Distinct Mechanosensitive Properties of Capsaicin-Sensitive and -Insensitive Sensory Neurons

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Mechanical stimulation of the somata of cultured neonatal rat dorsal root ganglia (DRG) neurons evoked inward cationic currents that displayed distinct properties between different subsets of cells. The presumptive nociceptor population, defined by capsaicin sensitivity, showed higher thresholds for the induction of an inward current and lower peak currents than other mechanosensitive neurons. A subset of capsaicin-sensitive IB4-positive sensory neurons was refractory to mechanical stimulation. All mechanically activated currents were blocked by gadolinium (IC_{50} ~8 μM) and ruthenium red (IC_{50} ~3 μM). Disruption of the actin cytoskeleton by acute application of 10 μM cytochalasin B inhibited currents much more effectively in capsaicin-insensitive (61%) than capsaicin-sensitive neurons (20%). Extracellular calcium also attenuated mechanosensitive currents and to a greater degree in capsaicin-insensitive neurons than capsaicin-sensitive neurons. These data demonstrate that the somata of different types of cultured sensory neurons have distinct mechanosensitive phenotypes that retain properties associated with nerve terminal mechanosensors *in vivo*.

Key words: mechanosensation; ion channel; DRG; capsaicin; nociception; IB4; ASIC

Dorsal root ganglia (DRG) contain the cell bodies of sensory neurons. Most DRG neurons respond to mechanical stimulation and can be broadly classified as low threshold mechanoreceptors and high threshold nociceptors. Mechanical stimulation is thought to directly activate mechanosensitive ion channels expressed on the receptive endings of these neurons. However, the molecular identity of these ion channels remains uncertain, and their physiological and pharmacological properties are poorly understood (Lewin and Stucky, 2000).

Recent studies suggest a role for members of the acid-sensing ion channel (ASIC) family in mechanosensation. Animals lacking the gene for ASIC2/BNC1 show reduced firing frequencies in rapidly adapting and, to a lesser extent, slowly adapting mechanoreceptors in response to mechanical stimulation (Price et al., 2000). Null mutant ASIC3/dorsal root acid-sensing ion channel (DRASIC) mice show diminished mechanosensitivity of A-fiber mechanonociceptors and an enhanced response of rapidly adapting mechanoreceptors (Price et al., 2001). However, to date there is no evidence that ASICs can be directly gated by pressure.

Receptors normally found on the peripheral terminals of DRG neurons *in vivo* are often expressed on the cell bodies of these neurons *in vitro*. For example, high temperatures (>42°C) applied to somata of sensory neurons in culture activate an inward cationic current (Cesare and McNaughton, 1996). These currents are mediated by the capsaicin receptor (VR1) that is expressed on nociceptor terminals (Tominaga et al., 1998). Therefore the somata of cultured sensory neurons were examined as a system for the study of mechanically activated (MA) currents.

McCarter et al. (1999) have shown previously that cultured DRG neuron somata respond to mechanical stimulation with an inward cationic current. In this study we extend these findings to show that there is diversity among the responses of DRG neurons to pressure *in vitro* that correlates with aspects of their *in vivo* properties.

MATERIALS AND METHODS

Cell culture. Neonatal Sprague Dawley rats were decapitated, and 25–35 DRG were taken from each animal. DRG were enzymatically digested in 1 mg/ml collagenase D (Roche), and neurons were isolated by mechanical trituration. Cells were cultured on poly-L-lysine- and laminin-coated dishes in the presence of NGF (100 ng/ml) and used the day after preparation. Superior cervical ganglia (SCG), from postnatal day (P) 14–21 rats, were prepared in the same way.

Electrophysiology. Medium-sized neurons (diameter >30 μm) or small neurons (diameter <30 μm) with cell bodies that were not in contact with those of other neurons were selected for recording. Recordings were made using an Axopatch 200B amplifier at a holding potential of −70 mV. Data were acquired at 20 kHz using PClamp software (Axon Instruments).

Experiments were performed in the perforated-patch configuration. MA currents recorded using this technique were stable for considerably longer (up to 30 min) than those recorded using the conventional whole-cell configuration. The pipette solution contained (in mM): 110 methane-

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sulfonic acid, 30 KCl, 1 $MgCl_2$ and 10 HEPES, pH 7.35 (pH was corrected using KOH; final K^+ concentration 140 mM); 200 μg/ml amphotericin B was added immediately before recording. Series resistance was typically 5–10 MΩ and was compensated for by 40–60%. Standard extracellular solution contained (in mM): 140 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 4 D-glucose (added fresh), and 10 HEPES, pH 7.4. Solutions were changed in ~2–3 sec using a system whereby multiple tubes flowed into a single output that perfused the cell.

Neurons were mechanically stimulated using a heat-polished glass electrode (tip diameter ~5–6 μm) positioned at an angle of 60° to the surface of the culture dish. Pressure was applied using a computer-controlled Piezo-electric crystal drive (Burleigh). The probe was positioned such that a 10 μm movement did not visibly contact the cell but a 12 μm stimulus produced an observable membrane deflection. A 12 μm probe movement was defined as a 2 μm stimulation, 14 μm was defined as a 4 μm stimulus, and so on. Stimulation was at a rate of 0.5 μm/msec, and the stimulus lasted 200 msec. To assess the mechanical sensitivity of a cell, a series of six mechanical steps in 2 μm increments were applied at 15 sec intervals, which was sufficient time for full current recovery. For other experiments, cells that showed a reproducible response to mechanical stimuli (>200 pA stimulated at 20 sec intervals) were selected for further experimentation.

Gadolinium ($GdCl_3$), amiloride, ruthenium red, cytochalasin B, and gentamicin (all from Sigma) were dissolved in extracellular solution. Concentration-inhibition curves for ruthenium red and Gd^{3+} were fitted using the Langmuir equation. Capsaicin (Calbiochem) was dissolved in DMSO (10 mM) and applied at 1 μM after mechanical stimulation of the cell (a clear, inward current >50 pA was defined as a positive response). Capsaicin was used to distinguish presumptive nociceptive and non-nociceptive neurons, because at P1–2 there is no clear correlation between action potential properties and DRG neuronal phenotype (Ritter et al., 2000). pH 5.2 solution was also used to stimulate neurons; this was applied before capsaicin.

IB4 labeling was achieved by incubating the cells in IB4-Alexa 488 (Molecular Probes) 3 μg/ml in standard external solution for 10 min before recording. Cells were then washed in external solution three times. For control experiments responses were recorded, and then 4.5 μg of IB4-Alexa in 100 μl was added to the 1.5 ml bath solution for 10 min before perfusion of the cells with control solution.

RESULTS

Perforated-patch recordings from cultured DRG neurons have demonstrated that most of these cells are mechanically sensitive. In 92% (66 of 72) of medium-sized neurons tested, mechanical stimulation evoked an inward current. Of cells that responded, the majority (86%; 57 of 66) had evoked currents characterized by an initially rapidly adapting (RA) phase followed at high stimulation intensities by a sustained component (Fig. 1*A*, *right*). The remaining 14% (9 of 66) of neurons expressed MA currents that were slowly adapting (SA) ($t_{0.5}$ of adaptation >200 msec) (Fig. 1*A*, *left*). Mechanical stimulation of SCG neurons did not evoke an inward or outward current ($n = 10$).

Division of medium-sized neurons into presumptive nociceptive and non-nociceptive populations according to their sensitivity to capsaicin (1 μM) revealed clear differences between these groups. Of those neurons that did not respond to capsaicin (Caps−), 95% (38 of 40) responded to mechanical stimulation, whereas 88% (28 of 32) of capsaicin-sensitive (Caps+) neurons responded. All neurons that displayed SA MA currents were insensitive to capsaicin and considered a separate population for analysis.

The three groups showed differing sensitivities to mechanical stimulation (two-way ANOVA, repeated measures; $p < 0.001$) (Fig. 1*B*). The most striking difference was in the amplitude of RA MA currents between Caps+ and Caps− populations of cells (Fig. 1*B*). In Caps− cells with RA MA currents, currents were consistently much larger than those in the Caps+ population (Fig. 1*B*). The amplitude of SA MA currents showed large variability but was intermediate between RA groups. At the maximum stimulation the mean amplitudes of MA currents were as follows: Caps−, RA 2.62 ± 0.50 nA; Caps−, SA 1.28 ± 0.38 nA; and Caps+ 0.57 ± 0.11 nA. All cells that responded to peak mechanical stimulation with currents >2 nA were Caps− (16 RA, 2 SA).

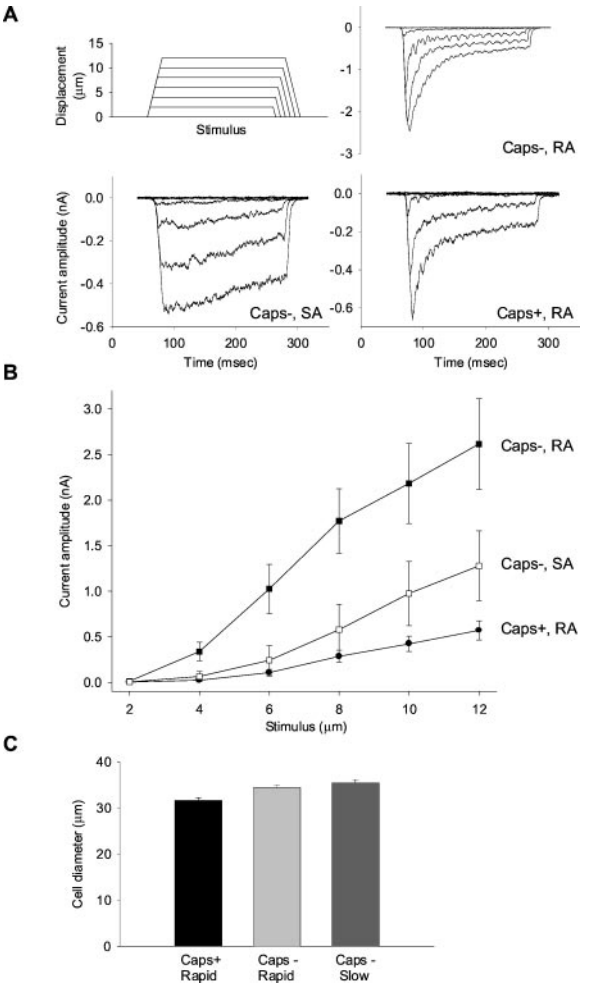

Figure 1. Subpopulations of DRG neurons demonstrate different levels of mechanosensitivity. *A*, Representative traces from three subpopulations of DRG neurons. *Right panel*, *Top* and *bottom traces* show RA currents from Caps− and Caps+ neurons, respectively. The *left panel* shows an SA current; note the differences in scale. *B*, The mean amplitude of RA MA currents in Caps− neurons (■, $n = 31$) was significantly larger than in Caps+ neurons (●, $n = 32$). MA currents of Caps− neurons with SA responses (□, $n = 9$) were intermediate (two-way, repeated measures ANOVA; $p = 0.001$). *C*, Caps+ neurons (*black bar*; diameter 31.7 ± 0.6 μm) were significantly smaller than Caps− neurons with either SA (*dark gray bar*; 35.5 ± 0.7 μm) or RA (*light gray bar*; 34.4 ± 0.6 μm) MA currents by 10.8 and 8.0%, respectively (*t* test; $p < 0.001$ and $p = 0.002$, respectively).

At low levels of stimulation, significantly more Caps− than Caps+ neurons displayed MA currents, consistent with a lower threshold of mechanical activation. With a 4 μm displacement, 47% of Caps−, RA neurons responded with currents >50 pA, which was significantly more than Caps+ neurons (13%) and Caps−, SA neurons (11%) (χ^2; $p < 0.05$).

Differences in mechanosensitivity were not attributable to cell size (Fig. 1*C*). Although Caps+ cells (diameter 31.7 ± 0.6 μm) were significantly smaller than Caps− neurons with RA MA currents (34.4 ± 0.7 μm), the difference was only 8.0% (*t* test; $p = 0.02$). This small difference is unable to account for the large differences in current amplitude. Furthermore, MA current ampli-

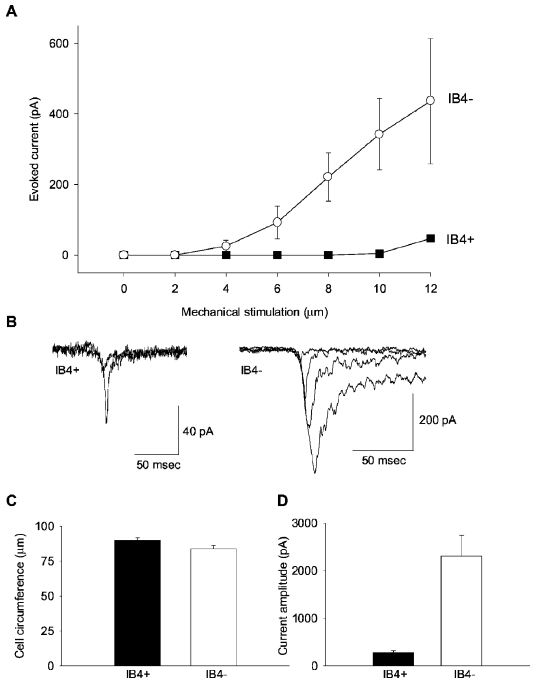

Figure 2. IB4+ and IB4− nociceptors respond differently to mechanical stimulation. *A*, The mean amplitude of MA currents in IB4−, Caps+ neurons (○, $n = 8$) was significantly larger than those seen in IB4+, Caps+ neurons (■, $n = 9$) (two-way, repeated measures ANOVA; $p < 0.001$). IB4+, Caps+ neurons were mostly refractory to mechanical stimulation, whereas the responses seen in IB4−, Caps+ small neurons were similar to those seen in medium-sized Caps+ neurons. *B*, Representative traces from an IB4+ and an IB4− cell. *C*, There was no significant difference in the diameter of IB4+ (*black bar*; 28.6 ± 0.6 μm) and IB4− (*white bar*; 26.7 ± 0.7 μm) neurons (*t* test; $p = 0.053$). *D*, The mean amplitude of responses evoked by 1 μM capsaicin was significantly larger in IB4− cells (2306.3 ± 437.8 pA; $n = 8$) than in IB4+ cells (301.1 ± 41.4 pA; $n = 9$) (*t* test; $p < 0.001$).

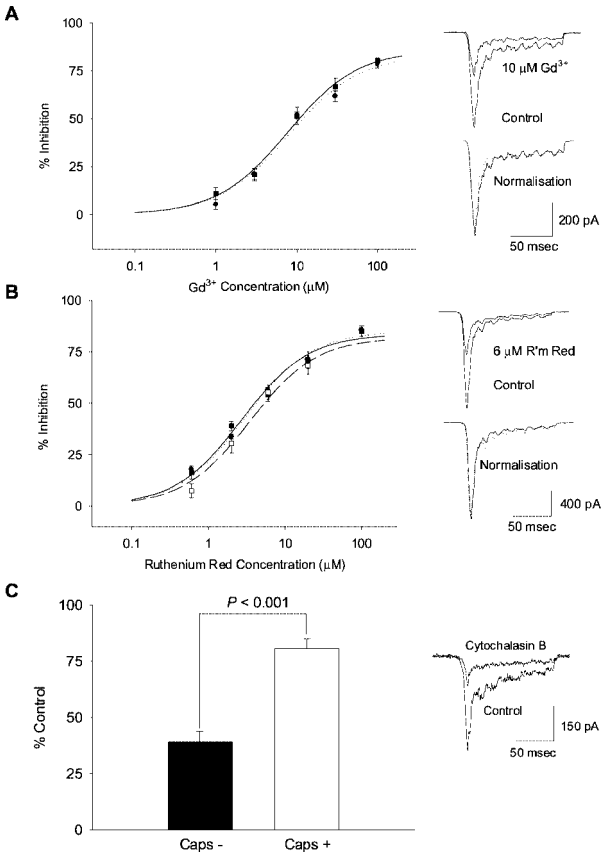

Figure 3. Ruthenium red, gadolinium, and cytochalasin B inhibit MA currents. *A*, *Left*, The concentration dependence of block of RA MA currents in Caps+ (●) and Caps− (■) neurons by Gd^{3+} (1–100 μM) was indistinguishable. The derived IC_{50} values were 7.99 μM (Caps+) and 7.77 μM (Caps−). $n = 3$–6 for each data point. *Right*, Typical *trace* of inhibition by Gd^{3+}. Also shown is the *trace* normalized to peak current showing equal blockade of transient and sustained components of the currents. *B*, Ruthenium red (0.6–100 μM) blocked RA and SA MA currents with similar efficacy. Derived IC_{50} values were 2.97 μM (Caps+, RA; ●), 2.71 μM (Caps−,RA; ■), and 3.45 μM (SA; □). $n = 2$–7 for each data point. *Right*, Typical *trace* of inhibition by ruthenium red and currents normalized to peak current. *C*, *Left*, Cytochalasin B (10 μM) had an inhibitory effect on MA currents that was more pronounced in Caps− neurons. In Caps− neurons (*black bar*), MA currents were inhibited by 60.9 ± 4.7% ($n = 6$) (significantly less than control; $p < 0.001$) and by 19.5 ± 4.5% in Caps+ cells (*white bar*; significantly less than control; $p < 0.05$). Comparison of the drug effect in the two groups showed that the effect of cytochalasin B was significantly greater in the Caps− neurons ($p < 0.001$). *Right*, Typical *trace* of inhibition by cytochalasin B in a Caps− neuron.

tude did not correlate with cell diameter either overall or within groups.

We examined the mechanically evoked responses of smaller capsaicin-sensitive neurons and distinguished cells according to whether they bound IB4, an isolectin that labels a subpopulation of nociceptive neurons (Molliver et al., 1997). We found that the responses of Caps+, IB4− cells did not significantly differ from those of medium-sized Caps+ neurons. However, those Caps+ neurons that were labeled with IB4 showed little or no response to mechanical stimulation (Fig. 2*A*,*B*). There was large variation in the size of MA currents in Caps+/IB4− cells, but all were mechanically sensitive. It was not possible to determine whether IB4+ neurons respond to higher levels of mechanical stimulation, because stimuli >12 μm are liable to dislodge the cell from the substrate or disrupt the seal. To control for the possibility that IB4 blocks the underlying ion channels, mechanical responses were recorded in six Caps+ neurons before staining with IB4. Of these, two that were unlabeled responded to a 10 μm mechanical displacement with currents >100 pA and to a 12 μm stimulus with currents of 254 and 389 pA, respectively. Conversely, of the four neurons that were subsequently labeled with IB4, two did not respond to mechanical stimulation, and of the other two the maximal evoked current was 160 pA. There was also a large difference in the amplitude of currents evoked by 1 μM capsaicin between these two populations; IB4+ neurons had a mean current amplitude of 0.30 ± 0.04 nA, whereas IB4− cells had a larger mean response of 2.31 ± 0.44 nA (*t* test; $p < 0.001$) (Fig. 2*C*). IB4+ and IB4− cells were not significantly different in size (Fig 2*D*).

Gd^{3+} and ruthenium red both reversibly blocked MA currents with IC_{50} values of <10 μM (Fig. 3). The blockade of RA MA currents in Caps− and Caps+ cells by Gd^{3+} (1–100 μM) was very similar (Fig. 3*A*): the derived IC_{50} values were 7.99 μM (Caps+) and 7.77 μM (Caps−). Likewise the blockade of MA currents by ruthenium red (0.6–100 μM) was similar in all three subpopulations of neurons (Fig. 3*B*). Derived IC_{50} values were 2.97 μM (Caps+, RA), 2.71 μM (Caps−, RA), and 3.45 μM (SA). Amiloride up to 500 μM and gentamicin up to 100 μM did not block MA currents.

The role of the cytoskeleton in the activation of mechanically evoked responses was examined by acutely applying the inhibitor of actin polymerization cytochalasin B (10 μM) (Fig. 3*C*). This

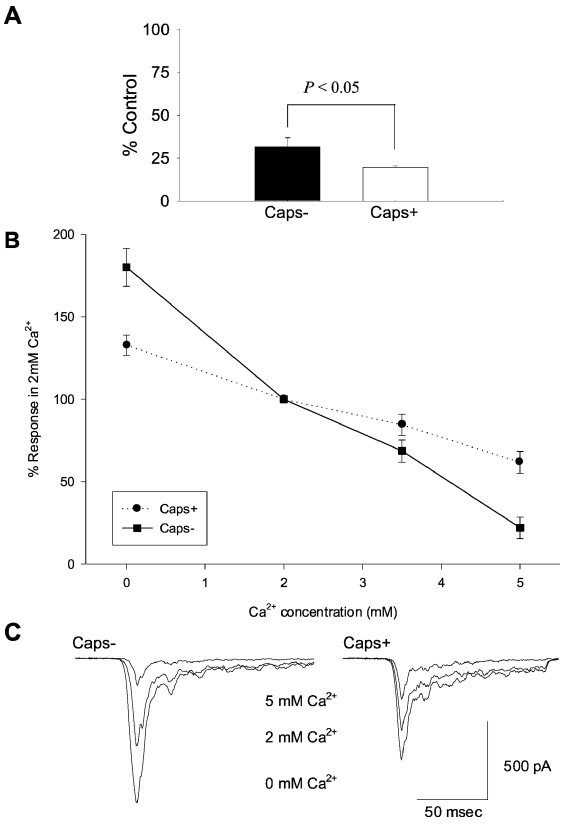

Figure 4. External Ca^{2+} had a modulatory effect on MA current amplitude. *A*, Removal of Na^+ from the external solution (control: $[Ca^{2+}]$ = 2 mM) caused a reduction in MA currents of 80.3 ± 0.9 and 68.3 ± 5.1% in Caps+ (*white bar*; $n = 5$) and Caps− (*black bar*; $n = 5$) neurons, respectively. The level of reduction was significantly different between the two groups (*t* test; $p < 0.05$). *B*, The amplitude of MA currents was largest in nominally Ca^{2+}-free solution, and increasing external Ca^{2+} concentration inhibited MA currents. The effect of Ca^{2+} was significantly greater in Caps− neurons than in Caps+ neurons (two-way ANOVA; $p < 0.01$) (n = 3–8 for each data point). *C*, Representative *traces* from a Caps− (*left panel*) and Caps+ (*right panel*) neuron showing MA currents evoked in nominally 0, 2, and 5 mM Ca^{2+}.

significantly inhibited MA currents in Caps+ neurons by 19.5 ± 4.5% ($n = 6$; *t* test; $p < 0.05$) and in Caps− neurons by 60.9 ± 4.7% ($n = 6$; $p < 0.001$). Notably, the effect of cytochalasin B was substantially larger in Caps− neurons (*t* test; $p < 0.001$).

The ionic basis of MA RA currents was investigated using ionic substitution experiments. Replacement of external Na^+ with the impermeant cation *N*-methyl-D-glucamine led to a large reduction in the amplitude of all currents (Fig. 4*A*). However, there was a significantly larger reduction in the amplitude of currents in Caps+ cells (80.3 ± 0.9%) than in Caps− cells (68.3 ± 5.1%) (*t* test; $p < 0.05$). Manipulation of the external Ca^{2+} concentration revealed that MA current amplitude was inversely correlated to Ca^{2+} concentration (Fig. 4*B*,*C*). This effect was significantly more pronounced in Caps− neurons than in Caps+ neurons (two-way ANOVA; $p < 0.05$). In nominally Ca^{2+}-free external solution, current amplitude increased, relative to 2 mM Ca^{2+}, by 80.1 ± 11.5 and 32.8 ± 6.1% in Caps− and Caps+ cells, respectively. Consistent with a blocking effect of Ca^{2+}, increasing Ca^{2+} concentration to 5 mM led to a reduction in current amplitude of 38.3 ± 6.6 and 78.0 ± 6.6% in Caps+ and Caps− cells, respectively.

The putative mechanosensory ASICs are activated by low pH (Waldmann and Lazdunski, 1998). However, we found that neither the amplitudes nor the kinetics of MA currents were related to those of proton-gated currents ($n = 14$). Interestingly, the amplitude of currents evoked by mechanical stimulation with an external pH of 5.2 or 6.4 did not differ from those recorded at pH 7.4 (n = 10).

DISCUSSION

Presumptive non-nociceptive and nociceptive neurons, defined by their capsaicin sensitivity, showed clear differences in their mechanosensitive properties. These differences were consistent with the *in vivo* phenotypes of DRG neurons. Caps− neurons expressed larger mechanically evoked responses than Caps+ neurons, and a large proportion of these cells responded to low levels of stimulation, whereas the majority of Caps+ neurons responded only to higher levels of pressure. *In vivo* non-nociceptive mechanoreceptors detect multiple forms of pressure change and are characterized by low thresholds to mechanical activation. Conversely, nociceptors, have high thresholds of mechanical activation (Lewin and Stucky, 2000).

Approximately one-fourth of capsaicin-insensitive neurons responded to mechanical stimulation with SA currents. This mixture of response kinetics is of interest given the diversity of non-nociceptive mechanoreceptor subtypes seen *in vivo* (Koltzenburg et al., 1997). The identity of the cells with SA MA currents is unclear. They could correspond to slowly adapting mechanoreceptors or, given their intermediate mechanosensitivity, to Aδ neurons. Not all nociceptive neurons are capsaicin sensitive [~75% are VR1 positive (Guo et al., 1999)]; therefore, the Caps− population must include some nociceptive neurons that may account for those cells that did not respond or responded weakly to mechanical stimulation.

There was a clear divergence in mechanical sensitivity between IB4+ and IB− cells in the Caps+ population. IB4+ cells were mostly refractory to mechanical stimulation, whereas IB4−/Caps+ cells responded to pressure. However, it is known that IB4+ neurons respond to high levels of mechanical stimulation *in vivo* (Gerke and Plenderleith, 2001). It is possible that IB4+ cells have higher mechanical thresholds that were not reached because of the danger of detaching the cell from the substrate or losing the seal. Another possible explanation is that these neurons respond to high levels of pressure via the release of a chemical mediator. One candidate for this role is ATP. Approximately 90% of IB4+ neurons display P2X3 or P2X2/3 currents (Burgard et al., 1999), and there is evidence that P2X3 receptors are central to mechanosensation in the bladder through activation by mechanically evoked ATP release (Vlaskovska et al., 2001). The observed differences in mechanical sensitivity and in the amplitude of capsaicin-evoked responses between IB4+ and IB4− neurons also extend findings by Stucky and Lewin (1999) showing differences in heat sensitivity and voltage-activated sodium currents between these populations.

DRG MA currents are blocked by Gd^{3+} and ruthenium red. Gd^{3+} blocks mechanosensitive ion channels in a range of systems (Hamill and Martinac, 2001); it blocks DRG MA currents with an IC_{50} of ~8 μM in both Caps+ and Caps− neurons. Interestingly, there is evidence that Gd^{3+} antagonizes ASIC3/2a-mediated currents (Babinski et al., 2000) and also inhibits transient low-pH evoked responses in DRG (our unpublished observations). Ruthenium red, a known antagonist of VR1 (Caterina et al., 1997), blocked MA currents with an IC_{50} of ~3 μM for both populations of RA currents and SA currents. IC_{50} values for ruthenium red obtained here are at least 10-fold higher than those found for

VR1 (Dray et al., 1990). The similarity in the pharmacology of MA currents among different subpopulations of neurons suggests that closely related ion channels mediate these currents. DRG MA currents are not blocked by gentamicin, suggesting they are not mediated by the ion channels that underlie cochlear mechanotransduction (Jaramill and Hudspeth, 1991).

MA currents are primarily mediated by Na^+ ions, but there is a significant contribution from Ca^{2+}, more so in the Caps− neurons. Furthermore, Ca^{2+} had a modulatory effect on the amplitude MA currents. Up to 5 mM, MA current amplitude was inversely related to the external Ca^{2+} concentration. Moreover, currents in Caps− cells appeared more sensitive to changes in external Ca^{2+} levels. These data suggest that Ca^{2+} has a blocking effect on the underlying ion channels. This may be attributable to slow permeation by Ca^{2+} effectively blocking the passage of Na^+. Similarly, Ca^{2+} ions have been demonstrated to block Na^+ channels where they bind to a site within the pore (Campbell and Hille, 1976).

It is proposed that the mechanically activated currents observed here are caused by activation of mechanosensitive ion channels that underlie mechanotransduction at the sensory terminal *in vivo*. However, it is likely that the cytoarchitecture of the sensory terminal, the surrounding extracellular matrix, and association of the terminal with auxiliary cell types (e.g., Pacinian corpuscles, Merkel cells, etc.) will modify the transmission of forces to the transduction site (Loewenstein and Skalak, 1966). All or some of these factors may well affect the kinetics of mechanically evoked responses *in situ*. We found that the actin cytoskeleton plays an important role in channel gating by treating cells with cytochalasin B. Inhibition of actin polymerization inhibited MA currents in Caps+ neurons and to a much greater degree in Caps− neurons. This suggests that differential tethering of mechanosensitive ion channels to the cytoskeleton may be important in setting the sensitivity of the channels.

Recent data suggest that members of the ASIC family of ion channels play a critical role in mechanosensation (Price et al., 2000, 2001). The ionic basis of MA currents is similar to those of some proton-activated ASIC currents (Waldmann and Lazdunski, 1998). Furthermore, Immke and McCleskey (2001) have shown recently that ASIC-mediated currents in ischemia-sensing neurons are modulated in a manner similar to MA currents by changes in external Ca^{2+} (and Mg^{2+}) concentration, and Berdiev et al. (2001) have shown that Ca^{2+} blocks recombinant ASIC2 channels. We found that the amplitude of MA currents is not correlated with the amplitude of low pH-evoked responses in DRG and that MA currents are not regulated by acidification of the external solution. However, ASIC2a (Price et al., 2000; Garcia-Anoveros et al., 2001) and ASIC3 (Price et al., 2001) are present on the endings of Aβ fibers *in vivo*, and these neurons are not activated by low pH. This observation raises the possibility that ASICs may be able to exist in a proton-insensitive state that is mechanosensitive (Welsh et al., 2002). Those ion channels that are mechanosensitive may not respond to protons because of their inclusion in a complex of molecules that masks the proton-binding site. These two states may display differing sensitivities to amiloride blockade.

In conclusion, these results confirm that cultured DRG neurons are a system in which mechanotransduction can be studied and have revealed a number of physiological and pharmacological properties of mechanosensitive currents. Further analysis of this system should enable the molecular identity of DRG mechanosensitive ion channel components to be established

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