Differential Response Properties of IB4-Positive and -Negative Unmyelinated Sensory Neurons to Protons and Capsaicin

SAHERA DIRAJLAL, LAURA E. PAUERS, AND CHERYL L. STUCKY
Department of Cell Biology, Neurobiology and Anatomy, Medical College of Wisconsin, Milwaukee, Wisconsin 53226-0509

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Differential response properties of IB4-positive and -negative unmyelinated sensory neurons to protons and capsaicin. J Neurophysiol 89: 513–524, 2003; 10.1152/jn.00371.2002. Activation of unmyelinated (C-fiber) nociceptors by noxious chemicals plays a critical role in the initiation and maintenance of injury-induced pain. C-fiber nociceptors can be divided into two groups in which one class depends on nerve growth factor during postnatal development and contains neuropeptides, and the second class depends on glial cell line-derived neurotrophic factor during postnatal development and contains few neuropeptides but binds isolectin B4 (IB4). We determined the sensitivity of these two populations to protons and capsaicin using whole cell recordings of dorsal root ganglion neurons from adult mouse. IB4-negative unmyelinated neurons were significantly more responsive to protons than IB4-positive neurons in a concentration-dependent manner. Approximately 86% of IB4-negative neurons responded to pH 5.0 with an inward current compared with only 33% of IB4-positive neurons. The subtypes of proton-evoked currents in IB4-negative unmyelinated neurons were also more diverse. Many IB4-negative neurons exhibited transient, rapidly inactivating proton currents as well as sustained proton currents. In contrast, IB4-positive neurons never displayed transient proton currents and responded to protons only with sustained, slowly inactivating inward currents. The two classes of neurons also responded differently to capsaicin. Twice as many naïve IB4-negative unmyelinated neurons responded to 1 μM capsaicin as IB4-positive neurons, and the capsaicin-evoked currents in IB4-negative neurons were approximately fourfold larger than those in IB4-positive neurons. Interestingly, proton exposure altered the capsaicin responsiveness of the two classes of neurons in opposite ways. Brief preexposure to protons increased the number of capsaicin-responsive IB4-positive neurons by twofold and increased the capsaicin-evoked currents by threefold. Conversely, proton exposure decreased the number of capsaicin-responsive IB4-negative neurons by 50%. These data suggest that IB4-negative unmyelinated nociceptors are initially the primary responders to both protons and capsaicin, but IB4-positive nociceptors have a unique capacity to be sensitized by protons to capsaicin-receptor agonists.

INTRODUCTION

Activation and sensitization of C-fiber nociceptors is a key driving force underlying the pain that occurs with tissue injury. The increased excitation of nociceptors during injury has been attributed to the generation of a variety of chemicals by local inflammatory and immune cells (Levine and Reichling 1999). One prominent chemical in the injured milieu is hydrogen ions and accumulation of hydrogen ions results in tissue acidosis. Mild-to-severe tissue acidosis occurs with painful clinical disorders, which include inflammation, ischemia, muscle fatigue, hematomas, and bone cancer (Griffiths 1991; Häbler 1929; Hood et al. 1988; Luger et al. 2001; Pan et al. 1988; Revici et al. 1949), and the degree of pain in humans has been correlated with the level of tissue acidosis (Issberner et al. 1996). Protons can activate the receptive terminals of nociceptors in situ (Steen et al. 1992), and protons exert their effects on sensory neurons by inducing at least two diverse types of inward currents. One current is a transient, rapidly desensitizing current and the second is a sustained, slowly inactivating inward current (Bevan and Yeats 1991; Krishal and Pidoplichko 1980). Evidence suggests that the sustained proton current in nociceptors may result from proton-induced activation of the capsaicin receptor VR1 (Caterina et al. 2000; Tominaga et al. 1998), although incomplete overlap in the responsiveness of individual nociceptors to protons and capsaicin has also been reported (Steen et al. 1992).

C-fiber nociceptors are notably diverse in their capacity to be activated and sensitized by noxious and inflammatory chemicals. Two broad classes of C-fiber nociceptors have recently attracted attention because of their distinct neurochemical characteristics and neurotrophic factor responsiveness. The first group expresses trkA receptors for nerve growth factor (NGF), depends on NGF for survival during postnatal development, and contains neuropeptides such as calcitonin gene-related peptide and substance P. The second class expresses receptors for glial cell line-derived neurotrophic factor (GDNF), depends on GDNF for survival during postnatal development, and is relatively “peptide poor” but expresses a surface carbohydrate group that binds isolectin B4 (IB4) (Averill et al. 1995; Bennett et al. 1996, 1998; Molliver et al. 1997). The central terminals of the two groups project to distinct regions of the dorsal spinal cord. IB4-binding C-fibers project principally to inner lamina II (substantia gelatinosa) whereas the IB4-negative “peptide rich” C-fibers terminate more superficially in lamina I and outer lamina II (Molliver et al. 1995; Nagy and Hunt 1982; Silverman and Kruger 1990). These cellular and anatomical differences have contributed to a hypothesis that the two classes of C-fiber nociceptors serve distinct functional roles in inflammatory and neuropathic pain (Snider and McMahon 1998). How-
ever, clear evidence of different physiological roles in either acute nociception or persistent pain remains to be demonstrated. We have previously shown that IB₄-negative nociceptors from adult mouse have significantly larger heat-evoked inward currents compared with IB₄-positive nociceptors, suggesting that the peptidergic, NGF-responsive nociceptors may play the prominent role in acute responses to noxious heat (Stucky and Lewin 1999). However, IB₄-positive nociceptors also contribute to noxious heat transmission since specific ablation of IB₄-binding neurons in vivo causes thermal as well as mechanical nociceptive behavioral thresholds to increase (Vulchanova et al. 2001).

Because protons are elevated in diverse pathological conditions and because clear evidence of the nociceptive properties of GDNF- and NGF-dependent nociceptors is sparse, we investigated the response properties of IB₄-positive and IB₄-negative unmyelinated neurons to protons and the exogenous allogenic capsaicin.

METHODS

NEURONAL ISOLATION. Lumbar dorsal root ganglia (DRG) 1–6 were removed bilaterally from adult wild-type C57BL6 mice (age 2–3 mo) and placed in Ca²⁺/Mg²⁺-free HBSS (Gibco). The DRGs were incubated with 1 mg/ml collagenase IV (Sigma, St. Louis, MO) and 0.05% trypsin (Sigma-Aldrich) for 40 min at 37°C and dissociated into single cells by passing through flame-constricted Pasteur pipettes of decreasing diameter. The cells were washed and resuspended in DMEM/Hams-F12 medium containing 10% heat-inactivated horse serum, 20 mM glutamine, 0.8% glucose, 100 units penicillin, and 100 μg/ml streptomycin (Gibco, Invitrogen). Cells were plated on microgrid CELLlocate coverslips (square size 55 μm; Eppendorf) that were coated with poly-l-lysine (200 μg/ml) at a density of 1000–2000 cells per coverslip and maintained overnight at 37°C, 5% CO₂. Because NGF has been shown to alter the response properties of small-diameter neurons to nociceptive stimuli within minutes (Shu and Mendell 2001), no exogenous growth factors were added. One mouse per coverslip was studied and, after each recording, the chamber was washed with ethanol and water to eliminate any residual capsacain.

Electrophysiology. Whole cell recordings were made using fire-polished glass electrodes (2–5 MΩ resistance) pulled from filamented borosilicate glass on a micropipette puller (P-87; Sutter Instruments). The recording chamber (volume 400 μl) was continuously superfused (2–3 ml/min) with extracellular solution containing (in mM) 150 NaCl, 5.6 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 8 glucose; pH brought to 7.4 with 1 M NaOH; osmolarity = 320 mOsm. Cells were filled with solution containing (in mM) 135 KCl, 0.2 NaGTP, 4.1 MgCl₂, 2 EGTA, 10 HEPES, and 2.5 ATPNa₂; pH brought to 7.2 with 1 M KOH; osmolarity = 290 mOsm. All solutions were made fresh daily and filtered just before use. Neurons were visualized with a Nikon TE2000 inverted microscope and soma size was estimated by calculating the mean of the longest and shortest cross-sectional diameters with the aid of a calibrated eyepiece reticle. Immediately after each recording, neurons were incubated with 10 μg/ml IB₄ (Bandeiraea Simplicifolia BS-IB₄) conjugated to fluorescein isothiocyanate (IB₄-FITC; Sigma-Aldrich) for 10 min and rinsed for 2 min and the IB₄-FITC staining was visualized. The location of the neuron with respect to the grid on the coverslip was determined and the patch pipette was gently removed from the membrane. The cells were fixed with 4% paraformaldehyde for 10–15 min, washed, and saved for staining 1–4 days later with the antibody N52 (below). Recordings from neurons that were either lost when the pipette was removed or lost during N52 staining (approximately 25% of all recorded neurons) were deleted from the data reported here.

For all chemical tests with pH and capsaicin, solutions were applied locally and rapidly (10 s duration) to the neuron of interest using silica 28-gauge syringes of 0.25 mm ID (World Precision Instruments). The tip of each syringe was placed 50 μm from the cell soma using a manipulator (Narishige) and the gravity-fed solutions were controlled by manual switching of one-way stopcock valves (Cole-Parmer). Solutions containing protons were buffered to 7.0, 6.0, or 5.0 with HCl. These pH values were chosen because identified C-fiber nociceptors in the isolated skin-nerve preparation are maximally activated by pH 5.2 (Steen et al. 1992). HEPES-based buffer was used for solutions of pH 7.4 and 7.0, and MES-based buffer for pH 6.0 and 5.0. For the proton concentration–response curve, each neuron was tested successively with pH 7.0, 6.0, and 5.0 for 10 s each and a 2-min wash was given between the different proton tests. Another group of naive neurons was tested only with pH 5.0 for 10 s. To test the effect of amiloride on the responses of neurons to protons, a pH 5.0 stimulus was applied for 10 s, the neuron was then superfused with buffer containing 100 μM amiloride for 3 min, and a second pH 5.0 test containing 100 μM amiloride was applied for 10 s. The neuron was then washed with extracellular buffer for 3 min and a third pH 5.0 test was performed to determine the recovery of the proton response.

For experiments with capsaicin, 1 μM capsaicin (in HEPES buffer) was prepared fresh each day from a 10 mM capsaicin stock solution dissolved in 1-methyl-2-pyrrolidinone (Sigma-Aldrich), a solvent that has no effect on the physiology of sensory neurons (Evans et al. 1999; Nicol et al. 1997). To test the effect of capsaicin on naive unmyelinated neurons, a group of neurons was superfused with 1 μM capsaicin for 10 s. To determine the overlap between proton and capsaicin responsiveness, as well as to determine the effect of proton exposure on capsaicain responsiveness, a separate group of neurons was treated with pH 5.0 for 10 s, washed for 2 min, and then exposed to a 1 μM capsaicin for 10 s. One neuron per coverslip was studied and, after each recording, the chamber was washed with ethanol and water to eliminate any residual capsacain.

Data recording and analysis

Membrane voltage was clamped using an EPC-9 amplifier run by Pulse software (version 8.50, HEKA Electronic, Lambrecht, Germany). Data were sampled at 500 Hz. Whole cell configuration was maintained at −60 mV. Seals ranged from 1.5 to 6.0 GΩ. Pipette and cell capacitance were compensated using the computer-controlled circuitry. Electrical access to the cell was monitored every minute throughout each recording by measuring the size of the uncompensated cell capacitance transients and ensuring that the transients did not change by more than 10%. Series resistance ranged from 3 to 9 MΩ and a neuron was discarded if series resistance changed by more than 10% during the recording. After establishing whole cell configuration, the recording was switched to current-clamp mode. Action potentials were generated by injecting current from 0.02 to 1.8 nA for 40 ms. The presence of an inflection on the falling phase of the action potential falling phase was determined using Igor software (version 4.01, WaveMetrics) to calculate the rate of change in voltage during the action potential. The recording mode was then switched back to voltage clamp to measure changes in inward currents evoked by chemical stimuli. The magnitude of inward current was determined using PulseFit software. Neurons were considered to be proton or capsacain sensitive if either chemical elicited an inward current of ≥100 pA in peak amplitude. For neurons that responded to low pH with a transient, rapidly desensitizing inward current, the time course for activation (current onset to peak) and for desensitization (current peak to 75% of recovery) was determined using PulseFit software. To correct for differences in cell size, all inward current values are expressed as a function of cell capacitance (pA/μF). For statistical measures, groups were compared using χ² test, an unpaired two-tailed t-test, or a paired two-tailed t-test using Instat (GraphPad Software). Error bars indicate ±SE.
Staining

We used the antibody clone N52, which recognizes the high-molecular-weight (200 kD) neurofilament protein to distinguish neurons that are likely to have myelinated axons (A-fibers) in vivo from neurons likely to have unmyelinated axons (C-fibers). The presence of the 200-kD neurofilament protein in sensory neurons has been correlated with the presence of myelination (Lawson and Waddell 1991) and the antibody N52 has been used extensively to identify myelinated neurons in rat (Amaya et al. 2000, Beland and Fitzgerald 2001; Bennett et al. 1998; Chen et al. 1998; Michael and Priestley 1999) and has more recently been used in mouse (Matsuo et al. 2001; Orozco et al. 2001). To determine the expression of the 200-kD neurofilament protein in recorded neurons, the fixed neurons were rinsed for 15 min in PBS, pH 7.4, with 0.1% Triton X-100. Nonspecific staining was reduced by incubating the coverslips for 1 h (RT) in 4% normal goat serum (Jackson ImmunoResearch Laboratories) diluted in PBS with 0.1% Triton X-100. Neurons were incubated with the mouse monoclonal anti-neurofilament 200-kD antibody N52 (1:30,000; Sigma-Aldrich) overnight at 4°C, washed, and incubated with Texas Red-conjugated goat anti-mouse IgG (1:1000; Jackson ImmunoResearch Laboratories) for 1 h (RT). Coverslips were inverted onto slides over Fluoromount-G mounting medium (Southern Biotechnology Associates).

To determine the size distribution of all neurons in the cultures and the size of neurons that were IB4 positive or N52 positive, separate staining experiments were performed on mouse lumbar 1–6 DRG neurons that were dissociated, plated for 15–24 h, and then fixed (n = 3 animals in different preparations). A double-stain was performed with the mouse N52 anti-neurofilament antibody combined with biotinylated IB4 (Sigma-Aldrich). Staining for N52 was performed as above, and after the anti-mouse Texas Red-conjugated secondary antibody was rinsed off, the cells were incubated with 4% normal mouse serum (Sigma-Aldrich) for 1 h (RT). Cells were then incubated with biotin-labeled IB4 (10 μg/ml, Sigma-Aldrich) for 1 h (RT) and then (FITC)-conjugated IgG monoclonal mouse anti-biotin for 1 h (1:200; RT; Jackson ImmunoResearch Laboratories). Controls for each staining in which the N52 primary antibody and biotinylated IB4 were omitted were performed in parallel.

Immunostaining analysis

For analysis of double-staining with N52 and IB4, images were obtained with a Spot II color camera (Diagnostics) attached to an upright fluorescence microscope (Nikon Optiphot). Images were analyzed with Metamorph software (version 2.5, Universal Imaging). Neurons that appeared granular under phase-contrast optics (approximately 15% of the population) were excluded from our analysis.
because their cytoplasm and nucleus stained intensely with the secondary antibodies alone. All remaining neurons were measured for soma size and intensity of staining. The average brightness intensity was determined for each cell body. Neurons were considered to be positive for either N52 or IB4 if they had mean brightness values greater than any control value measured from nongranular neurons stained with the secondary antibody alone. In addition, all neurons classified as N52 positive had bright neurofilaments coursing through the soma and all neurons classified as IB4 positive had an intense bright ring of stain around the soma membrane. Neurons that had been characterized electrophysiologically were located with respect to the grid and N52 staining was analyzed as indicated above.

RESULTS

IB4 and N52 label separate populations of mouse DRG neurons

Figure 1A shows a confocal image of two neurons that had undergone double-staining for IB4 and N52. Note that the small neuron is labeled only with IB4 whereas the large neuron stained positively only for N52. Figure 1B shows a pie chart of the fraction of all neurons isolated from mouse lumbar DRGs that labeled with each of these markers. Approximately 47% of all neurons labeled with N52 and therefore are likely to have had myelinated axons in vivo, and 53% were N52 negative and probably unmyelinated. Among the N52 negative neurons, 54% were IB4 positive and 46% were IB4 negative. These proportions of mouse neurons that labeled with IB4 or N52 are very similar to those in adult rat (McMahon and Bennett 1999). Only 2% of the isolated mouse DRG neurons stained positively for both IB4 and N52, and this minimal overlap is consistent with the isolated mouse DRG neurons stained positively for both IB4 and N52, and this minimal overlap is consistent with the pattern of 200-kD neurofilament staining very similar to that reported for DRG sections from adult rat (Lawson and Waddell 1991; Perry et al. 1991). Nearly all large mouse DRG neurons (≥30 μm diam) stained positively for N52. However, as with rat DRG neurons, a few smaller mouse neurons (<20 μm diam) were also N52 positive. N52-positive neurons will be referred to as “myelinated” and N52 negative neurons as “unmyelinated,” with the acknowledgment that N52 distinguishes between neurons that are likely myelinated versus unmyelinated in vivo.

Most unmyelinated mouse DRG neurons in isolation have an inflection on the somal action potential

The majority of unmyelinated mouse DRG neurons had a prominent inflection on the falling phase of the action potential (Fig. 2A, left) as 95% (100/105) of IB4-positive unmyelinated neurons and 89% (86/97) of IB4-negative unmyelinated neurons had an inflection on the somal action potential (Fig. 2B). Although a tight correlation between an inflection on the action potential and nociceptive response properties has been shown for myelinated fibers in rodents (Ritter and Mendell 1992), a similar tight correlation has not yet been demonstrated for unmyelinated fibers in rodents. However, nociceptive C-fibers in guinea pig have action potentials that are significantly longer in duration than nonnociceptive C-fibers (Djouhri et al. 1998). Therefore we have excluded neurons without an inflection to bias our data set toward unmyelinated neurons that are nociceptors. For the data reported below, all neurons had an inflection on the somal action potential and were negative for N52 staining.

![Figure 2](http://jn.physiology.org/Downloaded/from/10.22033.2)
Unmyelinated neurons from mouse have transient and sustained proton currents

Two distinct inward currents evoked by pH 5.0 were observed in mouse DRG neurons: a transient inward current that rapidly inactivated within 1 s and was large (>1 nA) in magnitude (Fig. 3, a), and a sustained inward current that inactivated slowly (>20 s) and typically outlasted the pH stimulus in duration (Fig. 3, b). Some unmyelinated neurons expressed a transient proton current that was followed by a sustained current as shown for all examples in Fig. 3, but other unmyelinated neurons exhibited only a sustained inward current (Fig. 4A). For each neuron, the profile of the inward current, whether transient followed by sustained or sustained alone, could be reproducibly evoked multiple times (data not shown).

The transient proton currents in all neurons were similar in duration (723 ± 39 ms; mean ± SE; duration measured at 25% below current onset to peak; n = 17; Fig. 3, insets). The time course for the transient current activation to peak was 134.6 ± 17.9 ms (range 54.4 to 267.3 ms) and the time course for desensitization from current peak to 75% of recovery was 312.3 ± 32.2 ms (range 48.7 to 517.2 ms; n = 17). In contrast, the sustained, slowly inactivating proton current profiles were far more heterogeneous in magnitude, duration, and profile from neuron to neuron (Fig. 4, A and B). We classified inward currents evoked by low pH into two categories: 1) transient

FIG. 3. Examples of whole cell voltage-clamp recordings in 6 different unmyelinated neurons that responded to pH 5.0 (10-s exposure) with a transient followed by sustained inward current. a: transient current. b: sustained component of the current. Insets: Transient component on an expanded time base. Note the similarity in the profile and duration of the transient component and the heterogeneity of the sustained components. Scale bars that indicate time and current magnitude are the same for all 6 neurons.

FIG. 4. A: examples of whole cell voltage-clamp recordings in 6 different unmyelinated neurons that responded to pH 5.0 (10-s exposure) with a sustained, nondesensitizing inward current (no transient component). Note that the sustained currents are heterogeneous in magnitude, duration, and profile. Scale bar that indicates time and current magnitude is the same for all 6 neurons. B: bar graph shows the distribution of the magnitudes of the sustained-only responses to pH 5.0 in unmyelinated neurons (n = 62).

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followed by sustained or 2) sustained only. None of the unmyelinated neurons responded to protons with a transient current alone as all transient inward currents were followed by a sustained current ≥100 pA.

Amiloride inhibits both the transient and sustained proton currents in mouse unmyelinated neurons

All known members of the ASIC family are sensitive to the epithelial sodium channel blocker amiloride at approximately 100 μM (McCleskey and Gold 1999). As expected, the transient rapidly desensitizing proton current in unmyelinated mouse neurons was almost completely blocked by 100 μM amiloride (average block 88%; range 69 to 100% block; n = 9; Fig. 5, A and B; P < 0.001; paired t-test). Interestingly, the sustained current that followed the transient current was also partially blocked (average block 58%; range 31 to 100% block; n = 9; Fig. 5, A and B; P < 0.05; paired t-test). Furthermore, the sustained-only proton currents in all but one neuron were inhibited by amiloride (average block 74%; n = 15; range block for 14 neurons 49 to 100% and 1 neuron potentiated by 17%; Fig. 5, C and D; P < 0.05; paired t-test). Thus, in mouse DRG neurons, both the transient and sustained proton currents were reversibly inhibited by amiloride.

IB4-negative unmyelinated neurons are more responsive to protons than IB4-positive neurons

Next, we classified the unmyelinated neurons as IB4 positive or IB4 negative and found that IB4-negative neurons were substantially more responsive to protons than IB4-positive neurons. Figure 6A shows a concentration–response relationship for the percentage of IB4-positive and -negative neurons that responded to protons. For this data set (32 neurons from 4 mice), each neuron was tested sequentially with pH 7.0, 6.0, and 5.0 for 10 s with a 2-min wash between low pH tests. Increasingly lower pH stimuli recruited responses from more neurons in both populations. Significantly more IB4-negative

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**FIG. 5.** A: examples of voltage-clamp recordings from one unmyelinated neuron that exhibited a transient inward current in response to pH 5.0 (top). The transient pH current was almost completely blocked by 100 μM amiloride (middle), and then recovered after a 3-min washout (bottom). B: bar graphs show that 100 μM amiloride almost completely blocks the transient component of the proton response (n = 9; ***P < 0.001; paired t-test). Furthermore, 100 μM amiloride significantly reduced the sustained current that follows a transient (n = 9; *P < 0.05; paired t-test). C: examples of voltage-clamp recordings from one unmyelinated neuron that exhibited only a sustained inward current in response to pH 5.0 (top). The sustained-only proton current was also significantly blocked by amiloride (middle) and recovered following a 3-min washout (bottom). D: bar graph shows that 100 μM amiloride significantly and reversibly blocked the sustained-only proton currents in unmyelinated mouse DRG neurons (n = 15; *P < 0.05; paired t-test).
neurons responded to protons than IB4-positive neurons in a concentration-dependent manner, and at pH 5.0, 86% (12/14) of IB4-negative neurons responded compared with only 33% (6/18) of IB4-positive neurons (P < 0.005; χ²). Similarly, in a separate data set in which neurons were tested only with pH 5.0 (102 neurons from 29 mice), IB4-negative neurons were significantly more responsive to low pH (77%; 36/47) than IB4-positive neurons (45%; 25/55; P < 0.005; χ²). With all pH 5.0 responses combined, the inward current evoked by pH 5.0 was twofold greater in IB4-negative neurons (81.3 ± 14.9 pA/pF; n = 48) than IB4-positive neurons (40.8 ± 11.2 pA/pF; n = 31; P < 0.05; unpaired t-test). Thus IB4-negative neurons are more responsive to protons in number and magnitude.

**IB4-negative neurons exhibit transient proton currents but IB4-positive neurons have no transients**

Among IB4-negative unmyelinated neurons that responded to pH 5.0, 35% (17/48) exhibited a large transient followed by sustained inward current. In contrast, none of the IB4-positive neurons that responded to pH 5.0 (0/31) exhibited a transient proton current (P < 0.0005; χ²; Fig. 6B). The pattern was similar for pH 6.0, for which three of six IB4-negative neurons exhibited a transient and sustained proton current whereas IB4-positive neurons had only sustained proton currents (n = 3).

The magnitude of the sustained-only current evoked by pH 5.0 in IB4-positive and -negative neurons was not significantly different (IB4 positive 40.8 ± 11.2 pA/pF; n = 31; IB4 negative 81.6 ± 20.6 pA/pF; n = 31; P = 0.09, unpaired t-test). In IB4-negative neurons, the magnitude of the pH 5.0-induced transient current was 80.6 ± 20 pA/pF (n = 17) and the magnitude of the sustained current that followed a transient was 21 ± 4.9 pA/pF (n = 17).

**IB4-negative unmyelinated neurons are more responsive to capsaicin than IB4-positive neurons**

Capsaicin is frequently used as a marker for nociceptive unmyelinated neurons and Fig. 7A shows a typical response of an IB4-positive and an IB4-negative neuron to 1 μM capsaicin. Naïve IB4-negative unmyelinated neurons (no prior chemical treatment) were more responsive to capsaicin than IB4-positive neurons in both number and magnitude. Whereas 61% of IB4-negative unmyelinated neurons responded to capsaicin, only 28% of IB4-positive neurons responded (P < 0.05 χ²; Fig. 7B, left). Furthermore, the capsaicin-evoked inward currents in IB4-negative neurons were on average 4.5-fold larger than those in IB4-positive neurons (P < 0.01, unpaired t-test; Fig. 7B, right).

**Sustained responses to protons and capsaicin do not overlap extensively in mouse unmyelinated neurons**

We then determined whether the same neurons that respond to protons also respond to capsaicin. Since one agonist must be presented first in this experiment, we chose protons because we observed minimal desensitization of either the sustained or transient proton currents with repeated 10-s exposures to pH 5.0 presented 2 min apart, but capsaicin induced a profound, long-lasting (>10 min) desensitization in our hands (data not shown). Therefore we applied pH 5.0 for 10 s, washed the neurons for 2 min, and applied 1 μM capsaicin for 10 s. Responsiveness to protons and capsaicin did not overlap completely for individual neurons, even for the sustained-only proton current. Among IB4-positive neurons that responded to pH 5.0 (all sustained-only inward currents), 70% also responded to capsaicin 2 min later (Table 1). The overlap was much less extensive for IB4-negative neurons for which only 21% of the neurons that responded to pH 5.0 with a sustained-only inward current also responded to capsaicin and 36% of the neurons that responded with a transient followed by sustained current also responded to capsaicin (Table 1). Conversely, many IB4-positive (43%) and IB4-negative (30%) neurons that exhibited no response to protons did respond to capsaicin (Table 1). These data indicate that the functional ion channels that protons and capsaicin activate to evoke a sustained, non-desensitizing inward current are not completely overlapping and/or prior treatment with protons affects subsequent responses to capsaicin.

**Protons sensitize IB4-positive neurons to capsaicin but desensitize IB4-negative neurons**

Next we determined whether proton exposure affects capsaicin responsiveness. We compared the capsaicin responsiveness of neurons previously exposed to protons to that of naïve neurons tested only with capsaicin. Prior treatment with pH 5.0
for just 10 s differentially altered the responsiveness of IB4-positive and -negative neurons to capsaicin. Exposure to protons increased the proportion of IB4-positive neurons that responded to capsaicin from 28 to 54% (\(P < 0.05; \chi^2\); Fig. 7C) and increased the magnitude of the capsaicin-evoked inward currents in IB4-positive neurons on average by threefold (no pH 124.7 ± 41.8 pA/pF; pH pretreated 155.1 ± 35.5 pA/pF; \(P < 0.05;\) unpaired \(t\)-test; Fig. 7D). Conversely, prior pH exposure decreased the proportion of IB4-negative neurons that responded to capsaicin from 61 to 28% (\(P < 0.05; \chi^2\); Fig. 7C) but had no effect on the magnitude of capsaicin response (no pH 183.9 ± 520 S. DIRAJLAL, L. E. PAUERS, AND C. L. STUCKY

**DISCUSSION**

This study demonstrates that IB4-positive and -negative unmyelinated sensory neurons have different response properties to the chemicals protons and capsaicin. The key differences are

### TABLE 1. Overlap between proton and capsaicin responses in unmyelinated neurons

<table>
<thead>
<tr>
<th>pH Responders That Responded to Cap, %</th>
<th>pH Responders That Did Not Respond to Cap, %</th>
<th>Non-pH Responders That Responded to Cap, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB4 positive</td>
<td>IB4 negative</td>
<td></td>
</tr>
<tr>
<td>All sustained pH responses</td>
<td>All sustained pH responses</td>
<td></td>
</tr>
<tr>
<td>70 (14/20)</td>
<td>30 (6/20)</td>
<td>43 (13/30)</td>
</tr>
<tr>
<td>27 (9/33)</td>
<td>73 (24/33)</td>
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<tr>
<td>Sust-only pH</td>
<td>Sust-only pH</td>
<td></td>
</tr>
<tr>
<td>21% (4/19)</td>
<td>79% (15/19)</td>
<td></td>
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<tr>
<td>Trans + Sust pH</td>
<td>Trans + Sust pH</td>
<td></td>
</tr>
<tr>
<td>36% (5/14)</td>
<td>64% (9/14)</td>
<td></td>
</tr>
</tbody>
</table>

Each neuron was tested with pH 5.0 for 10 s, washed for 2 min, and then tested with 1 μM capsaicin for 10 s. Columns show the overlap in proton and capsaicin responsiveness for individual neurons.
IB$_4$-negative unmyelinated neurons are two- to threefold more likely to respond to protons than IB$_4$-positive neurons; 2) many IB$_4$-negative unmyelinated neurons express transient, rapidly desensitizing proton currents whereas IB$_4$-positive neurons have no transient proton currents; 3) naive IB$_4$-negative unmyelinated neurons are significantly more responsive to capsaicin than IB$_4$-positive neurons in both number of neurons and magnitude of response; and 4) pretreatment with protons significantly increases both the number of capsaicin-responsive IB$_4$-positive neurons and the magnitude of the capsaicin-evoked currents. In contrast, proton-exposure decreases the number of IB$_4$-negative neurons that respond to capsaicin. These data suggest that IB$_4$-negative, peptide-rich C-fiber nociceptors are the major class that initially responds to both protons and capsaicin and, second, IB$_4$-positive peptide-poor C-fiber nociceptors are the class of C-fibers that is sensitized by protons to respond to capsaicin-receptor agonists while IB$_4$-negative peptide-rich nociceptors are desensitized by protons.

**IB$_4$-negative neurons are the major class of unmyelinated neurons that responds to protons and capsaicin**

We found that IB$_4$-negative unmyelinated neurons were twice as likely to respond directly to protons compared to IB$_4$-positive neurons. Furthermore, IB$_4$-negative neurons expressed a greater variety of inward current subtypes, including transient, rapidly desensitizing proton currents as well as sustained, slowly desensitizing proton currents. These data suggest that IB$_4$-negative, peptide-rich nociceptors initially initiate and contribute to maintaining the acid-evoked pain that occurs with inflammation, muscle ischemia, or bone cancer. As with protons, IB$_4$-negative unmyelinated neurons were twice as likely to respond to capsaicin and exhibited capsaicin currents that were on average fourfold larger than those in IB$_4$-positive neurons. These data indicate that IB$_4$-negative nociceptors are the major class of C-fiber nociceptors that responds to capsaicin-receptor agonists. The larger capsaicin-evoked currents in IB$_4$-negative neurons are consistent with our previous finding that IB$_4$-negative neurons have significantly larger currents evoked by noxious heat (Stucky and Lewin 1999). Together, the data indicate that IB$_4$-negative unmyelinated nociceptors are primarily responsible for the initial nociceptive response to protons, capsaicin, and noxious heat.

**Capsaicin responsiveness versus VR1 staining in unmyelinated neurons**

Naive IB$_4$-negative unmyelinated neurons were twice as likely to respond to capsaicin (61%) compared to IB$_4$-positive neurons (28%) and exhibited capsaicin-evoked inward currents that were approximately fourfold larger than those in IB$_4$-positive neurons. The percentage of naive IB$_4$-positive neurons in mouse that responds to capsaicin (28%) is higher than the recently reported very low percentage of IB$_4$-positive neurons in mouse DRG sections that stain with VR1 antibodies (2–3%) (Zwick et al. 2002). This discrepancy between VR1 expression and capsaicin responsiveness in IB$_4$-positive neurons from mouse is even more apparent considering our finding that a brief treatment with protons increases the percentage of capsaicin-responsive IB$_4$-positive neurons to 54%. A similar discrepancy is present in comparing the percentage of IB$_4$-negative neurons that respond to capsaicin in our study (61%) and the number of trkA-expressing neurons in mouse DRG sections that stain for VR1 (22%) (BD Davis, personal communication). One explanation is that the mouse may express receptors other than VR1 that respond to capsaicin. However, there is little evidence available to date that supports this possibility, since DRG neurons from VR1 knockout mice are reported to completely lack responses to capsaicin (Caterina et al. 2000; Davis et al. 2000). A more likely explanation is that, since the VR1 antibodies available were generated against rat VR1 (Zwick et al. 2002), they may not optimally recognize mouse VR1 protein.

**Capsaicin responsiveness versus heat responsiveness in unmyelinated neurons**

We previously showed that IB$_4$-negative neurons have heat-evoked currents that are twofold larger than those in IB$_4$-positive neurons (Stucky and Lewin 1999). The finding that IB$_4$-negative neurons have larger inward currents evoked by both heat and capsaicin is consistent with evidence that these two stimuli can act through the VR1 receptor. However, the percentage of IB$_4$-positive neurons that responds to noxious heat (49%) (Stucky and Lewin 1999) is higher than the percentage of naive IB$_4$-positive neurons that were capsaicin sensitive (28%). This mismatch is consistent with the idea that receptors other than VR1 also transduce moderate noxious heat in IB$_4$-positive nociceptors. The presence of other non-VR1 heat transducers is strongly supported by the fact that some responsiveness to moderate noxious heat remains in VR1-deficient mice (Caterina et al. 2000; Davis et al. 2000), the finding that only a few single ion channels in DRG neurons respond to both heat and capsaicin (Nagy and Rang 1999), and the recent identification of other heat-sensitive channels, including TRPV3 and TRPV4, that are insensitive to capsaicin (Güler et al. 2002; Peier et al. 2002; Smith et al. 2002).

**Mechanisms underlying transient proton currents in IB$_4$-negative neurons**

IB$_4$-negative unmyelinated neurons selectively expressed transient proton currents and these transient currents were reversibly blocked by amiloride. Transient proton currents are mediated by members of the amiloride-sensitive epithelial sodium channel (ENaC)-degenerin family, which currently includes five subtypes: ASIC1a (also called ASIC-α, BNC2) and its splice variant ASIC1b, ASIC2a (also known as BNC1; MDEG) and its splice variant ASIC2b, and ASIC3 (also called DRASIC or Dorsal Root Ganglion Acid Sensing Ion Channel) (Price et al. 1996; Waldmann et al. 1997; Waldmann and Lazdunski 1998). The mRNA for all ASIC subtypes is expressed in rat DRG neurons (Benson and Sutherland 2001; Price et al. 2000). In our study, amiloride almost completely blocked the transient proton component in IB$_4$-negative mouse neurons. A recent study indicates that the rapidly desensitizing, transient proton currents in native mouse DRG neurons are due to coexpression of ASIC1, ASIC2, and ASIC3, which assemble into heteromultimeric channels (Benson et al. 2002). Thus it is quite possible that the heterogeneity we observed in the sustained currents that follow a transient current in IB$_4$-neg-
tive neurons is due to different stoichiometric compositions of multiple ASIC family channels.

The transient, rapidly desensitizing proton currents found selectively in IB4-negative neurons may play a role in activating nociceptors following a sudden onset of acidification. A nociceptive role for the transient pH currents has been questioned because the transient current desensitizes very rapidly, whereas the pain due to inflammation or ischemia is persistent (Steen et al. 1995). However, evidence now indicates that at least ASIC3 (DRASIC) is involved in nociception because C-fiber nociceptors from mice lacking ASIC3 have reduced responses to acid, and these mice have reduced mechanical behavioral hyperalgesia evoked by acid injection into muscle (Price et al. 2001). Therefore, the transient proton currents in IB4-negative unmyelinated nociceptors may contribute to the initiation or maintenance of acid-induced hyperalgesia.

In addition to mediating responses to protons, ASIC channels have also been proposed to transduce mechanical stimuli in sensory neurons. ASIC channels in mammals share significant homology at the amino acid level to putative mechanotransduction proteins in C. elegans that are also members of the ENaC-degenerin family (Gillespie and Walker 2001). Recent in situ functional studies have provided evidence that two of the ASIC family members contribute to mechanotransduction in mouse sensory neurons. ASIC2a appears to set the sensitivity of myelinated rapidly adapting receptors (Price et al. 2000) whereas DRASIC contributes to mechanical transduction in myelinated (Aβ) nociceptors (Price et al. 2001). For unmyelinated nociceptors, no evidence directly indicates that specific ASIC family members are involved in mechanotransduction. However, an interesting correlation to our ASIC family members are involved in mechanotransduction.

In addition to mediating responses to protons, ASIC channels have also been proposed to transduce mechanical stimuli in sensory neurons. ASIC channels in mammals share significant homology at the amino acid level to putative mechanotransduction proteins in C. elegans that are also members of the ENaC-degenerin family (Gillespie and Walker 2001). Recent in situ functional studies have provided evidence that two of the ASIC family members contribute to mechanotransduction in mouse sensory neurons. ASIC2a appears to set the sensitivity of myelinated rapidly adapting receptors (Price et al. 2000) whereas DRASIC contributes to mechanical transduction in myelinated (Aβ) nociceptors (Price et al. 2001). For unmyelinated nociceptors, no evidence directly indicates that specific ASIC family members are involved in mechanotransduction. However, an interesting correlation to our finding that many IB4-negative unmyelinated neurons have transient ASIC-like currents whereas IB4-positive neurons have none is that IB4-negative neurons in isolation have substantially lower mechanical thresholds and larger mechanically gated currents than IB4-positive neurons (Drew et al. 2002). Thus one speculation is that the presence or absence of ASIC channels contributes to setting the mechanical response threshold for these two different classes of unmyelinated neurons.

### Mechanisms underlying sustained responses to protons

A likely candidate for the sustained-only proton current is the capsaicin receptor VR1. Transfection of VR1 into nonneuronal cells induces a sustained, non-desensitizing proton current that resembles a capsaicin current (Tomina et al. 1998). Furthermore, sustained slowly activating proton currents are virtually absent in DRGs from mice that lack VR1 (Caterina et al. 2000; Davis et al. 2000). Since VR1 presumably mediates sustained responses to both protons and capsaicin, we expected a complete overlap in sustained responses to both stimuli. Surprisingly, the overlap was not extensive and many neurons responded with a sustained current to either protons or capsaicin but not to both stimuli. Our results are consistent with a report that showed a lack of cross-sensitivity to protons and capsaicin in identified C-fiber nociceptors in situ (Steen et al. 1992).

Several mechanisms may explain the absence of overlap in sustained proton and capsaicin responses, including 1) different channels that underlie the sustained responses to protons and capsaicin and 2) proton exposure affects subsequent responses to capsaicin. As may be the case for noxious heat, other channels in addition to VR1 may mediate the sustained, non-desensitizing responses to protons. One possibility is ASIC3, since heterologous expression of human ASIC3 in oocytes has been reported to elicit sustained-only, non-desensitizing proton gated currents (Babinski et al. 2000). Interestingly, we found that the sustained-only proton currents were substantially blocked by amiloride. Indeed, some reports show that the sustained component of ASIC3 can be partially blocked by amiloride (Babinski et al. 1999, 2000). No available evidence indicates that VR1 can be inhibited by amiloride, although a nonspecific effect of amiloride on other channel types cannot be ruled out (Tang et al. 1988). Thus the sustained proton currents in unmyelinated neurons may be mediated by VR1 and/or ASIC3.

### Protons sensitize IB4-positive neurons to capsaicin but desensitize IB4-negative neurons

An alternative explanation for the lack of overlap is that proton exposure affects subsequent responses to capsaicin. Indeed, we found that when IB4-positive neurons were exposed to protons first, the number of capsaicin-responsive IB4-positive neurons increased by twofold and the magnitude of the capsaicin response increased by threefold. Conversely, when IB4-negative neurons were treated with protons first, the number of neurons that responded to capsaicin decreased by 50%. Substantial evidence indicates that protons can modulate responses to capsaicin. Several studies have clearly demonstrated that protons applied simultaneously with capsaicin potentiate the magnitude of capsaicin responses in rat sensory neurons (Kress et al. 1996; McLatchie and Bevan 2001; Petersen and LaMotte 1993). Our data indicate that IB4-positive neurons are the population that is potentiated by protons and the potentiation is evident by both recruitment of responsive neurons and an increase in response magnitude. Mechanistically, protons appear to potentiate capsaicin responses by increasing the probability that the VR1 channel will open in response to capsaicin (Baumann et al. 2000; Tominaga et al. 1998). Two elegant molecular studies indicate that hydrogen ions can cause protonation of several extracellular glutamate or histidine residues on VR1, thereby enhancing the probability of channel activation (Jordt et al. 2000; Kuzhihankadathil et al. 2001).

Our data extend these findings by showing two new pieces of information. First, brief prior exposure to protons followed by washout influences subsequent responses to capsaicin. Thus sensory neurons appear to retain a “memory” for proton-induced potentiation of VR1 agonists. Second, the IB4-positive neurons are the class of unmyelinated neurons that is sensitized by protons.

Proton exposure had the opposite effect on IB4-negative neurons, as protons decreased the probability that IB4-negative neurons would respond to capsaicin. A likely explanation is that since naive IB4-negative neurons are initially very responsive to protons and capsaicin, they may be more susceptible to proton-induced desensitization of the capsaicin receptor VR1. Thus proton exposure may be “antinociceptive” in IB4-negative nociceptors by decreasing the probability of VR1 channel activation by other capsaicin-receptor agonists.

In summary, our data indicate that IB4-negative unmyelinated nociceptors from mice lacking ASIC3 have reduced responses to acid, and these mice have reduced mechanical behavioral hyperalgesia evoked by acid injection into muscle (Price et al. 2001). Therefore, the transient proton currents in IB4-negative unmyelinated nociceptors may contribute to the initiation or maintenance of acid-induced hyperalgesia.
nated nociceptors are primarily responsible for the initial nociceptive response to protons when tissue is injured or ischemic. But IB4-positive nociceptors have a unique capacity to be sensitized by protons to respond to other noxious stimuli, such as capsaicin-receptor agonists. A recruitment in the number of responsive IB4-positive nociceptors as well as an increase in response magnitude would increase the barrage of nociceptive information transmitted to second order neurons in lamina II of the dorsal spinal cord. Therefore IB4-positive nociceptors may be an effective target for therapeutics designed to reduce the sensitization and recruitment of nociceptors that accompanies the many types of injury and disease in which protons are elevated in the peripheral targets of nociceptors.

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