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The DRASIC Cation Channel Contributes to the Detection of Cutaneous Touch and Acid Stimuli in Mice

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Summary

Cation channels in the DEG/ENaC family are proposed to detect cutaneous stimuli in mammals. We localized one such channel, DRASIC, in several different specialized sensory nerve endings of skin, suggesting it might participate in mechanosensation and/or acid-evoked nociception. Disrupting the mouse DRASIC gene altered sensory transduction in specific and distinct ways. Loss of DRASIC increased the sensitivity of mechanoreceptors detecting light touch, but it reduced the sensitivity of a mechanoreceptor responding to noxious pinch and decreased the response of acid- and noxious heat-sensitive nociceptors. The data suggest that DRASIC subunits participate in heteromultimeric channel complexes in sensory neurons. Moreover, in different cellular contexts, DRASIC may respond to mechanical stimuli or to low pH to mediate normal touch and pain sensation.

Introduction

Mammals readily distinguish among a variety of mechanical and painful cutaneous stimuli (Johnson and Hsiao, 1992; Perl, 1992). In the peripheral nervous system, this discriminatory ability results in part from the presence of distinct cutaneous sensory structures (for example, Meissner corpuscles, Merkel cell neurite complexes, and free nerve endings [Munger and Ide, 1988; Sinclair, 1981]). Moreover, different classes of sensory neurons carry information for distinct sensory modalities (for example, rapidly adapting mechanoreceptors, slowly adapting mechanoreceptors, and C-fiber nociceptors [Johnson and Hsiao, 1992; Koltzenburg et al., 1997; Perl, 1992]). However, little is known about the molecular identity and function of the proteins that transduce mechanical and noxious stimuli into electrical signals (Caterina and Julius, 1999; Lewin and Stucky, 2000). Earlier studies suggested that the receptors for many of these stimuli are ion channels (French, 1992; Hamill and McBride, 1996).

DEG/ENaC ion channels are attractive candidates to serve as receptors for cutaneous stimuli (Mano and Driscoll, 1999). Subunits of the DEG/ENaC protein family associate as homo- and heteromultimers to form voltage-insensitive cation ($Na^+ > K^+ > Ca^{2+}$) channels. Individual subunits share a common structure with two transmembrane domains, intracellular carboxy and amino termini, and a large, cysteine-rich extracellular domain thought to serve as a receptor for extracellular stimuli. Three lines of evidence suggest that DEG/ENaC channels may be mechanosensors. First, behavioral studies in *Caenorhabditis elegans* showed that mutations disrupting of the DEG/ENaC proteins MEC-4 and MEC-10 impair responses to touch (Driscoll and Chalfie, 1991; Huang and Chalfie, 1994). In addition, UNC-8 has been implicated in proprioception (Tavernarakis et al., 1997), and UNC-105 may be involved in detecting muscle stretch (Liu et al., 1996). Second, DEG/ENaC proteins have been localized in specialized mechanosensory structures. For example, the *Drosophila melanogaster* Pickpocket is localized in multiple dendritic mechanosensory neurons in the embryo (Adams et al., 1998). In the rat, β and γ subunits of the epithelial Na^+ channel (ENaC) have been localized in specialized mechanosensory endings in the skin (Drummond et al., 2000; Fricke et al., 2000). In addition, the Brain Na^+ Channel 1 (BNC1 [Price et al., 1996], also called MDEG [Waldmann et al., 1996], BNaC1 [García-Añoveros et al., 1997], and ASIC2 [Waldmann and Lazdunski, 1998]) is present in mechanosensory lanceolate nerve endings surrounding the hair shaft and other cutaneous sensory structures (Garcia-Añoveros et al., 2001; Price et al., 2000). Third, studies in mice with a disrupted BNC1 gene showed a markedly reduced sensitivity of low-threshold mechanoreceptors (Price et al., 2000). These data identified BNC1 as essential for the normal detection of light touch and suggested that it is a component of a mechanosensory complex.

The Dorsal Root Acid Sensing Ion Channel (DRASIC [Waldmann et al., 1997a], also called ASIC3 [Waldmann and Lazdunski, 1998]) is a particularly interesting candidate to participate in sensory transduction because in rats it is expressed predominantly in sensory neurons. Like BNC1 and the Acid Sensing Ion Channel, ASIC (Waldmann et al., 1997b), also called BaNaC2 (García-Añoveros et al., 1997), and ASIC1 (Waldmann and Lazdunski, 1998), DRASIC channels can be activated by a reduced pH. Acid stimulation of homomultimeric DRASIC channels expressed in heterologous cells generates biphasic currents with rapidly inactivating and sustained components (Waldmann et al., 1997a). These currents, as well as those generated by coexpression of DRASIC and an alternatively spliced form of BNC1, show kinetics and ion selectivity similar to those of H^+-gated channels in isolated DRG cell neurons (Lingueglia et al., 1997). Acidic pH can also stimulate some

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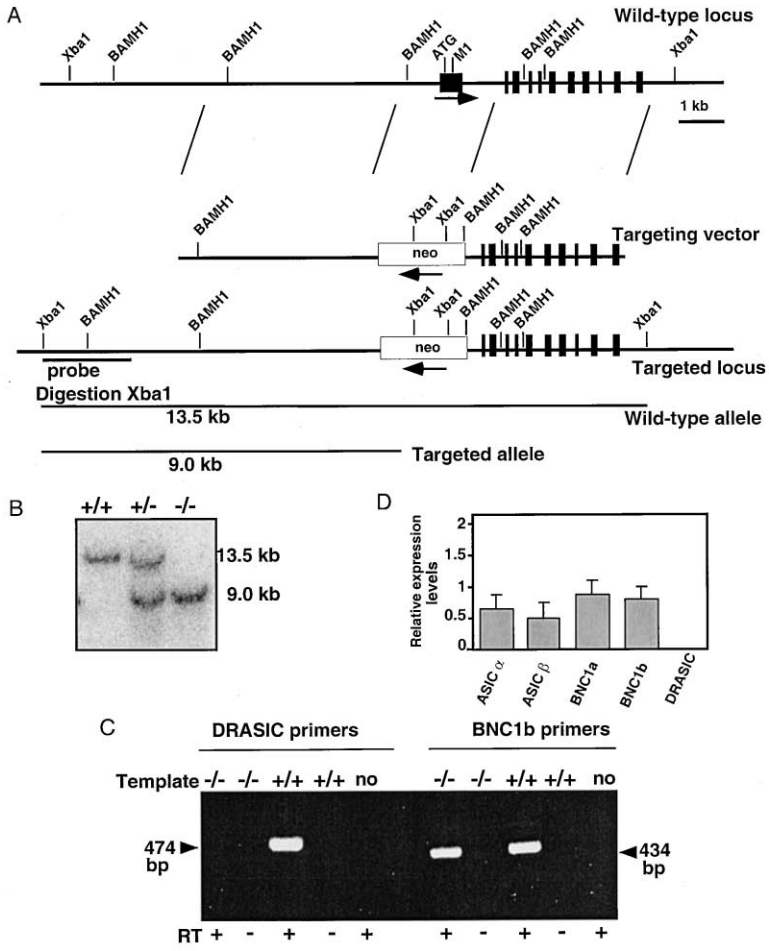

Figure 1. Generation of DRASIC-Deficient Mice

(A) Simplified restriction map of mouse DRASIC locus and structure of the targeting vector. The region of the gene containing the first exon, including the start ATG codon, was replaced with the PGK-neo cassette (neo). Exons are shown as black rectangles. Arrows denote the direction of transcription. The location of the probe used in Southern blot analysis is shown. Expected fragment sizes of wild-type (13.5 kb) and mutant (9.0 kb) alleles are indicated.

(B) Southern blot analysis of genomic DNA. DNA was digested with Xba1 and blotted with the probe shown in (a). The 13.5 kb band is derived from the wild-type allele, and the 9.0 kb band is derived from the targeted allele. +/+, wild-type; +/−, heterozygote; −/−, homozygote.

(C) RT-PCR analysis. cDNA derived from DRG total RNA was used as a template. The upstream primer was generated from the targeted exon 1 sequence, and the downstream primer was generated from exon 2. Although the DRASIC product of 474 bp is missing in the −/− mice, the BNC1b product of 434 bp is present in this same population of RNA. Reactions were run with and without reverse transcriptase (RT), as indicated; "no" indicates no RNA template.

(D) Abundance of mRNA levels for indicated channels in −/− mice relative to +/+ mice determined by real-time quantitative PCR. Data are mean ± SEM of three different experiments on three different groups of DRG RNA, each a pool from 5 or 6 +/+ or −/− animals. A one-sample t test showed no statistically significant difference in transcript abundance between +/+ and −/− animals ($p \geq 0.2$).

cutaneous nociceptors and produce pain in man (Reeh and Steen, 1996; Steen and Reeh, 1993). These findings have suggested that DRASIC might be a pH sensor in nociceptive neurons (McCleskey and Gold, 1999; Waldmann and Lazdunski, 1998), although whether acid is the only or even the predominant ligand for DRASIC is unknown.

Thus, we hypothesized that DRASIC might be a component of a mechanosensory complex and/or a proton sensor involved in nociception. To understand better the molecular basis of sensory receptors in general, and to discern the role of DRASIC channels in particular, we generated DRASIC null mice and examined their phenotype.

Results

Generation of DRASIC Null Mice by Homologous Recombination

To create a DRASIC null allele, we used homologous recombination to delete exon 1, which contains the start ATG necessary for initiation of protein synthesis (Figure 1A) (McDonald et al., 1999). The deleted sequence also encodes the first transmembrane segment and part of the extracellular domain. Two lines were generated and studied.

Genotyping 508 progeny of F1 heterozygote crosses identified 132 wild-type (+/+), 256 heterozygous (+/−), and 120 homozygous (−/−) offspring (Figure 1B). This ratio is consistent with Mendelian inheritance and indicates that disruption of the DRASIC gene is not lethal. Using an RT-PCR assay, we detected no DRASIC mRNA in DRG isolated from DRASIC −/− mice (Figure 1C). DRASIC null mice appeared to be normal and did not differ from their littermates in development, size, or fertility.

We considered the possibility that disrupting the DRASIC gene might alter expression of another acid-activated DEG/ENaC channel. These genes are located on different mouse chromosomes; DRASIC is on 9, BNC1 on 11, and ASIC on 15. We used real-time PCR of DRG RNA to compare these genes' transcript levels, including levels of alternatively spliced transcripts of the ASIC gene (ASICα and ASICß [Chen et al., 1998]) and the BNC1 gene (BNC1a and BNC1b [Lingueglia et al., 1997; Price et al., 1996]). Disruption of the DRASIC gene did not significantly alter the mRNA levels for any of the other genes.

DRASIC Is Localized in Specialized Sensory Nerve Endings

Previous studies in the rat showed that DRASIC transcripts were present in DRG sensory neurons (Babinski et al., 1999; Chen et al., 1998; Waldmann et al., 1997a).

Therefore, we asked if DRASIC protein is present in DRG and which neurons contain it. Most large-diameter sensory neurons are low-threshold mechanoreceptors that detect innocuous stimuli such as light touch or muscle stretch (Lawson, 1992). Most small neurons are nociceptive; they detect noxious thermal, chemical, or mechanical stimuli (Cesare and McNaughton, 1997; Lawson, 1992; Stucky and Lewin, 1999). In wild-type mice, we detected DRASIC immunoreactivity in most of the large-diameter and in most of the small-diameter DRG neurons (Figure 2A). We also labeled DRG neurons with an antibody against substance P, a marker for a subset of small-diameter DRG nociceptive neurons (Lawson et al., 1997; McCarthy and Lawson, 1989). Most small neurons staining for substance P also stained for DRASIC. No specific DRASIC staining was detected in DRG from −/− animals.

For DRASIC to serve as a component of a mechanoreceptor or nociceptor complex, the protein must be present in peripheral sensory nerve terminals. Meissner corpuscles are specialized cutaneous sensory structures present in glabrous skin and are located at the apex of the dermal papillae (Ide, 1976; Munger and Ide, 1988). Figure 2B shows DRASIC (green) colocalization with neuronal markers (red) in nerves of the Meissner corpuscle (lamellar cells, blue). There was no specific DRASIC immunostaining in −/− animals (not shown). In hairy skin we also found DRASIC in the palisades of lanceolate nerve endings that run longitudinally to and surround the hair shaft (Figure 2C) (Halata, 1993). DRASIC immunostaining (green) colocalized with neuronal markers (red), placing DRASIC at the putative site of mechanoreception in guard hair follicles. There was only minimal DRASIC immunostaining of Pilo-Ruffini endings. Nerve endings in Meissner corpuscles and lanceolate fibers are rapidly adapting (RA) mechanoreceptors (Sinclair, 1981). RA mechanoreceptors have large myelinated fibers and respond to very light touch (low threshold), and when a supramaximal constant stimulus is applied, they respond briskly during the movement of the skin but show no sustained activity in the continued presence of the nonmoving stimulus (Johnson and Hsiao, 1992; Koltzenburg et al., 1997).

We also detected DRASIC in slowly adapting (SA) mechanoreceptors. Like RA mechanoreceptors, SA mechanoreceptors are large, myelinated, low-threshold fibers. However, with application of a supramaximal stationary stimulus they generate sustained activity that adapts only slowly during the stimulus (Koltzenburg et al., 1997). Merkel cell-neurite complexes are SA mechanoreceptors (Airaksinen et al., 1996; Burgess and Perl, 1973; Darian-Smith, 1984; Martin and Jessell, 1991). Merkel cells immunopositive for cytokeratin-20 (blue) were found in the basal layer of the epidermis (Figure 2D). Neuronal innervation (red) of the Merkel cells was identified predominantly at the basal aspect of the structure, near the epidermal-dermal border. DRASIC (green) partially colocalized with the neuronal marker, and it was often in or adjacent to the Merkel cells. Controversy persists about the role of Merkel cells versus nerve endings in SA mechanosensation, although most data suggest that the nerve ending is sufficient for SA mechanosensitivity (Diamond et al., 1988; Ikeda et al., 1994; Kinkelin et al., 1999). Ultrastructural localization will be required to precisely localize DRASIC in the Merkel cell-neurite complex.

We found DRASIC immunostaining in some, but not all, free nerve endings running in the epidermal layer of the mouse paw pad (Figure 2E). These nerve endings could belong to myelinated or unmyelinated nociceptors (Kruger et al., 1981).

The DRASIC channel thus appears well placed to participate in mechanotransduction. Its colocalization in the DRG with substance P, a marker of nociceptive C fibers, and its presence in free nerve endings in the skin indicate that it may also participate in the transduction of nociceptive stimuli.

Mechanoreceptor Function Is Altered in the Absence of DRASIC

We tested the hypothesis that DRASIC is required for normal sensory mechanotransduction by using an in vitro skin nerve preparation (Carroll et al., 1998; Koltzenburg et al., 1997; Price et al., 2000). This method examines the mechanosensory properties of individual saphenous nerve afferents innervating the skin. Fibers are identified based on their conduction velocities (Aβ fibers, >10 m/s; Aδ fibers, 1–10 m/s; and C fibers, <1 m/s) and their response to various stimuli (see Table 1 and Experimental Procedures). As shown in Table 1, Aβ fibers are large myelinated fibers that include the RA and SA mechanoreceptors, which respond to light touch, as described above. Aδ fibers are thin myelinated fibers that include down hair (D-hair) receptors and A fiber mechanonociceptors (AM). D-hair receptors have low mechanical thresholds and show a brisk, rapidly adapting response at the onset and removal of a constant force stimulus. AM fibers respond to high-threshold mechanical stimuli, and they adapt only slowly to a constantly applied stimulus. C fibers possess unmyelinated axons and respond to noxious mechanical stimuli (C-mechanonociceptors, C-M fibers) or to noxious mechanical, heat, and acid stimuli (C-mechanoheat, C-MH fibers, also called polymodal C fibers).

To test the effect of peripheral mechanical stimuli, we used a standard series of displacements, as previously described (Price et al., 2000). Strikingly, the sensitivity of RA mechanoreceptors increased more than two-fold in null mice (Figure 3A). RA mechanoreceptors from wild-type and null animals showed similar conduction velocities and median mechanical thresholds measured with von Frey filaments (Table 1 and legend).

In contrast to RA mechanoreceptors, SA mechanoreceptor sensitivity was not significantly altered by the loss of DRASIC (Figure 3B, $p = 0.14$, two-way ANOVA). The conduction velocity of the sampled SA mechanoreceptors was, however, slightly lower in DRASIC-deficient mice (Table 1). The explanation is not known at this time.

Without DRASIC the sensitivity of AM mechanonociceptors to mechanical stimuli fell significantly (Figure 3C). In addition, AM mechanonociceptors from −/− mice exhibited a significantly higher median von Frey threshold than those from wild-type animals (Figure 3F). Thus, the consequence of losing DRASIC is opposite in different neurons; in RA mechanoreceptors the absence of DRASIC enhanced mechanosensitivity, whereas in AM fibers it reduced mechanosensitivity.

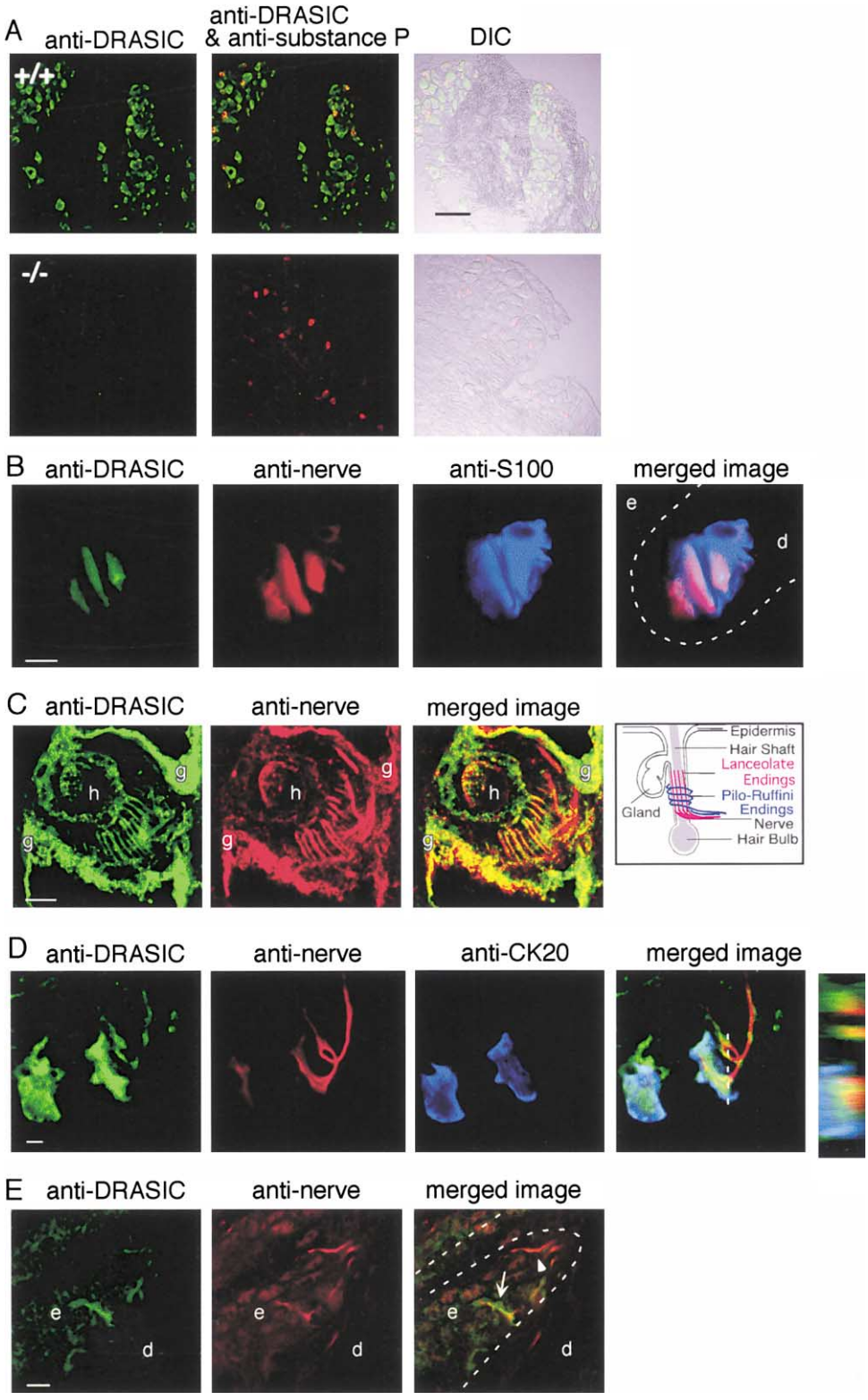

Figure 2. Immunocytochemical Localization of DRASIC in Sensory Neurons of +/+ and −/− Mice

(A) DRG. Cryosections (10 μm) of DRG were exposed to antibodies directed against DRASIC (green) and/or substance P (red), as indicated. A differential-interference contrast image (DIC) superimposed with fluorescence is shown on right. The scale bar represents 100 μm.

(B) Meissner-like corpuscles. Oblique sections through toe pads were stained with antibodies against DRASIC (green), nerve markers (red), and a lamellar cell marker (blue). Images are a series of 20 optical sections taken at 0.5 μm intervals. The dashed line in the right panel shows the junction between epidermis (e) and dermis (d). The scale bar represents 5 μm.

(C) Guard hair follicles. Oblique sections through hair follicles stained for DRASIC (green) and nerves (red). Inset shows the neural network

Table 1. Properties of Individual Fibers from Saphenous Nerve

Aβ Fibers	+/+ (n = 78)	−/− (n = 100)	P Value
RA mechanoreceptors			
%	45	52	NS
Conduction (m/s)	17.8 ± 0.68	16.7 ± 0.41	NS
SA mechanoreceptors			
%	55	48	NS
Conduction (m/s)	16.7 ± 0.53	14.0 ± 0.30	<0.005
Aδ Fibers	**+/+ (n = 59)**	**−/− (n = 58)**	**P Value**
AM receptors			
%	67	60	NS
Conduction (m/s)	4.86 ± 0.41	4.74 ± 0.48	NS
D-hair receptors			
%	33	40	NS
Conduction (m/s)	6.66 ± 0.38	6.12 ± 0.23	NS
C Fibers	**+/+ (n = 87)**	**−/− (n = 95)**	**P Value**
C-mechanoheat (C-MH)			
%	64	58	NS
Conduction (m/s)	0.59 ± 0.02	0.56 ± 0.01	NS
C-mechanonociceptors (C-M)			
%	36	42	NS
Conduction (m/s)	0.64 ± 0.02	0.65 ± 0.02	NS

The statistical significance of the proportion of fibers in a subset was tested by the χ^2 test, and differences in conduction velocity were tested by an unpaired t test. The median von Frey threshold (vFT) was evaluated by a Mann-Whitney U test and was not different for animals from either genotype, except for AM fibers (see Figure 3F). The number of fibers is indicated. Data for each fiber type and genotype were collected from at least ten animals, with approximately equal distribution of male and female mice.

The proportions of AM fibers and D-hair receptors were normal in animals of both genotypes (Table 1). Low-threshold D-hair receptors from knockout mice responded normally to mechanical stimuli (Table 1, Figure 3D). Subsets of C fibers in DRASIC +/+ and −/− mice were found in similar proportions (Table 1), had similar conduction velocities (Table 1), showed similar mechanosensitivity (Figure 3E), and exhibited similar median von Frey thresholds (Figure 3F). Thus, loss of DRASIC did not change all mechanosensory function; the effects were selective and specific.

Loss of DRASIC Alters Acid-Evoked Currents in Mechanosensory Neurons

It has been known for 20 years that acid evokes large cation currents in isolated large-diameter sensory neurons (Krishtal and Pidoplichko, 1981). Yet, the peripheral fibers of large-diameter sensory neurons do not respond to low pH; instead they respond to innocuous mechanical stimuli (Lewin and Stucky, 2000; Steen et al., 1992). This apparent discrepancy has been unresolved because the molecular identity and physiologic function of H^+-gated currents in these neurons has remained unknown. We hypothesized that DRASIC might contribute to these currents.

Consistent with this hypothesis, the lack of DRASIC altered several properties of H^+-gated currents from isolated large-diameter DRG neurons. Representative whole-cell, acid-evoked current records are shown in Figure 4A. As previously reported (Bevan and Yeats, 1991), acid stimulated two distinct inward currents, a rapidly inactivating Na^+ current induced by pH below 7 and a sustained cation current obtained with a further drop in pH. A similar proportion of cells from DRASIC wild-type and null mice responded to pH (legend to Figure 4). Thus, the absence of DRASIC did not eliminate H^+-gated currents.

However, the lack of DRASIC markedly slowed the rate of desensitization in the continued presence of an acid stimulus (Figures 4A and 4B). The average amplitude of the transient current also increased in −/− neurons (Figure 4C). These changes will substantially increase the charge flow during the initial transient phase of the current. Current in −/− neurons was also less sensitive to a pH 6.5 stimulus than that in +/+ neurons (Figure 4D). Comparing the properties of pH-gated cur-

innervating guard hair follicle. "h" indicates a hair follicle shaft; "g" indicates a sebaceous gland. Sebaceous glands stain nonspecifically with primary and secondary antibodies. Micrographs are a stacking of 16 images, each 0.8 μm thick. The scale bar represents 10 μm.

(D) Merkel cell/neurite complexes. Oblique sections through patches of hairy skin stained with antibodies against DRASIC (green), nerve marker (red), and a Merkel cell marker (blue). The right panel shows an X-Z image of the region indicated by a dashed line in the adjacent panel; DRASIC staining was often most prominent between nerve and Merkel cell markers. The image represents a series of 17 sections. The scale bar represents 5 μm.

(E) Fine epidermal nerve fibers. Oblique sections through toe pads were stained for DRASIC (green) and nerve marker (red). Images represent a series of three optical sections taken at 0.5 μm intervals. The dashed line in the right panel shows the junction between epidermis (e) and dermis (d). The scale bar represents 5 μm. An arrow indicates a free nerve ending positive for DRASIC immunostaining, and an arrowhead indicates a nerve ending negative for DRASIC.

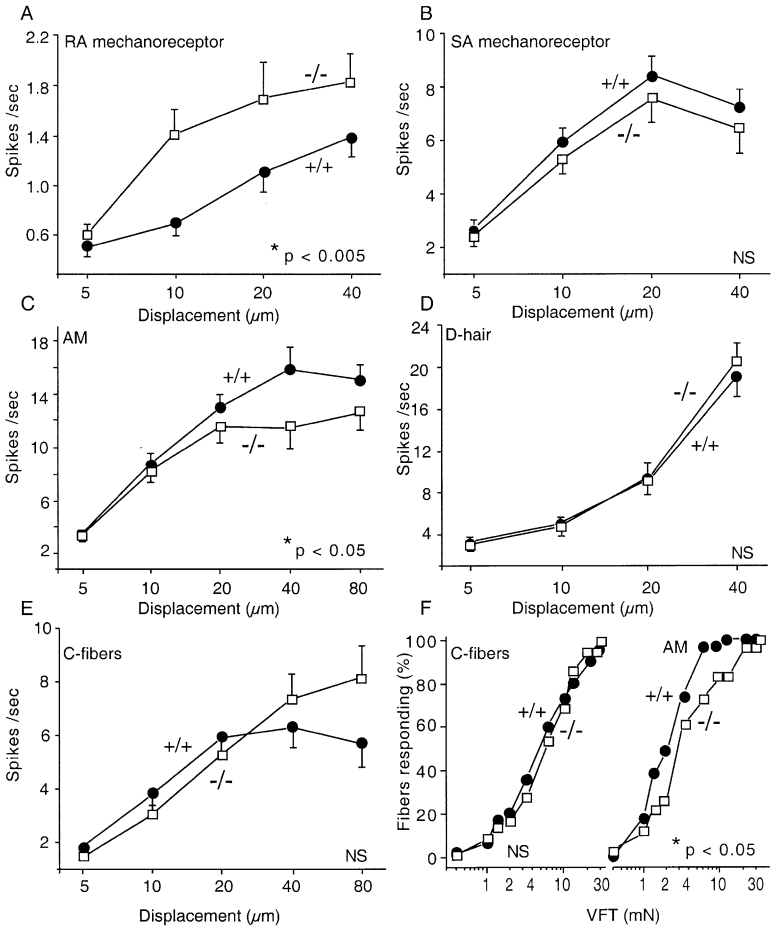

Figure 3. Sensitivity of Wild-Type and DRASIC Null Mechanoreceptors Measured by Single-Fiber Recording with the Skin-Nerve Preparation

Stimulus-response function of RA mechanoreceptor fibers (A), SA mechanoreceptor fibers (B), slowly conducting myelinated mechanonociceptors (AM) (C), D-hair afferents (D), and C-fiber nociceptors (E). Closed circles indicate +/+ fibers, and open squares indicate −/− fibers. The number of fibers is given in Table 1. Statistical significance, assessed by a two-way ANOVA, is indicated. The frequency of firing (spikes/s) was calculated over the entire 10 s of the stimulus except in the case of D-hair afferents, for which only the first 2 s were used because some afferents exhibited spontaneous discharge, perhaps due to skin stretch. (F) Cumulative distribution mechanical thresholds as determined with von Frey monofilaments required to activate C-fiber mechanonociceptors and myelinated mechanonociceptors (AM). "vFT" is the von Frey threshold. Data were obtained from 25–77 single fibers for each receptor type and genotype.

rents from homomultimeric DRASIC channels to those of BNC1 and ASIC helps one to assess these changes. For example, DRASIC desensitizes more rapidly than either BNC1 or ASIC (Sutherland et al., 2001); thus, its loss is consistent with slowed inactivation in −/− neurons. DRASIC is also more sensitive to pH than BNC1 or ASIC (Sutherland et al., 2001); this is consistent with the reduced response to a pH 6.5 stimulus in −/− neurons. These data indicate that DRASIC contributes to the pH-gated currents of DRG neurons.

Acid-Evoked Responses in Nociceptive Sensory Neurons

We also tested the hypothesis that loss of DRASIC might affect cutaneous nociceptors. A limited set of nociceptors in the skin respond vigorously to lowered pH (Caterina et al., 2000; Price et al., 2000; Steen et al., 1992). In the mouse, these neurons normally comprise around 30% of all C-MH fibers. Therefore, we tested C-MH fibers for sustained responses to a 2 min acid application. Approximately the same proportion of C-MH fibers exhibited a sustained discharge to pH 5 in DRASIC null and wild-type animals (29% and 33%, respectively). However, the magnitude of this response was clearly reduced for C-MH fibers from DRASIC-deficient mice (Figure 5A). Further reducing the stimulus to pH 4 produced no greater response from +/+ fibers (Figure 5B), suggesting a saturating relationship between pH and the response. However, the pH 4 stimulus increased the response of −/− fibers so that they showed activity similar to that of +/+ fibers.

Previous studies have shown that C-MH fibers respond to capsaicin (Steen et al., 1992). Therefore, we also tested the acid responsiveness of DRG neurons that were identified by their response to capsaicin. Studies in the rat suggest that most capsaicin-responsive neurons are nociceptors (Szolcsanyi et al., 1988). Figures 5C and 5D show that genotype did not alter the response to capsaicin. Moreover, the response to a pH 5 solution was not altered in the capsaicin-responsive neurons (Figures 5C and 5D). This result is consistent with the earlier observation that the majority of the pH-gated current in nociceptive neurons results from the activity of VR1 receptors (Caterina et al., 2000). We occasionally observed transient pH-activated currents in these neurons, but their frequency was too low to allow us to draw firm conclusions. Additional studies will be required to further elucidate the contribution of DRASIC and VR1 to the pH responsiveness of these neurons.

DRASIC and Noxious Heat Sensitivity

We used a relatively high stimulus (peak corium skin temperature of 52°C) to identify heat-sensitive C fibers (C-MH fibers). Approximately the same proportion of C-MH fibers responded to this noxious stimulus in DRASIC-deficient and wild-type mice (Table 1). Surpris-

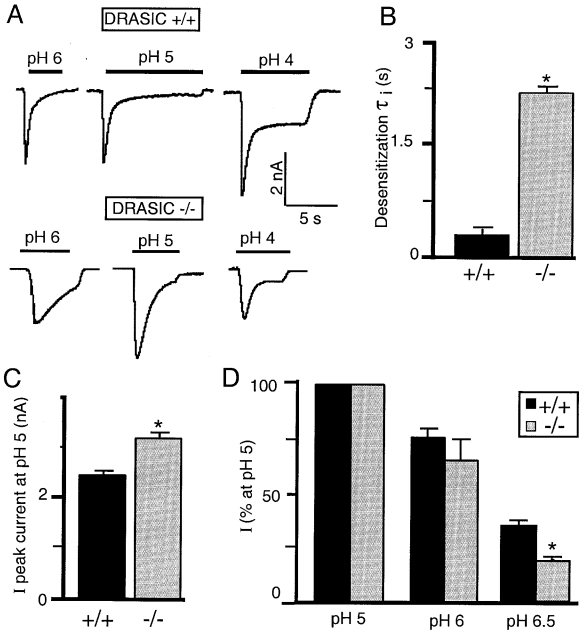

Figure 4. pH-Dependent Whole-Cell Currents in Medium- to Large-Diameter DRG Sensory Neurons from DRASIC +/+ and −/− Mice

(A) Representative tracings of whole-cell current response to acid. The holding voltage was −70 mV; a downward deflection indicates an inward current. The pH of the bathing solution was 8 except where indicated by bars. Sixty-one percent of +/+ neurons (33 ± 1 μm, n = 53) and 63% of −/− neurons (33 ± 1 μm, n = 62) responded to a pH 5 stimulus. Forty-four percent of +/+ neurons (35 ± 1 μm, n = 44) and 48% of −/− neurons (35 ± 1 μm, n = 29) responded to a pH 6 stimulus.

(B) Desensitization time constants for currents evoked at pH 6. Desensitizing currents were fit with the single exponential equation $Y = a^{*}\mathrm{Exp}(-t/\tau)$, where Y is the current amplitude and t is the elapsed time. n = 12 cells from four +/+ mice; n = 9 cells from three −/− mice. An asterisk indicates $p < 0.001$ in the t test.

(C) Amplitude of peak current evoked by pH 5. n = 53 cells from ten +/+ mice; the mean cell diameter was 33 ± 1 μm. n = 62 cells from ten −/− mice; the mean diameter was 33 ± 1 μm. An asterisk indicates $p < 0.005$ in the t test.

(D) Effect of pH on transient current. The current is shown as a percentage of that measured at pH 5. All data are given as the mean ± SEM. n = 20 cells at pH 6.5, and n = 8–10 cells at pH 6. Data were obtained from six to ten mice of each genotype. An asterisk indicates $p < 0.05$ compared to wild-type in the t test.

ingly, the overall neural response from all C-MH fibers tested was 29% lower in DRASIC-deficient mice compared to controls (Figure 5E). This reduced response was largely accounted for by a reduced number of evoked spikes from fibers with the most robust responses.

Altered Behavioral Responses to Somatosensory Stimuli in DRASIC Null Mice

The requirement for DRASIC in normal mechanoreceptor function and acid nociception led us to examine the behavioral responses of DRASIC null mice to somatosensory stimuli. We used calibrated von Frey monofilaments of increasing force to evoke paw withdrawal, but we did not observe a significant difference in mice of different genotypes (Figure 6A). However, this assay does not measure the minimal force required for the mouse to perceive touch. Instead, it defines a force strong enough to evoke withdrawal, and the test is thought to reflect the response of Aδ or C fibers to noxious mechanical stimuli (Mogil et al., 1999). After tissue injury, there is an increased sensitivity to mechanical stimuli at the site of injury, i.e., primary hyperalgesia. Earlier work indicates that this mechanical hyperalgesia results, at least in part, from activation of large-diameter afferents (RA and SA mechanoreceptors) that is misinterpreted in the central nervous system (Cervero and Laird, 1996; Treede et al., 1992; Woolf and Doubell, 1994). Therefore, to increase the sensitivity to light touch, we induced mechanical hyperalgesia with carrageenan before testing with von Frey monofilaments. Both DRASIC +/+ and −/− mice developed mechanical hyperalgesia (compare Figures 6A and 6B). However, compared to +/+ animals, the −/− mice showed a small but significantly increased sensitivity to mechanical stimuli (Figure 6B). The increased mechanical sensitivity of RA mechanoreceptors in DRASIC null mice (Figure 3A) might in part explain the enhanced behavioral hyperalgesia to low-intensity mechanical stimuli.

The response of DRASIC null mice to a noxious thermal stimulus was not statistically different from that of wild-type mice, either in the presence or absence of carrageenan-induced inflammation (Figures 6C and 6D). Previous studies have shown that heat activates nociceptors, both Aδ and C fibers (Yeomans and Proudfit, 1996) and that these nociceptors sensitize in response to carrageenan inflammation (Kocher et al., 1987). These data thus suggest that activation of DRASIC is not necessary for development of primary heat hyperalgesia. Acid injected into the skin causes pain in humans, stimulates nociceptors in vitro, and produces characteristic behaviors such as paw licking in rats (Gorman et al., 1983; Wheeler-Aceto et al., 1990). Therefore, we injected pH 3 acetic acid into the hindpaw of the skin and measured paw licking, a measure of spontaneous pain. However, the frequency of paw licking was not statistically different between −/− and +/+ mice (Figure 6E).

Previous work shows that injecting acidic pH into skeletal muscle produces pain in humans (Issberner et al., 1996) and induces secondary mechanical hyperalgesia outside the site of injury in rats (Sluka et al., 2001). This secondary mechanical hyperalgesia is thought to result from acid activation of nociceptors innervating muscle, which in turn generates secondary changes in the dorsal horn of the spinal cord (Sluka et al., 2001). After injection of pH 4 saline into the gastrocnemius muscle, DRASIC null mice displayed significantly less hyperalgesia when they were compared to wild-type mice (Figure 6F). These data suggest that acid-induced secondary hyperalgesia involves the activation of DRASIC.

Discussion

Our data suggest that DRASIC forms a key component of receptor complexes that detect some cutaneous touch and painful stimuli. Several observations support this conclusion. First, in the DRG both large-diameter, low-threshold mechanoreceptors and small-diameter nociceptors contained DRASIC. Moreover, in the periph-

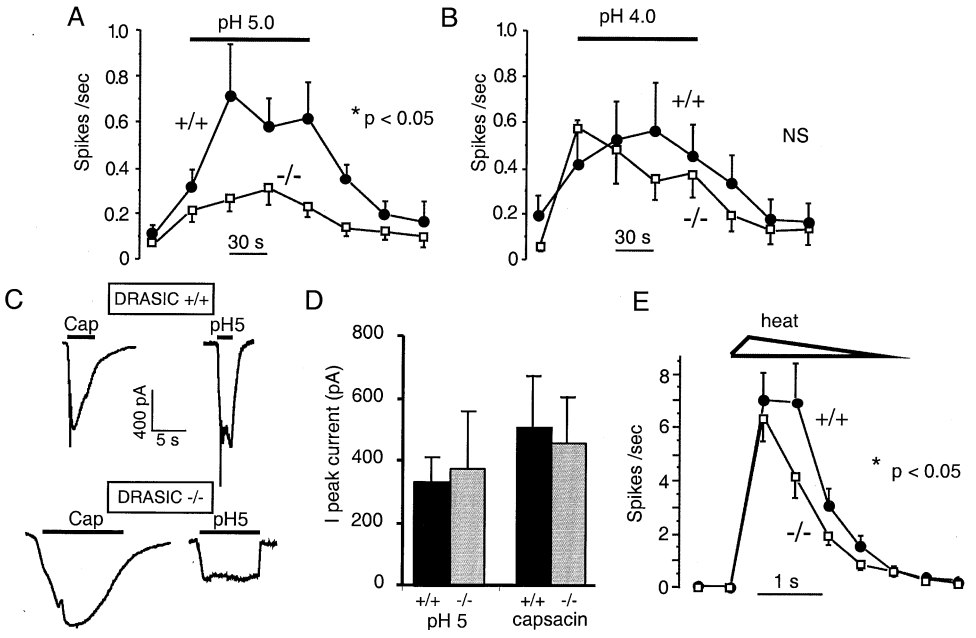

Figure 5. Response of Sensory Neurons to Acidic pH and Noxious Heat

(A) Response of C-MH fibers to 2 min applications of low pH solutions to the receptive field. The response of DRASIC null fibers to pH 5 application was substantially attenuated ($p < 0.05$), ($n = 21–23$ C fibers per genotype).

(B) A second stimulus of pH 4.0 was applied to some C fibers ($n = 11–14$ per genotype). There was no significant difference between the genotypes with the pH 4 stimulus.

(C) Representative whole-cell current tracings of isolated DRG sensory neurons showing responses to capsaicin and pH 5 solution. The holding voltage was −70 mV. The bathing solution was pH 8, and additions of capsaicin and pH 5 (1 μM) are indicated.

(D) Mean amplitude ± SEM of peak current evoked by pH 5 and capsaicin. Of 44 +/+ neurons tested, 18% responded to capsaicin, and of 39 −/− neurons tested, 21% responded to capsaicin. Of these neurons, six out of eight showed a sustained response to acid in +/+ neurons, and five out of eight did so in −/− neurons. n = 6 cells from six +/+ mice; the mean cell diameter was 25 ± 1 μm. n = 5 cells from five −/− mice; the mean diameter was 25 ± 2 μm.

(E) Response of single C-MH nociceptors to a standardized noxious heat stimulus ($n = 49–57$ per genotype); the peak temperature was 52°C. The time course of the spike response in C-MH fibers lacking the DRASIC channel was significantly shorter as compared to that in wild-type C-MH fibers (two-way ANOVA, $p < 0.05$).

ery DRASIC resides in nerve endings at sites where mechanical and noxious stimuli are converted into electrical activity. Second, loss of DRASIC from these sites induced very specific abnormalities in that it increased the sensitivity of some sensory modalities and decreased the sensitivity of others. We also observed an abnormal behavioral response to mechanical stimuli. Third, two different DEG/ENaC subunits, DRASIC and BNC1, made distinct contributions to mechanosensation. Like DRASIC, BNC1 is present in lanceolate nerve endings and Meissner corpuscles that form RA mechanoreceptors (Garcia-Añoveros et al., 2001; Price et al., 2000). But whereas the loss of DRASIC enhanced RA mechanosensitivity, loss of BNC1 reduced RA mechanosensitivity.

Although we did not detect statistically significant changes in the transcript levels of related channels, we cannot exclude secondary changes in protein levels of BNC1, ASIC, or some other channel subunit in DRASIC null neurons. For example, removing DRASIC subunits from a heteromultimeric channel might alter the stability of the complex, interactions with other proteins, or targeting. Based on our studies of BNC1 null animals (Price et al., 2000), it seems unlikely that the specific changes we observed in DRASIC null animals could be explained only by an increase or decrease in BNC1 expression. For example if BNC1 subunits were secondarily decreased, we would have expected a decrease in RA mechanosensor sensitivity. Alternatively, if BNC1 subunits were increased, we would not have expected the decrement in AM mechanonociceptors or C-MH fiber function. Similar arguments can be made based on the phenotype of ASIC null animals (unpublished). However, attribution of the physiologic alterations solely to the loss of DRASIC should be taken with the caveat that we cannot exclude secondary changes in other proteins.

Diverse Roles of DRASIC in Mechanosensation

The loss of DRASIC produced very specific but strikingly diverse effects in cutaneous mechanosensation. There are at least two main alternatives to explain how such alterations might occur. (1) Within an individual neuron, DRASIC might form homomultimeric channels, and other DEG/ENaC subunits might form separate channels. (2) DRASIC subunits might contribute to heteromultimeric channels with other DEG/ENaC subunits and the subunit composition of channels and associated proteins might vary in different neurons. Our data suggest the latter alternative. First, the mechanosensitivity of RA fibers increased in DRASIC null animals, whereas that of AM fibers fell. It is difficult to explain both observations solely by the loss of a single type of homomultimeric channel. Second, the kinetics of acid-evoked currents in large-diameter sensory neurons slowed *and*

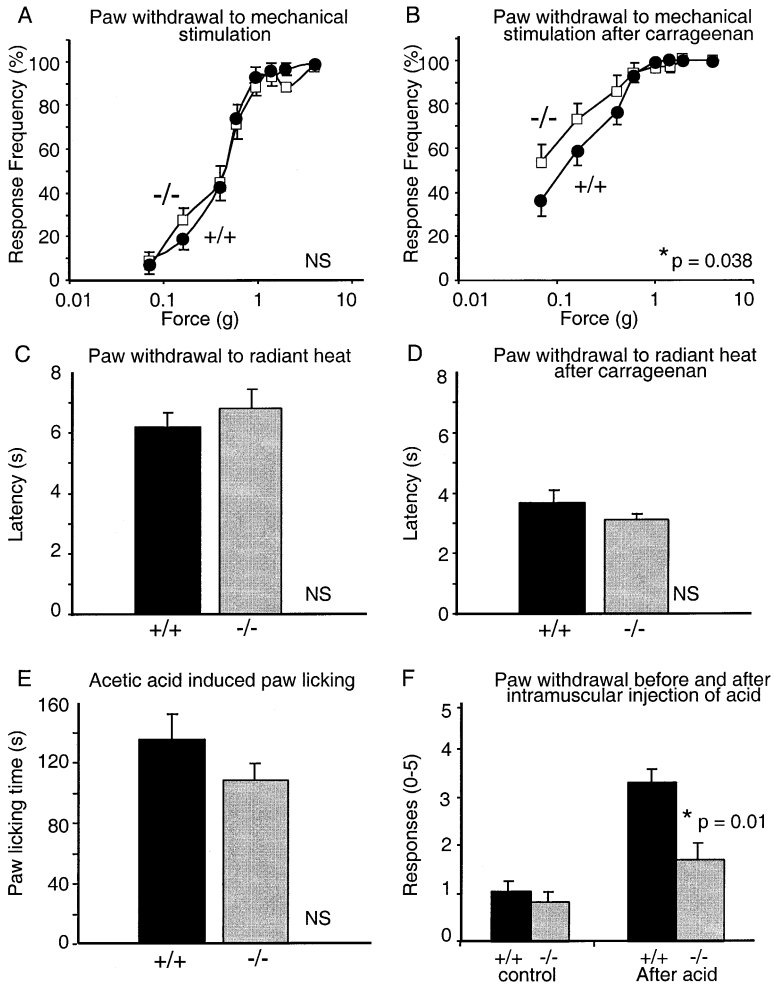

Figure 6. Analysis of Touch- and Pain-Related Behaviors

(A and B) Frequencies of paw withdrawal to mechanical stimulation with von Frey monofilaments before and 3 hr after carrageenan-induced inflammation (n = 11 for both genotypes). With inflammation there was a significant increase in the response frequency for the DRASIC −/− mice when compared to wild-type controls ($p < 0.05$, two-way ANOVA). There was no significant change in paw edema in +/+ and −/− mice (data not shown). Data are means ± SEM.

(C and D) Paw withdrawal latency to thermal stimulation with radiant heat before and 3 hr after carrageenan injection (n = 11 for both genotypes). Both +/+ and −/− groups showed a decrease in withdrawal latency after carrageenan ($p < 0.0001$). However, there was no significant difference between these groups either before or 3 hr after inflammation (repeated-measures ANOVA).

(E) Time spent licking paw during a 30 min period following injection of 0.6% acetic acid. The time was similar for +/+ (n = 18) and −/− (n = 17) mice (one-way ANOVA).

(F) The frequency of mechanical withdrawal (0–5, the number of times an animal withdrew its paw in five trials) from a 0.37 mN bending force before and 4 hr after intramuscular injection of pH 4.0 sterile saline. After the injection of acidic saline, the frequency of withdrawal increased in +/+ mice (n = 11) compared to −/− mice (n = 7) ($p < 0.01$, repeated-measures ANOVA). Littermates were used for all studies; approximately equal distributions of both sexes were used for all studies except those in panel (f), in which only males were studied.

the amplitude increased. If DRASIC formed a homomultimeric channel, it is possible that loss of that current could uncover current from another channel with slower kinetics. However, in such a case the amplitude of current would fall.

Although our data are most readily explained by DRASIC subunit incorporation into heteromultimeric complexes, we cannot exclude the possibility that DRASIC homomultimers predominate in some neurons. Nevertheless, our interpretation is consistent with evidence that other DEG/ENaC subunits form heteromultimers. For example, three subunits, α, β, and γ, form ENaC channels (Canessa et al., 1994; McDonald et al., 1995). Genetic evidence in *C. elegans* also suggests that MEC4 and MEC10 form heteromultimers (Huang and Chalfie, 1994). Our interpretation would also account for the fact that other DEG/ENaC subunits reside where they could combine with DRASIC to generate heteromultimeric channels; BNC1 is present in lanceolate fibers and several other specialized mechanosensors (Garcia-Añoveros et al., 2001; Price et al., 2000); ASICβ is expressed in large DRG neurons (Chen et al., 1998), and β and γENaC are present in sensory endings associated with Meissner corpuscles, Merkel cells, and hair follicles (Drummond et al., 2000; Fricke et al., 2000). The diversity of molecular components, including DEG/ENaC subunits and associated scaffolding and matrix proteins (as suggested by genetic studies in *C. elegans* [Mano and Driscoll, 1999]) offers the opportunity to construct sensory receptors with substantial functional heterogeneity.

Incorporation of DRASIC into heteromultimers may also explain our finding that deleting DRASIC did not completely eliminate any one type of mechanosensory response. Perhaps the remaining DEG/ENaC subunits form channels, albeit with altered properties in the absence of DRASIC. It is also interesting that disruption of related genes in *C. elegans* can show subtle defects; UNC-8 null worms show only mild locomotion defects, and a mechanosensory defect has not yet been reported in UNC-105 null worms (Tavernarakis et al., 1997; Liu et al., 1996). In addition, other types of channels may contribute to mechanosensation; examples include Trp family members such as the vanilloid-related osmotically activated channel (Liedtke et al., 2000), TREK-1 (Maingret et al., 1999), and the NompC channel present in ciliated *Drosophila* mechanosensory neurons (Walker et al., 2000).

Role of DRASIC in Acid-Evoked Nociception

Earlier studies proposed that DEG/ENaC channels participate in acid-evoked nociception (McCleskey and Gold, 1999; Waldmann and Lazdunski, 1998). Our data support this idea. We found DRASIC in small-diameter, substance P-positive DRG neurons and in free nerve

endings in the epidermis. In addition, single-fiber recordings from −/− animals revealed a subpopulation of C fibers with a blunted response to acid. However, the behavioral responses to acid injection in the paw and a noxious thermal stimulus were not altered. Likewise, the response to acid was similar in isolated capsaicin-responsive neurons. The DRASIC channel was, however, required for development of secondary mechanical hyperalgesia induced by acid injected into skeletal muscle. This stimulus activates nociceptors innervating skeletal muscle and evokes a pronounced central sensitization and mechanical hyperalgesia to stimulation of the paw (Sluka et al., 2001). The reduced hypersensitivity in DRASIC null mice is probably due to a reduction in the activation of muscle acid-sensitive nociceptors.

Previous work showed that disruption of the VR1 gene in mice reduced H^+-gated currents in capsaicin-sensitive DRG neurons and reduced the response to acid in single C fibers (Caterina et al., 2000). Those studies suggested that VR1 is the predominant acid-responsive nociceptor. Our current data and our earlier work (Price et al., 2000) are consistent with the conclusion that VR1, rather than DRASIC and BNC1, is primarily responsible for cutaneous acid-induced nociception. However, our data indicate that DRASIC may be required for a full-blown receptor response to acid. Interestingly, as with VR1 knockout mice, C-MH fibers from DRASIC knockout animals showed a reduced response to noxious heat. This was an unexpected finding because the heterologously expressed DRASIC channel itself is not gated by noxious heat (our unpublished data). These data raise the interesting possibility of a functional interaction between VR1 and DRASIC channels; this might explain the similarities between DRASIC and VR1 null phenotypes (Caterina et al., 2000). These results also suggest that channels containing DRASIC may be targets for pharmacologic intervention for the treatment of pain.

Potential Relationship between the Kinetic Response of DRASIC to Acid and Properties of Mechanosensitive Neurons

The identity and function of the acid-evoked currents that occur in large-diameter sensory neurons have been unknown (Krishtal and Pidoplichko, 1981). It has seemed puzzling that acid activates these currents because acid does not elicit activity from the peripheral extensions of these cells (Lewin and Stucky, 2000; Steen et al., 1992). Instead, those fibers are tuned to detect primarily innocuous mechanical stimuli. Our data do not explain this paradox. However, they bring it into sharp focus by showing that DRASIC contributes to these acid-evoked currents and that DRASIC is located in the periphery, where it is required for normal mechanoreceptor function. Why then are the peripheral nerve endings not activated by acid? First, we cannot exclude the possibility that under some conditions the peripheral nerve endings might be pH-activated. For example, if the pH changes are not fast enough, the channel could activate and then desensitize without causing sufficient depolarization to generate action potentials. Second, perhaps in mechanosensory nerve endings DRASIC is tethered to extracellular and intracellular proteins that confer touch sensitivity (Mano and Driscoll, 1999). Binding matrix proteins might mask pH-sensitive sites on the channel and/or preclude H^+-activation. In other words, the absence of such specialized connections in isolated cells may allow DRASIC to respond to acid. We suggest that proton activation may serve as a signature of DRASIC function, even in cells for which protons are not the physiologic ligand.

If this idea is correct, the kinetic response to protons may provide some insight into mechanosensor function. As described above, because loss of DRASIC slowed desensitization, the peak H^+-gated current and charge transfer increased. Intriguingly, the number of spikes evoked by standard displacement stimuli also increased dramatically in RA mechanoreceptors. These mechanoreceptive neurons only respond to movement of the receptive field, and sustained mechanical displacement evokes no further activity. Thus, it is possible that channels gated by mechanical displacement are susceptible to rapid desensitization just as are the transient H^+-gated currents observed in the cell soma. We hypothesize that changes in the gating kinetics of a DEG/ENaC channel complex may explain both the phasic neural response to mechanical stimuli and the phasic current response to protons in the cell body. Further assessment of these ideas may depend on an in vitro assay of mechanosensation that incorporates DRASIC, other DEG/ENaC subunits, and associated intracellular and extracellular tethering proteins.

Conclusion

Our analysis of mice lacking the DRASIC channel reveals this subunit to have a necessary function in low- and high-threshold mechanoreception, acid nociception, and heat nociception. The extraordinary diversity of function for this channel strongly suggests that it normally exists as part of a heteromultimeric complex, the composition of which varies systematically among functionally distinct sensory neurons.

Experimental Procedures

Gene Targeting

Gene-targeting experiments were done essentially as previously described (McDonald et al., 1999). A 4.5 kb Not1/Xho1 fragment, located 5′ of exon 1, and a 3.5 kb Asc1/Kpn1 fragment, located 3′ of exon 1, were subcloned into the targeting vector, P1339 LoxP-PGK-Neo (a gift from Dr. T.J. Ley, Washington University), on either side of the neomycin resistance cassette sequence. The linearized targeting vector was introduced into ES cells by electroporation. Two different ES cell lines carrying a targeted DRASIC allele were injected into C57Bl-6 blastocysts to generate chimeras.

Real-Time Quantitative PCR

We used real-time quantitative RT-PCR to determine whether there were differences in transcript levels for BNC1a, BNC1b, ASICα, ASICß, and DRASIC (referred to as target transcripts) in DRG from +/+ and −/− mice. cDNA was prepared from three different groups of +/+ and −/− DRG RNA; each group was a pool from 5 or 6 +/+ or −/− mice. Each assay was run in quadruplicate. We used the comparative Ct method to quantify the abundance of target transcripts in −/− RNA relative to +/+ RNA, as previously described (PE Applied Biosystems User Bulletin 2).

Immunocytochemistry

A polyclonal anti-DRASIC antibody was raised in rabbits (Pocono Rabbit Farm, Canadensis, PA) against amino acids 79–153 (numbering as in [Waldmann et al., 1997a]) of the extracellular domain of mouse DRASIC expressed as a GST fusion protein in *E. coli*. The anti-DRASIC antibody was affinity purified with Affigel 10 resin (Bio-Rad, Hercules, CA) linked to purified fusion protein.

Immunocytochemical analysis of DRG tissue and hair follicles was done as previously described (Drummond et al., 2000; Price et al., 2000).

For immunocytochemical staining of Meissner-like corpuscles and free nerve endings, paw pad tissue was isolated and fixed as described for DRG. For identification of Meissner-like structures, sections were further incubated in 5% normal rabbit serum followed by unconjugated goat anti-rabbit Fab fragment (20 μg/ml) (Jackson Immuno Research), which in turn was followed by incubation with rabbit antibody to S100 (1:100) (Sigma Chemicals, St. Louis, MO), a marker for Meissner corpuscle lamellar cells and peripheral Schwann cells.

Immunocytochemical staining of Merkel cell/neurite complexes was modified from Lemaster et al. (LeMaster et al., 1999). In brief, 40 μM sections of hairy skin were incubated with mouse anti-cytokeratin 20 (1/10) (DAKO, Carpinteria, CA), a marker for Merkel cells, and were subsequently labeled with biotinylated anti-mouse IgG (Vector Laboratories) and Texas red-conjugated streptavidin (20 μg/ml) (Vector Laboratories). Sections were further incubated with rabbit anti-DRASIC and sheep anti-nerve cocktail primaries as described for staining in hair follicles (Price et al., 2000). We used a Biorad MRC1024 laser-scanning confocal microscope and software (Hercules, CA) to examine samples.

Culture of Dorsal-Root Ganglia Sensory Neurons

A procedure previously described for rat DRG (Benson et al., 1999) was used, with a few modifications (Price et al., 2000), to dissociate and culture mouse DRG.

Patch-Clamp Analysis

For whole-cell patch-clamp, DRG neurons were bathed with extracellular solution containing, in mM, 128 NaCl, 5 $MgCl_2$, 1.8 $CaCl_2$, 5.4 KCl, 5.55 glucose, 20 HEPES (pH 8). Bath solutions below pH 6 were buffered with 10 mM MES and 10 mM HEPES. The pipette solution contained, in mM, 120 KCl, 10 NaCl, 2 $MgCl_2$, 5 EGTA, 10 HEPES, 1 ATP (pH 7.4). Data were recorded with an AXOPATCH 200 amplifier (Axon Instruments, Foster City, CA). Data analysis and curve fitting were performed with Excel, Igor, and SigmaPlot software. For the patch-clamp studies, ten mice (five male and five female) from each genotype were studied. Littermates of the F2 generation were compared, and multiple litters were used.

Single-Fiber Recording

An in vitro skin/nerve preparation was used to record from functionally single primary afferents, as described previously (Carroll et al., 1998; Koltzenburg et al., 1997). At least 24 wild-type and DRASIC null animals from multiple F2 crosses were studied. Single-fiber data obtained from wild-type fibers were similar to those previously obtained in our studies of BNC1 (Price et al., 2000) and in our studies of C57/Bl6 mice (our unpublished data). The receptive properties of single sensory fibers were systematically examined with a range of quantitative stimuli. A standard ascending series of displacement stimuli was applied to the receptive field at 30 sec intervals. Each displacement was maintained for 10 s, and a sustained discharge throughout the stimulus was characterized as slowly adapting (Price et al., 2000).

The C fiber nociceptive neurons (axonal conduction velocity $<$ 1.0 m/s) were characterized in a similar manner, but in addition, an extra subset of C fibers was tested for noxious heat and acid pH sensitivity. A small metal ring was used to isolate the receptive field of the fiber, and a heated Ringer solution was then rapidly applied to the receptive field. The measured intradermal temperature increased to approximately 52°C for about 3 s, a stimulus that activates all heat-sensitive nociceptors. One minute after the heat stimulus, the ring solution was replaced with oxygenated ringer solution with its pH adjusted to 5.0 for exactly 2 min. Electrophysiological data were collected with a Powerlab 4.0 system (AD instruments), and spikes were discriminated offline with the spike histogram extension of the software.

Behavioral Studies

von Frey Filament Test of Mechanical Sensitivity

von Frey monofilaments were applied to the plantar surface of each hindpaw for 1 s. Each hindpaw was studied five times with an ascending series of monofilaments. The frequency (%) of paw withdrawal was calculated for each monofilament as described (Mansikka et al., 1999).

Radiant Heat Test of Thermal Sensitivity

Radiant heat was applied from below to the plantar surface of each hindpaw until the mouse withdrew its paw (Hargreaves et al., 1988). An average of three measurements was used to determine mean withdrawal latency.

Carrageenan Mechanical and Thermal Hypersensitivity

Carrageenan (2%; type IV; Sigma) was dissolved in sterile saline and injected subcutaneously into the right plantar hindpaw of anesthetized (halothane, 2%–4%) mice (Mogil et al., 1999). Three hr after carrageenan injection, mechanical withdrawal frequencies and withdrawal latencies to radiant heat were determined, as described above.

Response to Acid Injection in Skin

Acetic acid (0.6%, 20 μl) in saline was injected into the dorsal surface of the right hind paw in awake mice (Wheeler-Aceto et al., 1990). The total number of seconds spent licking the injected paw was recorded for 30 min.

Response to Acid Injection into Muscle

We injected mice with 20 μl sterile saline, adjusted to pH 4.0 with HCl, in one gastrocnemius muscle and used von Frey filaments to analyze the mice for mechanical sensitivity in the paw on the injected side, as previously described for rats (Sluka et al., 2001). We measured the number of withdrawals out of five trials and repeated this ten times at each testing period. We averaged the trials to get a response number between 0 and 5. Because differences in sensitivity to a variety of painful stimuli have been reported among mouse strains (Mogil et al., 1999), we used littermate $+/+$ animals as controls for $-/-$ mice. Moreover, in our animals we did not observe differences in baseline sensitivity between animals of the two genotypes, as has been reported for different pure strains of mice (Mogil et al., 1999). All behavioral studies were performed with littermates of the F2 generation. Approximately equal numbers of male and female mice were used for all studies, except for those in which acid injection into the muscle was used; in these studies, only males were used. For all behavioral studies, investigators were blinded to genotype.

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