poorly understood. The current model of mechanoreceptor transduction is that mechanical stimuli directly gate ion channels in the membrane of the sensory ending and the resulting membrane depolarization is followed by action potential firing. The underlying mechanosensitive ion channels are thought to be largely voltage insensitive and their activation under normal physiological conditions leads to a graded receptor potential (Hu et al. 2006). Electrophysiological investigations of transduction events in vivo using intracellular recording techniques have been successfully carried out in spider slit organ mechanoreceptors and more recently in nematode body touch receptors (Hoger et al. 1997; O’Hagan et al. 2005). These studies have shown that, at least in invertebrates, some mechanosensitive ion channels are relatively sodium selective and insensitive to membrane voltage. Such studies have proven much more difficult to carry out with mammalian mechanoreceptors or nociceptors. The results from extracellular recordings of the receptor potential in Pacinian corpuscle and muscle spindle afferents are consistent with the existence of similar mechanosensitive channels in vertebrates (Diamond et al. 1958; Hu et al. 2006; Ottoson 1964).

In recent years several groups have identified mechanosensitive currents that can be measured with the whole cell patch-clamp method after mechanical stimulation of DRG neurons in culture (Cho et al. 2002; Di Castro et al. 2006; Drew et al. 2002, 2004; Hu and Lewin 2006; McCarter et al. 1999; Wetzel et al. 2007). Detailed studies of such mechanosensitive currents have shown that their latencies for activation are in the range of 300–800 μs, which is consistent with the mechanical stimulus directly gating ion channels in the membrane (Hu and Lewin 2006). Two major types of mechanosensitive current have been identified that have distinctive pharmacological...
sensitivities one of which, a slowly adapting current, is found exclusively in putative nociceptive neurons. The second major type of mechanosensitive current inactivates very rapidly but, like the slowly adapting current, has a very short latency and a fast activation time constant (Hu and Lewin 2006). Interestingly, like the mechanoreceptor current analyzed in invertebrates (Hoger et al. 1997; O’Hagan et al. 2005), the rapidly adapting mechanosensitive current is also relatively selective for sodium ions (Hu and Lewin 2006). It has long been known that in vivo the time taken for a suprathreshold mechanical stimulus to initiate a spike in an individual mechanoreceptor can be in the order of a millisecond or less (Bell et al. 1994). This parameter—which we have termed “mechanical latency”—has rarely been analyzed in detail and no systematic measurements have been made to examine how mechanical latency in mechanoreceptors and nociceptors behaves as a function of stimulus strength. Mechanical latency may be dependent on several factors, including stimulus strength, transduction time, and the excitability of the membrane adjacent to the site of transduction.

In the present study we have characterized a large population of mechanoreceptors and nociceptors in the mouse saphenous nerve using defined displacement stimuli of varying amplitude and velocity. We show that systematic measurement of the mechanical latency for each receptor type can provide an additional physiological signature of the receptor type and that mechanical latency in C-fibers is very strongly and uniquely sensitive to changes in temperature. Finally, we have asked whether short-term inflammation (2–6 h) induced by carrageenan can lead to robust changes in the mechanical latency and suprathreshold response of nociceptors.

 METHODS

All the experiments in this study were carried out either on inbred C57Bl/6N mice (obtained from Charles River, Sulzfeld, Germany) or on a laboratory-bred hybrid mouse strain. A total of 111 mice were used in these studies. Normally, when generating mutant mice the blastocysts from a donor strain, C57Bl/6N, are injected with gene-manipulated embryonic stem cells derived from 129/sv strain mice to obtain chimeric animals (Joyner 2000). Thus first-generation (F1) mutant and wild-type mice are a genetic mixture between 129/sv and C57Bl/6N strains. The hybrid wild-type mice used in this study were derived from the progeny of multiple intercrosses of F2-generation mice derived from C57Bl/6N–129/sv chimera mice. The wild-type hybrid mice were maintained as an inbred strain in the lab. During the course of the studies described here almost identical experimental data were obtained from C57Bl/6N and hybrid mouse strains. The stimulus response functions from single units derived from the two mouse strains were analyzed and plotted separately (Figs. 2 and 3).

 Electrophysiology

The skin nerve preparation was used essentially as previously described (Koltzenburg et al. 1997; Martínez-Salgado et al. 2007). Mice were killed by placing the animal in a CO2-filled chamber for 2–4 min followed by cervical dislocation. Animal housing and care, as well as protocols for killing, are registered with and approved by the appropriate German federal authorities (State of Berlin). The saphenous nerve and the shaved skin of the hind limb were dissected free and placed in an organ bath. The chamber was perfused with a synthetic interstitial fluid (SIF buffer) the composition of which was (in mM): NaCl, 123; KCl, 3.5; MgSO4, 0.7; NaH2PO4, 1.7; CaCl2, 2.0; sodium gluconate, 9.5; glucose, 5.5; sucrose, 7.5; and HEPES, 10 at a pH of 7.4 (Koltzenburg et al. 1997). The skin was placed with the corium side up in the organ bath 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. A computer-controlled nanomotor (Kleindiek, Reutlingen, Germany) 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 probe was a stainless steel metal rod and the diameter of the flat circular contact area was 0.8 mm as in previous studies (Koltzenburg et al. 1997; Martínez-Salgado et al. 2007; Wetzel et al. 2007). 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. The precise timing of the ramp part of each displacement stimulus could be measured and the phasic response of the receptor precisely determined. The phasic response of the receptor was defined as arising from those spikes occurring during the ramp displacement and those occurring within a time frame after cessation of the ramp that included the conduction delay for that receptor. All experiments were carried out with an organ bath temperature of 32°C unless indicated otherwise.

Following the isolation of each receptor a sharp tungsten metal electrode was placed in the receptive field and an electrically evoked spike was evoked with suprathreshold current pulses with durations of 50, 150, or 500 μs. As a rule only short-duration, low-amplitude current injection was required for myelinated fibers and longer pulses of more intense current were required for unmyelinated C-fibers. For each isolated fiber the conduction velocity was calculated from the electrical latency for the spike. Next the mechanical threshold was estimated for each afferent by evoking spikes with a series of calibrated von Frey filaments. The computer-controlled mechanical stimulation was then maneuvered onto a spot within the receptive field where the most reliable responses could be obtained with a von Frey filament. Using small movements (48 μm) of the nanomotor the mechanical stimulus was advanced onto the receptive field until one spike was evoked. The amplitude of the stimulus was then systematically reduced and the probe moved into a z-axis position, so that the smallest stimulus possible (usually 12 μm) reliably evoked at least one spike when the probe was advanced. The starting position of the mechanical stimulator was therefore just above threshold for each recorded unit. The afferent was then confronted with an ascending series of displacement stimuli, sent as a preprogrammed series of commands to the nanomotor. The standard ramp speed used in the ascending series had a velocity of 1,435 μm/s. The same neurons were tested with an intercalated series of stimuli with constant amplitudes of either 48 or 96 μm, but with increasing velocities from 26 μm/s up to a maximum of 2,945 μm/s (Fig. 1). Mechanical latency was measured for each individual recorded afferent by measuring the delay between the onset of each ramp stimulus and the first spike minus the conduction delay measured for the same fiber at the same temperature.

It could be argued that the force generated by the series of displacement stimuli described earlier may show a complex nonlinear relationship to the probe displacement. We obtained a custom-made force-measurement device (Kleindiek) and measured the force at the tip when the probe was used to apply a series of increasing ramp- and hold stimuli ranging from 50 to 500 μm (Supplemental Fig. S1).1 This calibration experiment demonstrates several things. First, the mechanical stimulator delivers reliable displacement stimuli and there is no evidence of drift so that the starting position for subsequent stimuli is altered in relation to preceding stimuli. Thus the force returns to the baseline value after application of each stimulus.

1 The online version of this article contains supplemental data.
C-mechanoheat [C-MH]), or as nociceptors, which are low-threshold mechanoreceptors, or A-fiber axons and 235 single units with unmyelinated C-fiber axons was used in our analysis because spike firing was most reliable with this protocol. Nociceptors were in general tested only with the displacement series (gray-filled stimuli).

Second, the force shows little hysteresis because the force exerted at the end of the ramp-and-hold stimulus shows only minimal sag compared with the start of the ramp (Supplemental Fig. S1). Finally, the force increases during the ramp in a manner that follows the displacement signal.

To test the heat responsiveness of the mecanosensitive C-fiber units preheated SIF buffer was applied on the receptive field isolated with a small metal ring and the actual temperature of the surface of the skin was measured with a thermocouple. We recorded from the same units perfused with buffer at two temperatures, 24 and 32°C, and also from two populations of afferents at the two indicated test temperatures. The latter protocol avoids possible desensitization that might occur with repeated stimulation. There was no difference, however, in these two data sets and so they were merged.

Carrageenan inflammation

Animals were deeply anesthetized with urethane (1.5 g/kg, administered intraperitoneally) and four injections of 2% wt/vol carrageenan (total volume, 400 µl) were made intradermally into the area of skin innervated by the saphenous nerve. After 3–4 h the animal was killed by exposure to a rising concentration of CO2 gas and the skin-nerve preparation was made as described earlier. In all cases we observed pronounced inflammation and edema throughout the innervation area of the saphenous nerve after carrageenan injections. We assume that mechanical hyperalgesia resulted from the carrageenan inflammation, although this was not examined directly in this study. The ongoing discharge of C-fibers was measured in controls and inflamed skin by calculating the mean activity during all interstimulus periods. All means are shown ±SE.

RESULTS

We recorded from a total of 596 single units with myelinated A-fiber axons and 235 single units with unmyelinated C-fiber axons in the saphenous nerve. A detailed breakdown of the units recorded, their classification, conduction velocity, and median von Frey threshold is summarized in Tables 1 and 2. It should be noted that there are slight discrepancies between the total numbers quoted in the tables and the numbers quoted in individual stimulus response measurements. This is mostly explained by the fact that not all single units that could be classified as a particular receptor type were successfully characterized with the entire stimulus series illustrated in Fig. 1. As in previous studies we classified all the myelinated units (conduction velocity >1.0 m/s) into one of four receptor types (Koltzenburg et al. 1997; Price et al. 2001; Wetzel et al. 2007); fast conducting Aβ-fibers (CV >10.0 m/s) were classified either as rapidly adapting mechanoreceptors (RAMs) or as slowly adapting mechanoreceptors (SAMs). Slowly conducting Aδ-fibers (CV <10 m/s) were classified either as D-hair receptors, which are low-threshold mechanoreceptors, or A-fiber mechanonociceptors (AMs), which are nociceptors. Each low-threshold mechanoreceptor (SAM, RAM, or D-hair) was subjected to a standardized series of increasing ramp-and-hold displacement stimuli with a constant-velocity ramp (1.435 µm/s) interspersed with two series, one low (48 µm) and one high constant-amplitude (96 µm) stimulus with varying ramp velocities (Fig. 1). Nociceptive fibers were subjected only to a series of increasing ramp-and-hold stimuli (shaded stimuli in Fig. 1) because these afferents have no significant sensitivity to moving stimuli. The units with C-fiber axons were almost all classified as either polymodal, responding to both mechanical and noxious thermal stimuli (C-mechanoheat [C-MH]), or as Table 1. Proportions and some physiological properties of different primary afferent mechanoreceptors recorded in C57Bl/6N and hybrid mice

<table>
<thead>
<tr>
<th>Receptor Type</th>
<th>C57Bl/6N</th>
<th>Hybrid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percentage Total</td>
<td>CV, ms</td>
</tr>
<tr>
<td>Aβ-fibers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAM</td>
<td>38% (52/138)</td>
<td>14.3 ± 0.95</td>
</tr>
<tr>
<td>SAM</td>
<td>62% (86/138)</td>
<td>15.9 ± 0.94</td>
</tr>
<tr>
<td>Aδ-fibers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>72% (102/142)</td>
<td>5.9 ± 0.30</td>
</tr>
<tr>
<td>D-hair</td>
<td>28% (40/142)</td>
<td>5.4 ± 0.36</td>
</tr>
<tr>
<td>C-fibers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-units</td>
<td>(86)</td>
<td>0.50 ± 0.02</td>
</tr>
<tr>
<td>C-M</td>
<td>30% (24/81)</td>
<td>0.55 ± 0.04</td>
</tr>
<tr>
<td>C-MH</td>
<td>70% (57/81)</td>
<td>0.45 ± 0.02</td>
</tr>
</tbody>
</table>

CV values are means ± SE. Conduction velocity and median von Frey thresholds (with quartile ranges) for each mechanoreceptor type are shown.
The stimulus response functions for low-threshold mechanoreceptors (SAM, RAM, and D-hair receptors) were plotted. The data sets from C57Bl/6N mice and the hybrid strain were plotted separately (open circles and upright triangles, respectively, in Figs. 2 and 3). Statistical analysis of the two data sets did not include data from units that did not fire any spike for that stimulus (Fig. 2, A–C); however, there was no significant effect of displacement on firing rate in SAM and D-hair receptors (repeated-measures ANOVA). The decreased firing rate with increasing displacement amplitude for the RAM fibers was statistically significant \( P < 0.0001 \), repeated-measures ANOVA) and probably reflects adaptation during the movement phase, as increasing amplitude necessitates a longer duration ramp. The fact that low-threshold mechanoreceptors do not increase their firing rate for increasing displacement stimuli suggests that firing rate codes the speed of the ramp (which is constant at 1.435 \( \mu \)m/s) rather than its amplitude. Therefore we also plotted firing rate against stimulus velocity with constant amplitude (velocity series, 96 \( \mu \)m) for the same mechanoreceptors. The data were also analyzed for the 48-\( \mu \)m amplitude velocity series but the firing rates were much more variable than those found with the larger-amplitude stimulus. All three types of low-threshold mechanoreceptors code increasing ramp velocity with increased firing rates (Fig. 2, D–F). The two mechanoreceptor types (RAM and D-hair receptors), which respond only to the moving stimuli, code the velocity of the ramp. For D-hair receptors the slope of the stimulus response was 0.033 \( \pm \) 0.0004 (linear regression analysis \( r^2 = 0.94; P < 0.0005 \)), for RAM fibers the slope was 0.022 \( \pm \) 0.0008 (linear regression analysis \( r^2 = 0.99; P < 0.0001 \)), and for SAM fibers the slope was 0.028 \( \pm \) 0.003 (linear regression analysis \( r^2 = 0.91; P < 0.0001 \)). Thus velocity-sensitive mechanoreceptors increase their firing by on average 10 spikes/s for changes in velocity between 220 and 330 \( \mu \)m/s. Interestingly, all these mechanoreceptors can detect extremely slowly moving stimuli as low as 10 \( \mu \)m/s, but D-hair receptors are the most reliable at detecting such slow movements (Dubreuil et al. 2004). Thus a larger proportion of D-hair receptors respond to the slowest moving stimuli, between 26 and 100 \( \mu \)m/s, than do RAMs (see Fig. 2, E and F, insets). Note that the firing rate/velocity plots do not include data from units that did not fire any spike for that stimulus (Fig. 2, D–F). There is no indication of desensitization of the RAM receptor response with repeated stimuli with increasing ramp speeds. The slope of the firing rate/velocity plot for SAM fibers is relatively shallow compared with that of RAM and D-hair receptors; this reflects the fact that SAM receptors also have a tonic firing response that cannot be separated from the phasic response of the receptor (Fig. 2D). Thus the mean firing rate for the slowest velocity stimulation is approximately the same as the mean firing rate to a 10-s-duration ramp-and-hold stimulation of the same amplitude (96 \( \mu \)m) (Fig. 2G). We next plotted the mean firing rate for a series of increasing displacements for all three receptor types that respond to static displacements, i.e., SAM, AM, and C-fibers (Fig. 2, G–I). Each receptor type has a characteristic stimulus response function with AM receptors having the highest mean firing rates, consistent with the findings of other groups (Slugg et al. 2000).

### Mechanical latency

For each receptor type we have systematically measured the latency between onset of the mechanical stimulus and the first spike corrected for conduction delay (Fig. 3A). We have termed this parameter mechanical latency and we measured its relationship to stimulus strength. We found that the mean mechanical latency behaved very differently in response to changes in stimulus strength in different receptor types (Fig. 3). For receptors with a purely phasic response (RAM and D-hair receptors) the mean mechanical latency was very small (10–12 ms) and the shortest latencies were found with the smallest stimuli used (Fig. 3, C and D). The mean mechanical latency in RAM and D-hair receptors appeared to increase with increas-
ing displacement amplitude but this was not significant (repeated-measures ANOVA; Fig. 3, C and D). Interestingly, mechanical latency in receptors with a slowly adapting response (SAM, AM, and C-fibers) behaved very differently from RAM and D-hair receptors to changes in stimulus strength. Thus for SAM and AM fibers mechanical latencies were initially very long and shortened to a plateau value as stimulus strength increased (Fig. 3, B, E, and F). The shortest mean mechanical latency and the stimulus strength at which it was observed were very characteristic for each receptor type: for example, the minimum mean mechanical latency observed for C-fibers (114 ms) was much longer than that found for SAM fibers (19 ms) and was observed at higher stimulus strengths for C-fibers, 192 μm compared with 48 μm for SAM fibers (Fig. 3, B and F). Thus for each receptor type we could estimate a minimum mean mechanical latency based on the response of a population of units to an ascending series of displacement stimuli. As with the data on the stimulus response properties we observed no quantitative difference in the response properties of receptors recorded from hybrid and C57Bl/6N mice (Fig. 3). For the analyses presented in the rest of this study we did not separate data obtained from hybrid or C57Bl/6N mice because the physiological properties of the receptors were, in all aspects studied, identical.

Mechanosensitivity of identified nociceptors
We grouped the data from all C-fiber nociceptors for the analysis shown in Figs. 2 and 3. However, we noticed after recording from a large number of C-M and C-MH nociceptors...
that the mechanosensitivities of these two types of nociceptors were not identical. Thus when the stimulus response function of C-M and C-MH fibers was plotted separately it was clear that C-M fibers have significantly larger mean responses to suprathreshold mechanical stimuli than do C-MH fibers (Fig. 4A). Thus at the highest stimulus strengths used (indentation > 96 μm) C-M fibers fired on average more than twice as many spikes/s as did C-MH fibers. The mean conduction velocities of these two populations of C-fibers were indistinguishable as was their mean von Frey threshold (Table 1). The data shown in Fig. 4A are derived from a subpopulation of C-fibers, recorded from C57Bl/6N mice, measured at 32°C under identical conditions in the same experimental series, although the same phenomenon of greater C-M sensitivity was consistently observed for all the C-fibers characterized in this study.

Recent studies have suggested that in rodents there is a subpopulation of fast conducting Aβ-fibers that have the physiological properties of nociceptors (Djouhri and Lawson 2004; Woodbury and Koerber 2003). Here we asked whether it is possible to identify a population of faster conducting Aβ nociceptors based purely on their stimulus response characteristics. We tentatively identified Aβ nociceptors as those SAM receptors (conduction velocity > 10 m/s) that did not fire an action potential during the movement phase of the ramp-and-hold stimulus until a stimulus strength of ≥ 48 μm. We reasoned that since SAM receptors classically have a robust phasic response to small-amplitude indentation then fibers failing to respond may represent a distinct group of receptors. We found that 28% of the SAM fibers recorded (15/53 units) fulfilled the above-cited criteria and, consistent with the idea that they represent a distinct nociceptors-like population, they had significantly higher median von Frey thresholds than those of the remaining SAMs (Fig. 4C). However, the stimulus response plots of putative SAM nociceptors compared with SAM and bona fide AM fibers were not significantly different (Fig. 4B). It thus appears to be difficult to reliably identify SAM nociceptors based solely on their response to suprathreshold mechanical stimuli.

**Mechanoreceptor sensitivity: effect of changing temperature**

In this study we compared the physiological properties of mechanoreceptors and nociceptors at two different temperatures: 24 and 32°C. The receptor was tested with a standard stimulation protocol at each temperature (increasing displacements omitting the velocity stimuli shown in Fig. 1). In a second series of experiments the response of a population of units tested at either temperature were compared. The outcome of these two experiments was essentially identical and so the data sets were pooled. We found no significant effect of changing the temperature on the stimulus response of RAM.
(n = 20 at 32°C and n = 8 at 24°C), SAM (n = 46 at 32°C and n = 24 at 24°C), D-hair (n = 22 at 32°C and n = 9 at 24°C), and AM fibers (n = 61 at 32°C and n = 28 at 24°C) (see Fig. 5C and Supplemental Fig. S2, A, C, E, and G). Indeed when we calculated the Q_{10} values for suprathreshold responses (RAM, SAM, and D-hair receptors) we found Q_{10} values for all these receptor types ranging from 1.2 to 1.3. The conduction velocity of A-fiber afferents also slows with lower temperature and the Q_{10} values for conduction velocity in the same fibers ranged from 1.2 to 1.6. The temperature dependence of mechanical latency was also measured for the same set of receptors and the results for low-threshold mechanoreceptors (RAM, SAM, and D-hair receptors) indicated that this parameter is very insensitive to temperature changes (Q_{10} = 1.0–1.1). We did find that mechanical latency was significantly longer at lower temperatures in AM-fibers and the Q_{10} value for this effect was 1.4 (Supplemental Fig. S2 and Fig. 5C).

The situation with unmyelinated C-fibers, however, was substantially different from that observed with myelinated
afferents. We examined a large number of C-fibers \((n = 44\) at 24°C and \(n = 81\) at 32°C) and found significantly greater suprathreshold firing rates at 32°C compared with those at 24°C (Fig. 5A). The \(Q_{10}\) for the stimulus response function for all recorded C-fibers was 1.6. It might be argued that the apparent difference in sensitivity at the two test temperatures reflects an oversampling of C-M fibers in one population because these fibers have higher suprathreshold responses than those of C-MH fibers. However, the proportion of C-M and C-MH fibers studied at the two temperatures (32°C, 65% C-MH; 24°C, 67% C-MH) was not significantly different (\(\chi^2\) test, \(P > 0.8\)).

For each receptor type we also measured the mean minimum mechanical latency at the two test temperatures (Fig. 5, B and C). The mean minimum mechanical latency, which is the shortest mechanical latency found at a characteristic amplitude for each receptor type with a myelinated axon (Fig. 3, B–F, vertical arrows), did not differ between the two test temperatures (Fig. 5C). This was reflected in little or no difference in mechanical latency recorded at all stimulus strengths, with the notable exception of AM-fibers (Supplemental Fig. S2H). In contrast to the results for myelinated afferents the mean mechanical latency for C-fibers at almost all stimulus strengths at 24°C was substantially longer than that found at 32°C (Fig. 5, B and C). We calculated the \(Q_{10}\) for C-fiber mechanical latency from these data to be 5.1 at a displacement of 386 \(\mu\)m. The same C-fibers also have slowed conduction velocities at lower temperatures but the \(Q_{10}\) for this effect was just 1.4. Thus we have found that in nociceptors, in particular unmyelinated nociceptors, the speed of transduction and suprathreshold mechanosensitivity are highly sensitive to even small temperature changes. This marked temperature dependence was completely absent in low-threshold mechanoreceptors.

C-fiber nociceptors have exceptionally long mean mechanical latencies (114 ms measured with a 192-\(\mu\)m displacement and ramp velocity of 1,435 \(\mu\)m/s). The unique and marked temperature dependence of mechanical latency in C-fiber nociceptors led us to ask whether increasing the speed of the stimulus ramp could further reduce the mechanical latency. By increasing the speed of the ramp from 1,435 to 2,945 \(\mu\)m/s, the fastest possible velocity attainable with the nanomotor should, in theory, decrease mechanical latency by 52% if the latency is purely dependent on the time it takes for the stimulus to reach threshold for the receptor. This condition was met for all the mechanoreceptors tested (RAM, SAM, and D-hair receptors) and also for nociceptive AM-fibers (Fig. 6, A and B). Strikingly, the fastest ramp stimulus applied to C-fibers, 2,945 \(\mu\)m/s, produced no significant reduction in the mean mechanical latency compared with the next slowest ramp (1,435 \(\mu\)m/s). Thus the mechanical latency in C-fiber nociceptors is unusually long compared with other mechanoreceptors and nociceptors. Furthermore, increasing stimulus speed, which decreases the time to reach firing threshold, does not further decrease mechanical latency in C-fibers, suggesting that there is a minimum latency for C-fibers that is in the range of 100 ms.

Effects of short-term inflammation on suprathreshold responses of cutaneous nociceptors

It has long been hypothesized that mechanical hyperalgesia that follows tissue injury may be due to the sensitization of peripheral nociceptors to mechanical stimulation. The carrageenan model of paw inflammation has often been used as a model to induce both thermal and mechanical hyperalgesia (Hamilton et al. 2001; Kocher et al. 1987; Koltzenburg et al. 1999). Behavioral hypersensitivity to mechanical stimulation has been reported to last for several hours after intradermal injection of 2% wt/vol carrageenan. We tested the sensitivity of single identified primary afferents between 2 and 6 h after induction of the inflammation. The sample of primary afferents recorded is summarized in Table 2. We measured the rates of spontaneous activity during interstimulus epochs for all nociceptive fibers (Fig. 7A). We found that C-M fibers had higher spontaneous rates of firing than those of other nociceptors and this probably reflects firing that continues after the mechanical stimulus is terminated. There was no difference in the ongoing discharge rates between C-M fibers innervating control and carrageenan-inflamed skin (Fig. 7A). However, we did note a significant increase in spontaneous activity in polymodal C-MH nociceptors innervating carrageenan-inflamed skin compared with control (Fig. 7A). We used a series of ascend-
ing displacement stimuli starting at threshold to characterize the stimulus response of nociceptors in inflamed and noninflamed skin—the protocol did not include different velocity ramps because nociceptors are insensitive to movement. We found no difference between the stimulus response functions of AM receptors in the inflamed and noninflamed skin (Fig. 7B).

We also noted no significant difference in the stimulus response of C-fibers recorded in inflamed and noninflamed skin (Fig. 7C). We plotted the suprathreshold stimulus response of C-MH units separately but, as for the total population of C-fibers, there was no difference in the suprathreshold response properties of C-MH fibers in inflamed skin compared with control (Fig. 7D). Like others we did not see any marked change in the von Frey thresholds of nociceptors in inflamed skin (Koltzenburg et al. 1999), but it might be argued that thresholds are subtly reduced, a change that might be reflected in a shortening of the mechanical latency. However, we measured, at all stimulus strengths, mechanical latencies of afferents from control and inflamed skin but observed no significant change in this parameter (data not shown). We did not quantify in detail the sensitivity of C-MH fibers to noxious heat but we did measure the total spike response to local application of heated solutions applied to the receptive field. We noted that C-MH fibers in inflamed skin have a slight but nonsignificant increase in their spiking rate to noxious heat (control 15.4 ± 2.0 spikes/s compared with 17.0 ± 3.1 spikes/s for C-MH fibers in inflamed skin). The inflammation produced by carrageenan may well change the compliance of the skin and such effects might mask changes in the mechanosensitivity of nociceptors. However, we also measured the stimulus response properties of mechanoreceptors in inflamed skin and found no significant difference compared with mechanoreceptors in control skin (Fig. 7E).

**FIG. 7.** Effect of short-term carrageenan inflammation on mechanosensitivity of nociceptors. A: the mean spontaneous activity recorded between the stimulus epochs is plotted for C-fibers recorded in intact and carrageenan-inflamed skin. Note that an increased level of spontaneous activity was observed only for C-MH fibers and this effect was statistically significant (unpaired t-test, \(P < 0.05\)). B–E: the stimulus response functions of AM, C-fiber, C-MH fibers, and low-threshold SAMs are plotted for units in intact skin (open squares) compared with carrageenan-inflamed skin (filled squares). There was no significant difference in the response properties of any of these nociceptors between control and inflamed skin (repeated-measures ANOVA, \(P > 0.20\)).
DISCUSSION

In this study we have recorded a large number of mechanoreceptors and nociceptive sensory neurons that have their axons in the saphenous nerve of the mouse. The large sample size is best illustrated by the fact that the total sample of recorded single units with A-fiber conduction velocities is in the same range as the total number of myelinated fibers present in the saphenous nerve of the mouse (~600 axons). We characterized the stimulus response properties of all the recorded units with a standard series of stimuli using a computer-controlled mechanical stimulator (Fig. 1). The use of the same series of stimuli for all mechanosensitive afferents has allowed us to compare various aspects of the stimulus response behavior of different mechanoreceptor types. We plotted the stimulus response properties of mechanoreceptors and nociceptors from an inbred mouse strain (C57Bl6/N) compared with a hybrid strain (C57Bl/6N and 129Sv) of mice. We found the results from these two data sets to be in agreement with each other. However, we do not conclude that complex genetic factors cannot influence the mechanosensitivity of sensory neurons.

To test the hypothesis that gene variants have an impact on mechanosensitivity of primary afferents, comparisons should be made between two recombinant inbred strains, preferably with different behavioral responses to acute mechanical stimuli. Other types of pain behavior can vary considerably with genetic background (Mogil et al. 1999a,b). We characterized the mechanical latency at different stimulus strengths for mechanoreceptors and nociceptors. We found that the relationship between mechanical latency and stimulus strength is very characteristic for different receptor types. In this study we also asked whether moderate changes in temperature affect the stimulus response functions of mechanoreceptors and nociceptors. Interestingly, we found that C-fiber nociceptors are unique in that their stimulus response properties appear to be very sensitive to moderate changes in temperature; the properties of all other receptors were essentially unchanged within the temperature range studied. Finally, we tested whether major changes in the stimulus response properties of mechanoreceptors and nociceptors could be observed following short-term inflammation of the skin with carrageenan (2–6 h). We conclude that no substantial sensitization of nociceptors occurs with short-term inflammation produced with the carrageenan model.

We have plotted the stimulus response functions of both mechanoreceptors and nociceptors in the present study. In the case of mechanoreceptors (RAM, SAM, and D-hairs) we provide evidence that these receptors are best tuned to detect the velocity of moving stimuli. We calculated rates of firing during the ramp phase of the stimulus series and show that this increases steadily with increasing speed of the ramp (Fig. 2, D–F). The increased mean rates that we calculated with very rapid ramps often occurred with few total spikes because the ramp duration is so short. Nevertheless, it is likely that the nervous system uses such rate information, from populations of sensory receptors, to estimate stimulus velocity. We noted that RAM and D-hairs have very short mechanical latencies even at small stimulus strengths (Fig. 3); this phenomenon may be relevant to judgments of stimulus direction because some studies indicate that it is the timing of individual spikes that is important for judging stimulus direction (Johansson and Birznieks 2004).

In the present study we chose a standard interstimulus period of 30 s. In a previous study we showed that all types of cutaneous receptors (mechanoreceptors and nociceptors) respond with an essentially constant spike number to repetitive mechanical stimulation applied with an interstimulus period of 60 s using the same mechanical stimulator (Shin et al. 2003). This is contrast to results from C-fiber nociceptors recorded in primate skin, where it was reported that a significant attenuation of the response was seen with interstimulus intervals of ≥300 s (Slugg et al. 2000). Interstimulus effects may be less marked in nonprimate species, but it is nevertheless possible that different stimulus response properties may have been measured in our study had we used longer interstimulus times. In this study we found C-M fibers that lack noxious heat sensitivity had higher suprathreshold sensitivity to mechanical stimuli than did C-MH fibers. This is the first evidence that transduction of mechanical stimuli by this subpopulation of C-fibers may be distinctive from other polymodal nociceptors.

It has been recently shown that a proportion of sensory axons with fast conducting Aβ fibers in rodents may have some characteristics of nociceptors. For example, a proportion of Aβ fibers, estimated to be around 20% of the total, have unexpectedly broad action potentials and in some cases may have synaptic terminals in nocireceptive laminae in the spinal cord (Djouhri and Lawson 2004; Fang et al. 2005; Woodbury and Koerber 2003). We tried to identify Aβ-nociceptors purely on the basis of their mechanosensitivity and assumed that Aβ-nociceptors might be those SAM fibers that lack a marked phasic response. Such fibers were found to have von Frey thresholds that were on average higher than those found in conventional SAMs (Fig. 4C). Nevertheless, these putative Aβ-nociceptors have stimulus response function that are quantitatively indistinguishable from SAMs and von Frey threshold alone would be insufficient to identify such fibers. It should be noted that the effects of putative Aβ-nociceptors in producing painful sensation have not been tested and that mild A-fiber stimulation with small-amplitude electrical stimulation in humans has not been reported to lead to the sensation of pain.

Mechanical latency as measured here has so far not been systematically analyzed in previous studies on single units. It is clear that the ramp speed will to a large extent determine the mechanical latency because the faster the ramp, the more rapidly mechanical threshold is reached. For this reason we used very rapid ramp stimuli, for most experiments 1.435 μm/s, to measure the shortest possible mechanical latency for each receptor type. In general the receptors with the shortest mechanical latencies were found to be rapidly adapting (RAM and D-hair receptors), which can transform the smallest displacements (e.g., 12 μm) into action potentials in ≤11 ms (Fig. 3, C and D). There was a nonsignificant tendency for the mechanical latency in these receptors to increase with increasing stimulus amplitude, which may reflect desensitization in a subpopulation of the fibers studied. All other receptor types displayed decreasing mechanical latency with increasing displacement amplitude, a behavior that has also been noted for the receptor current in invertebrate receptors (O’Hagan et al. 2005). SAMs, in contrast, were found to have quite long mechanical latencies compared with RAMs and a relatively large stimulus strength of 48 μm was required for the fastest
response (~19 ms) (Fig. 3B). The shortest mean mechanical latencies found in nociceptors were between 5- and 10-fold longer than those found for most mechanoreceptors at between 49 and 114 ms. Mechanical latency probably reflects a two-step process at the receptor membrane; first suprathreshold mechanical stimuli induce a depolarizing receptor potential, presumably due to direct gating of mechanosensitive ion channels (Hu and Lewin 2006; Hu et al. 2006). Second, the depolarizing receptor potential must also be large enough to initiate action potentials. The biophysical properties of the ending and presence of voltage-gated channels may considerably influence the transformation of the receptor potential into action potentials and there may be a delay between the receptor potential and action potential initiation (Loewenstein and Skalak 1966). It is interesting to note that mechanosensitive currents that have been described in cultivated sensory neurons in vitro are generally very rapidly activated, regardless of whether they are found in putative nociceptors or mechanoreceptors (Drew et al. 2002, 2004; Hu and Lewin 2006). Indeed slowly adapting mechanosensitive currents that are pharmacologically distinct from rapidly inactivating mechanosensitive channels (Drew et al. 2007; Hu and Lewin 2006) are normally activated within 1 ms of stimulus onset (Hu and Lewin 2006). Nevertheless, mechanical stimulation of cultured sensory neurons showed that action potential initiation often occurs with a considerable delay (>50 ms) in nociceptors with slowly adapting mechanosensitive currents (Hu and Lewin 2006). The very long mechanical latencies that we have observed here for C-fiber nociceptors may be explained by a different complement of voltage-gated channels in nociceptors that tends to delay action potential initiation. For most receptors studied the mechanical latency decreased when the ramp speed was increased from 1,435 to 2,945 μm/s. The change in mechanical latency observed with increasing ramp speed is entirely accounted for by the fact that threshold is reached earlier; thus in theory even faster ramp speeds, >2,935 μm/s, should also further decrease mechanical latency (Fig. 6). A striking exception to this rule was the behavior of mechanical latency in C-fiber nociceptors; here, increasing ramp speed >1,435 μm/s did not lead to any reduction in mechanical latency (Fig. 6).

It has been consistently observed that the activation energy for mechanotransduction in vertebrate and invertebrate sensory receptors is very high compared with that of voltage-sensitive channels (Hoger and French 1999; Inman and Peruzzi 1961; Ishiko and Loewenstein 1961; Ottoson 1965). This is reflected by Q10 values >3.0, measured for the amplitude of receptor potentials in the Pacinian corpuscle and spider mechanoreceptors (Hoger and French 1999; Ishiko and Loewenstein 1961). In our study we tested the mean stimulus response functions at two test temperatures (24 and 32°C) separated by 8°C. The mechanosensitivity of all the receptor types but one, C-fiber nociceptors, did not differ significantly between these two test temperatures (data not shown). The temperature sensitivity of the stimulus response function for C-fibers was especially striking at higher stimulus strengths >100 μm, but the Q10 value for this effect was still relatively modest at 1.6 (Fig. 5A). The parameter that was most sensitive to temperature in C-fibers was mechanical latency, which was considerably elevated at all stimulus strengths when measured at 24°C compared with 32°C (Fig. 5, B and C). Indeed the Q10 value for this effect in C-fibers was very large at 5.1. Interestingly, the mechanical latency of other receptors was not significantly influenced by temperature (Fig. 5C and Supplemental Fig. S2), which suggests that mechanical latency in C-fibers reflects distinctive physiological properties of the ending. It has recently been shown that the nociceptor-specific voltage-sensitive sodium channel NaV1.8 has a unique sensitivity to temperature compared with other sodium channels (Zimmermann et al. 2007). It is possible that the unique behavior of mechanical latency in C-fiber nociceptors, both in terms of temperature dependence and long latency, are related to the presence of such channels in C-fiber nociceptors. Experiments using mice with null mutations in the genes coding for these channels would be required to test this hypothesis. Alternatively, since C-fibers often possess a slowly adapting mechanosensitive current not found in mechanoreceptors (Hu and Lewin 2006), the extreme temperature dependence of mechanical latency might also reflect a selective temperature dependence of this current. So far the temperature dependence of mechanosensitive currents and stretch-sensitive currents present in DRG cells has not been tested.

In the last part of this study we asked whether short-term inflammation induced with carrageenan can lead to changes in stimulus response properties of sensory receptors in the mouse skin. Earlier studies in the oral mucosa have indicated that the mechanosensitivity of nociceptors can be altered by acute carrageenan-induced inflammation (Cooper et al. 1991). The effects of inflammation on suprathreshold responses of skin nociceptors have only been rarely examined (Andrew and Greenspan 1999; Lewin and Moshourab 2004). In the only study to date where a quantitative analysis of suprathreshold responses was made using a computer-controlled stimulator increases in sensitivity were observed after a chronic Freund’s adjuvant inflammation (Andrew and Greenspan 1999). Several studies have noted signs of sensitization as reflected by slightly lowered von Frey hair thresholds of single fibers innervating inflamed skin (Kocher et al. 1987; Wenk et al. 2006). We also measured the von Frey threshold of populations of C-fiber afferents innervating inflamed and intact skin but found no significant differences (Table 2). In addition we found no indication of changes in the suprathreshold responses of nociceptors in carrageenan-inflamed skin (Fig. 7). We have used displacement stimuli in this study and we adjusted the mechanical stimulus to start from the smallest displacement that can evoke a spike, i.e., at threshold. It is therefore possible that subtle changes in the mechanical threshold could mask changes in the stimulus response function measured. The lack of effects on mechanosensitivity observed here may also be related to the time course or intensity of inflammation. It is possible that our measurements are confounded by changes in the compliance of the inflamed tissue that has been described (Andrew and Greenspan 1999; Cooper et al. 1991). Tissue compliance changes may also be expected to affect the coding properties of nonnociceptive afferents, although we did not observe any changes in the sensitivity of mechanoreceptors to suprathreshold stimuli (Fig. 7E).

In conclusion this study provides further quantitative data on the response properties of large numbers of different receptor types to a wide range of controlled mechanical stimuli. We show that the behavior of the mechanical latency in single sensory fibers is very characteristic for each receptor type. We also show that mechanical latency is...
extraordinarily long in C-fiber nociceptors (>100 ms) and does not shorten with increasing stimulus speed as is observed for other receptors. It remains to be determined why C-fibers are relatively slow in transducing mechanical signals into action potentials. However, the striking and unique temperature dependence of mechanical latency in C-fibers suggests that mechanoelectrical transformation in these fibers is quite different from that found in other mechanosensitive sensory neurons.

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REFERENCES


