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Characterization of Cutaneous Primary Afferent Fibers Excited by Acetic Acid in a Model of Nociception in Frogs

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Hamamoto, Darryl T. and Donald A. Simone. Characterization of cutaneous primary afferent fibers excited by acetic acid in a model of nociception in frogs. *J Neurophysiol* 90: 566–577, 2003. First published May 15, 2003; 10.1152/jn.00324.2003. Acetic acid applied to the hind limb of a frog evokes nocifensive behaviors, including a vigorous wiping of the exposed skin, referred to as the wiping response. The aim of this study was to examine the responses of cutaneous primary afferent fibers in frogs to acetic acid (pH 2.84–1.42) applied topically to the skin. Conventional electrophysiological methods were used to record neuronal activity from single identified primary afferent fibers with cutaneous receptive fields on the hind limb. Fibers were classified according to their conduction velocities and responses evoked by mechanical and thermal (heat and cold) stimuli. One hundred and twenty-two mechanosensitive afferent fibers were studied (44 Aβ, 60 Aδ, and 18 C fibers). Thirty-nine percent of all fibers were excited by acetic acid, but a greater percentage of Aδ (52%) and C fibers (44%) were excited than Aβ fibers (20%). Evoked responses of fibers increased with increasingly more acidic pH until the greatest responses were evoked by acetic acid at pH 2.59–2.41. Application of acetic acid at pHs $<$2.41 evoked less excitation, suggesting that fibers became desensitized. Similar percentages of nociceptors and low-threshold mechanoreceptors were excited by acetic acid. Thus primary afferent fibers were excited by acetic acid at pHs that have been shown to evoke the wiping response in our previous study. The results of the present study suggest that the model of acetic acid-induced nociception in frogs may be useful for studying the mechanisms by which tissue acidosis produces pain.

INTRODUCTION

Several lines of evidence suggest that tissue acidosis contributes to pain associated with inflammation (Keele and Armstrong 1964; Lindahl 1961; Reeh and Steen 1996; Revici et al. 1949). Mean pHs as low as 6.6 ± 0.2 (mean ± SE) have been measured in inflamed tissues in humans (Geborek et al. 1989; Goldie and Nachemson 1970; Harrison et al. 1986; Jebens and Monk-Jones 1959; Richman et al. 1981; Treuhaft and McCarty 1971; Ward and Steigbigel 1978) and animals (Edlow and Sheldon 1971; Hutchins and Sheldon 1972; Kofoed 1986; Punnia-Moorthy 1987). In humans, application of acidic solutions to skin (Keele and Armstrong 1964; Lindahl 1961), muscle (Issberner et al. 1996), and veins (Klement and Arndt 1991) evokes a sensation of pain. Continuous infusion of acidic buffer into skin decreased tissue pH from 7.4 to 6.2 (Steen et al. 1995a) and evoked a sensation of burning pain (Steen and Reeh 1993; Ugawa et al. 2002). The intensity of pain increased as intradermal pH decreased (Steen and Reeh 1993). Moreover, a combination of inflammatory mediators (bradykinin, serotonin, histamine, and prostaglandin E_2, each at 10^{-7} M) potentiated the algogenic effect of tissue acidosis (Steen et al. 1996). Thus tissue acidosis likely contributes to pain associated with inflammation.

Nociceptors are excited preferentially by stimuli that damage or potentially damage tissue (Sherrington 1906). Acidic stimuli have been found to excite some nociceptors (Belmonte et al. 1991; Gallar et al. 1993). A subpopulation of C polymodal nociceptors (~40%) in rats was excited by acidified buffer (pH 7.4–4.3) applied to the skin in an in vitro saphenous nerve-skin preparation (Steen et al. 1992). These C polymodal nociceptors encoded the acidity of the buffer down to pH 5.2. A combination of inflammatory mediators that potentiated the algogenic effect of tissue acidosis in humans (Steen et al. 1996) was found to increase the excitation of C polymodal nociceptors evoked by the acidified buffer (Steen et al. 1995b). Moreover, experimentally induced inflammation increased expression of acid-sensing ion channel (ASIC) isoforms in small dorsal root ganglion neurons (Voilley et al. 2001). Thus activation of C polymodal nociceptors by tissue acidosis can be potentiated by inflammatory mediators, perhaps in part by increased expression of acid-sensing ion channels, and likely contributes to pain associated with inflammation.

A behavioral model using frogs may be useful for studying the role of tissue acidosis in pain. Acetic acid applied topically to the hind limb results in nocifensive behaviors, including a vigorous wiping of the exposed skin, termed the wiping response (Pezalla 1983a). This model has been proposed as an alternative to mammalian models of nociception (Stevens 1992) and has been used to study the analgesic actions of pharmacological agents (e.g., opioids) (Pezalla and Stevens 1984; Stevens et al. 1994; Willenbring and Stevens 1996) and stress-induced analgesia (Pezalla 1983b; Pezalla and Dicig 1984). Using frogs as research subjects to study the contribution of tissue acidosis to nociception is attractive because frog skin is permeable to aqueous solutions (Boutilier et al. 1992). In contrast, mammalian skin is relatively impermeable to aqueous solutions, such as acids (Flynn 1989; Smith 1990). Thus

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acidosis in frog skin can be produced by applying acids topically (Hamamoto et al. 2000), which eliminates the need for an injection. An injection can mechanically injure the skin and may sensitize nociceptors (Perl 1976; Reeh 1986). Furthermore, frogs are not restrained during application of acetic acid. Restraining animals can produce stress-induced analgesia (Pezalla and Dicig 1984; Stevens et al. 1995) and confound the results of behavioral testing. Therefore this model of acetic-acid-induced nociception in frogs has advantages that make it attractive for studying the mechanisms by which tissue acidosis produces pain.

Little is known about the primary afferent fibers in frogs that are excited by tissue acidosis. Early electrophysiological studies in frogs reported that noxious mechanical, thermal, and chemical stimuli (including acetic acid) excited primary afferent fibers with slowly conducting axons (Adrian 1926, 1928; Hogg 1935). However, the percentage of fibers excited by acetic acid and their ability to encode the pH of the acid were not reported. More recently, acidic pH has been shown to induce inward currents in small- to medium-sized frog dorsal root ganglion neurons in vitro (Kuffler et al. 2002; Philippi et al. 1995). Because acetic acid likely evokes the wiping response in frogs by exciting nociceptors, further characterization of their response properties is needed. Thus the aim of this study was to examine responses of cutaneous primary afferent fibers evoked by topical application of acetic acid and thereby determine which types of afferent fibers may contribute to the wiping response in frogs.

METHODS

Animals

Northern grass frogs (*Rana pipiens*, 25–60 g, Sullivan, Nashville, TN) were housed in large metal cages, fed live crickets three times per week, and kept on a 12 h light/dark cycle. Room temperature was maintained at ~20°C. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Minnesota and conformed to the guidelines set forth by the International Association for the Study of Pain (Zimmermann 1983).

Acids

A series of 13 solutions with differing pHs were made by serially diluting glacial acetic acid (2 volumes of acid to 1 volume of distilled water) (Pezalla 1983a; Willenbring and Stevens 1996). The two most concentrated solutions (17.4 and 11.6 M) were not used because in previous experiments less-concentrated solutions always evoked the wiping response (Hamamoto et al. 2000). Thus 11 solutions of acetic acid (0.13–7.75 M, pH 2.84–1.42) were used. The pH of each solution was measured before and after each experiment and never drifted by >0.04 pH units.

Electrophysiological recording

Each frog was anesthetized with a subcutaneous injection of tricaine methane sulfonate (MS 222, Sigma Chemical, St. Louis, MO) at 0.1 mg/g body wt and then pithed. The frog was covered with wet gauze, and the skin was kept moist with water. Action potentials were recorded extracellularly from the sciatic nerve or the spinal nerves that join to form the sciatic nerve, using conventional microdissection and recording techniques. An incision was made in the skin overlying the nerve, and the skin was sewn to a plastic ring to form a basin. The overlying muscle was removed to expose the nerve, which was then separated from the surrounding connective tissue. The basin was filled with mineral oil that was at room temperature (~22°C). The nerve was placed on a dissecting platform, and the epineural sheath was opened. Small fascicles were cut and their proximal ends were placed on the platform for fine dissection.

Fine filaments were teased from fascicles using sharpened jeweler's forceps and placed on a silver wire electrode. Neuronal activity was amplified, filtered, displayed on an oscilloscope, and audio-monitored. Only single units that could be easily discriminated were studied. Action potentials from the fiber of interest were discriminated from those of other fibers and from background noise using an amplitude window discriminator. Neuronal activity and discriminated pulses were recorded by a computer using a customized data-acquisition system (Labview, National Instruments, Austin, TX). In most experiments, recordings were obtained from only one afferent fiber.

Identification and classification of primary afferent fibers

MECHANICAL STIMULATION AND IDENTIFICATION OF RECEPTIVE FIELDS. Receptive fields of primary afferent fibers were located using mechanical stimulation of the skin with a wet cotton swab. Controlled mechanical stimulation was delivered using von Frey monofilaments with bending forces that ranged from 0.05 to 137 mN (0.005–14.0 g). The precise location of the receptive field was determined using a suprathreshold von Frey monofilament and mapped onto a drawing of the hind limb. Next, the mechanical threshold of the fiber was ascertained by applying von Frey monofilaments with increasing bending forces to the most sensitive area of the receptive field. The threshold force was defined as the minimum force (mN) that evoked a response in ≥50% of the trials. The responses of each fiber to mechanical stimulation was further studied by evaluating its response to gentle brushing of the receptive field with a soft camel's hair brush and to gentle pinching with a curved forceps. Care was taken not to injure the skin during the pinching. Fibers that were excited by pinching but not brushing or that were differentially excited by pinching were classified as nociceptors.

CONDUCTION VELOCITY. Conduction velocity (CV) for each fiber was determined by electrically stimulating the receptive field with square-wave pulses (0.5 Hz, 0.1–0.5 ms) of constant current delivered to the skin at two times the current required to evoke an action potential in ≥50% of the trials (0.09–3.8 mA). Copper surface electrodes were used to avoid making holes in the skin that would allow the topically applied acid to directly enter. Conduction velocity was calculated by dividing the conduction distance (distance along the path of the nerve between the recording electrode and the middle of the receptive field) by the conduction latency (latency from the beginning of the stimulus artifact to the beginning of the action potential). Fibers were classified by their CV as Aβ fibers (CV ≥ 15.0 m/s), Aδ fibers (2.0 < CV < 15.0 m/s), or C fibers (CV ≤ 2.0 m/s). These classifications were based on analyses of compound action potentials in preliminary studies and review of the literature (Erlanger and Gasser 1930; Erlanger et al. 1924; also see DISCUSSION).

THERMAL STIMULATION. Thermal stimuli (heat and cold) were delivered by a feedback controlled Peltier device (surface area = 1 cm^2). Stimulus temperatures were recorded from a thermocouple located at the interface between the Peltier device and the skin. Beginning from an adapting temperature of 24°C, each thermal stimulus was applied for 5 s. There was 1 min between trials. To quickly ascertain if a fiber was excited by thermal stimuli, large increments in stimulus temperatures were used initially. These temperatures were 27, 37, and 47°C (ramp rate = 20°C/s) for heat stimuli and 20, 10, 0, and –10°C (ramp rate = 5°C/s) for cold stimuli. In later trials, −4°C was used as the coldest stimulus temperature because temperatures below –4°C occasionally froze the skin as identified by the abrupt rise in temperature at the surface of the skin produced by the exothermic crystallization of water in the skin (Beise et al. 1998). If a fiber was

excited by one of these initial temperatures, then the initial series of temperatures was interrupted and subsequent trials were performed to determine the threshold temperature and to determine if the fiber increased its response with increasing stimulus intensity. In these subsequent trials, stimuli were increased (for heat) or decreased (for cold) by increments of 2°C from an adapting temperature of 24°C. In some cases, fibers were excited only during cooling after a heat stimulus. These fibers were not considered to be heat responsive, but because cooling during the cold stimuli excited them, they were classified as cold responsive (see Fig. 1 for an example).

APPLICATION OF ACETIC ACID. Acetic acid was applied to the skin in a manner analogous to that used in the nocifensive behavioral assay, the Acetic Acid Test (Hamamoto et al. 2000; Pezalla 1983a). Five minutes after the end of the last cold stimulus, fibers were tested for sensitivity to acetic acid. Neuronal activity was recorded for a baseline period of 10 s. Next, a drop (30 μl) of one of the acetic acid solutions was applied to the center of the receptive field using a Pasteur pipette. After 5 s, the exposed skin was rinsed with distilled water for an additional 5 s. The time of application of acetic acid and the period during which the skin was rinsed were marked using a foot switch whose voltage output was recorded by the computer. Neuronal activity was recorded for a total of 60 s. Solutions of acetic acid were applied in order of decreasing pH, from pH 2.84 to 1.42, with 2 min between applications. Because exposure of the terminals of primary afferent fibers to noxious stimuli may alter fiber response properties, fibers were studied only if their receptive fields were ≥2 cm away from those of previously studied fibers.

Statistical analyses

χ^2 analyses were used to determine if the percentage of fibers excited by each stimulus (pinch, heat, cold, or acetic acid) differed between groups. Forces produced by the von Frey monofilaments are presented as medians (mN) and ranges. Kruskal-Wallis nonparametric statistical analyses were used to determine if mechanical thresholds differed among groups followed by Mann-Whitney *U* tests to compare mechanical thresholds between pairs of groups. Response thresholds for thermal stimuli (°C) are reported as means ± SE and were compared between groups using one-way ANOVAs followed by Duncan's multiple range test (Duncan's MRT) to compare mean response thresholds between pairs of groups. Comparisons of response thresholds for thermal stimuli and conduction velocities (m/s) between groups were made using *t*-tests.

The least acidic solution of acetic acid that evoked impulses during the 5 s period after application of the acid was defined as the threshold solution. The 5 s period was chosen because the nocifensive wiping response occurred within 5 s after application of acetic acid (Hamamoto et al. 2000; Pezalla 1983a). The pH of the threshold solution and the pH of the solution of acetic acid that evoked the greatest number of impulses are presented as means ± SE. Comparisons of pH of the threshold solutions (or the pH of the solutions that evoked the greatest number of impulses) between fiber types were made using one-way ANOVAs followed by Duncan's MRTs. When these comparisons were made between two groups, *t*-tests were used.

The number of impulses evoked during the 5 s period after application of a solution of acetic acid is reported as mean ± SE. Some fibers with low mechanical thresholds were excited by application of a drop of acetic acid and responded with one to three impulses. These responses occurred within 0.5 s of application, and the number of impulses did not increase as more acidic solutions of acetic acid were applied. Thus these few impulses were likely evoked by the mechanical stimulation produced by the contact of the acetic acid. Fibers excited in this manner were not classified as being excited by acetic acid.

Stimulus-response functions comparing the number of impulses to pH of acetic acid were constructed by calculating the mean (±SE) number of impulses evoked within 5 s of application of acetic acid at each pH. Fibers were grouped based on their CV, functional subtype, and response to pH. A two-way repeated-measures ANOVA followed by Duncan's MRT was used to test for differences in number of impulses between pHs of acetic acid within a group of fibers (repeated measure) and between groups of fibers following application of acetic acid at each pH. For all analyses, $P < 0.05$ was considered statistically significant.

RESULTS

Response characteristics of primary afferent fibers

GENERAL RESPONSE CHARACTERISTICS OF PRIMARY AFFERENTS FIBERS. Recordings were made from 122 mechanosensitive primary afferent fibers innervating skin on the hind limb of frogs. The majority of fibers (61%; 71/116) had their receptive field on the lateral surface of the lower leg (between the ankle and knee; e.g., Figs. 1*A*, 2*A*, and 3*A*). This area of the hind limb is where the acetic acid test is applied in behavioral studies (Hamamoto et al. 2000). The remaining fibers had receptive fields on the thigh (7%; $n = 8$), foot (17%; $n = 20$), and toes (15%; $n = 17$).

Fibers were classified according to their conduction velocities (Table 1). A greater percentage of C fibers were excited by pinching than were Aβ or Aδ fibers ($P < 0.01$) and mechanical thresholds were higher for C fibers than for Aβ and Aδ fibers ($P < 0.01$). The percentage of fibers that were excited by heat stimuli was greater for C fibers than for Aβ and Aδ fibers ($P < 0.01$). Although C fibers had higher response thresholds to heat than did Aδ fibers, this difference was not statistically significant. The percentage of fibers excited by cold stimuli did not differ between classes of fiber, but mean response threshold temperature was lower for C fibers than for Aβ or Aδ fibers ($P < 0.01$). Thus C fibers were more likely to be excited by nociceptive stimuli (pinch and heat) and required more intense

TABLE 1. *General response characteristics of primary afferent fibers*

	Aβ fibers	Aδ fibers	C fibers
No. of fibers	44	60	18
Conduction velocity, m/s	20.3 ± 0.8	9.3 ± 0.5	0.94 ± 0.17
Range	15.0–37.8	2.4–14.9	0.16–1.89
Percent of fibers excited			
Pinch, %*	2 (1/44)	33 (20/60)	78 (14/18)
Heat, %†	2 (1/44)	7 (4/56)	53 (8/15)
Cold, %	17 (7/41)	30 (16/53)	15 (2/13)
Response thresholds			
Mechanical‡			
Median, mN	0.76	3.51	25.3
Range	0.05–99	0.05–137	0.05–137
n	43	60	18
Heat, °C	41	33.0 ± 3.6	40.0 ± 1.1
n	1	4	7
Cold, °C§	18.0 ± 1.8	14.6 ± 1.2	2.0 ± 2.0
n	7	16	2

Values are means ± SE. Parentheses enclose numbers of fibers. * The percentage of fibers that responded to pinch was significantly different between each class of fiber ($P < 0.01$). † A significantly greater percentage of C fibers was excited by heat than were Aβ and Aδ fibers ($P < 0.01$). ‡ Median mechanical threshold was significantly higher for C fibers than for Aβ and Aδ fibers, and for Aδ fibers than for Aβ fibers ($P < 0.01$). § Mean response threshold temperature for cold stimuli was significantly lower for C fibers than for Aβ and Aδ fibers ($P < 0.01$).

mechanical and cold stimuli to evoke excitation than Aβ and Aδ fibers.

RESPONSES OF PRIMARY AFFERENTS FIBERS EVOKED BY ACETIC ACID. Figure 1 shows responses of a single Aβ fiber evoked by thermal stimuli and by solutions of acetic acid at various pHs. The receptive field of this Aβ fiber was located on the lateral aspect of the lower leg (Fig. 1*A*). This fiber had a conduction velocity of 19.7 m/s (Fig. 1*B*). The mechanical threshold of this fiber was 0.5 mN, and it responded to suprathreshold mechanical stimuli with a rapidly adapting burst of impulses at the onset and offset of the stimulus (not shown). As illustrated in Fig. 1, *C* and *D*, this Aβ fiber was excited by cooling in both heat and cold trials. Thus this Aβ fiber was classified functionally as a cold-responsive low-threshold mechanoreceptor. The response of this Aβ fiber to the acetic acid is shown in Fig. 1*E*. Application of the least acidic solution of acetic acid (pH 2.84) did not evoke any impulses. However, rinsing the skin produced a brief discharge of impulses perhaps because of the low mechanical threshold of this fiber. The least acidic solution of acetic acid that evoked discharges before the skin was rinsed had a pH of 2.72. The number of impulses increased following application of acetic acid at pH 2.59, which evoked the greatest number of impulses (15) for this fiber. Application of acetic acid solutions at more acidic pHs evoked fewer impulses; thus this Aβ fiber desensitized to application of acetic acid at pHs lower than pH 2.59. The mechanical stimulation produced by rinsing the skin with water produced a brief discharge in all of the acetic acid trials. However, acetic acid also evoked impulses that continued during the rinsing after application of acetic acid at pH 2.72, 2.59, and 2.49; in the case of acetic acid at pH 2.72 and 2.49, the impulses continued after rinsing had ended.

Responses of a single Aδ fiber to the solutions of acetic acid are shown in Fig. 2. Figure 2*A* shows that the receptive field was located on the lateral surface of the lower leg. Figure 2*B* illustrates the conduction latency (3.9 ms) of this fiber to electrical stimulation of its receptive field; the conduction velocity was 11.0 m/s. This fiber had a very low mechanical threshold (0.05 mN) and exhibited a rapidly adapting response after suprathreshold mechanical stimuli. Heat stimuli ≤47°C and cold stimuli down to −4°C did not excite this fiber. However, as demonstrated in Fig. 2*C*, this

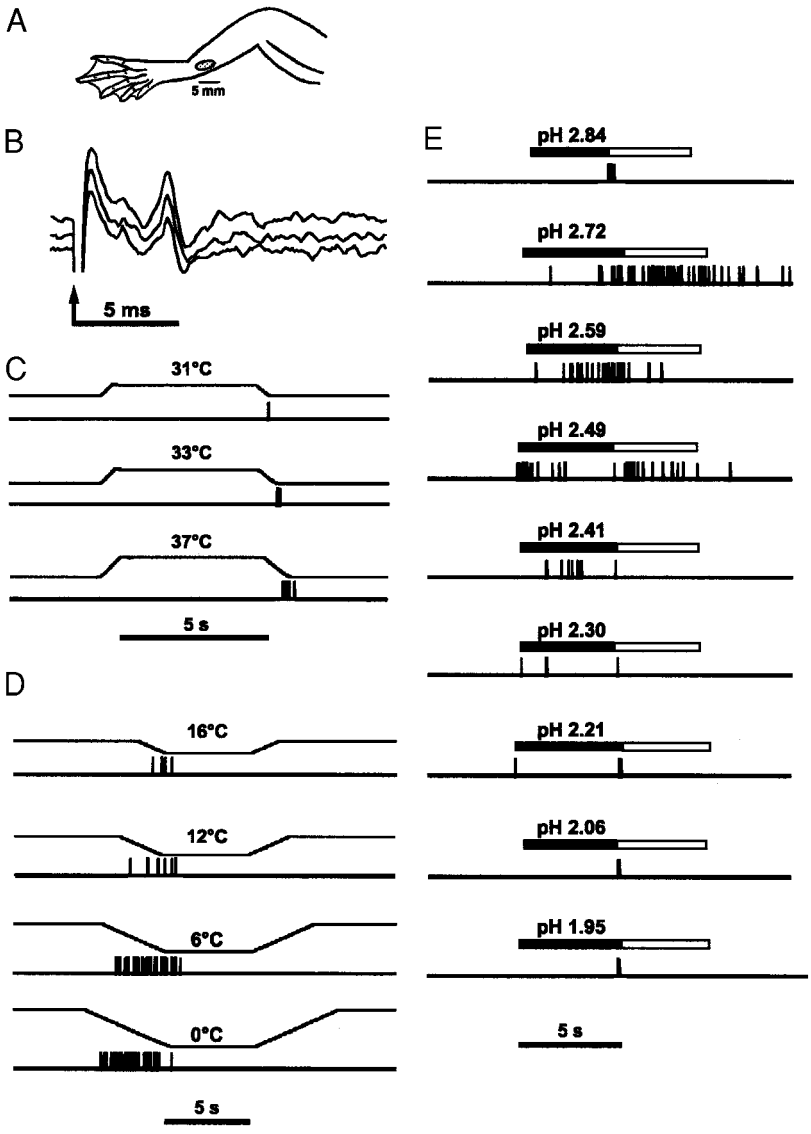

FIG. 1. Evoked responses of a representative example of a single Aβ mechanoreceptor. The response threshold of this fiber to mechanical stimuli (von Frey monofilament) was 0.5 mN, and a constant mechanical stimulus evoked impulses only at the onset and offset of the stimulus. *A*: the receptive field of this fiber was located on the lateral aspect of the hind limb. *B*: 3 examples of the constant conduction latency (3.2 ms) used to calculate the conduction velocity (19.7 m/s) of this fiber. The arrow indicates the stimulus artifacts. *C* and *D*: this fiber was excited by cooling in both the heat and cold trials, thus this fiber was functionally classified as a cold-responsive low-threshold mechanoreceptor. Each vertical tick represents 1 discriminated impulse. The lines above the evoked responses are analog traces of stimulus temperature, which were each 5 s in duration. The numbers above the traces indicate stimulus temperatures. *E*: evoked responses of this fiber to solutions of acetic acid. The greatest number of impulses was evoked by acetic acid at pH 2.59. The filled bars represent the periods during which the solutions of acetic acid were in contact with the receptive field. The open bars represent the periods during which the receptive field was rinsed with distilled water.

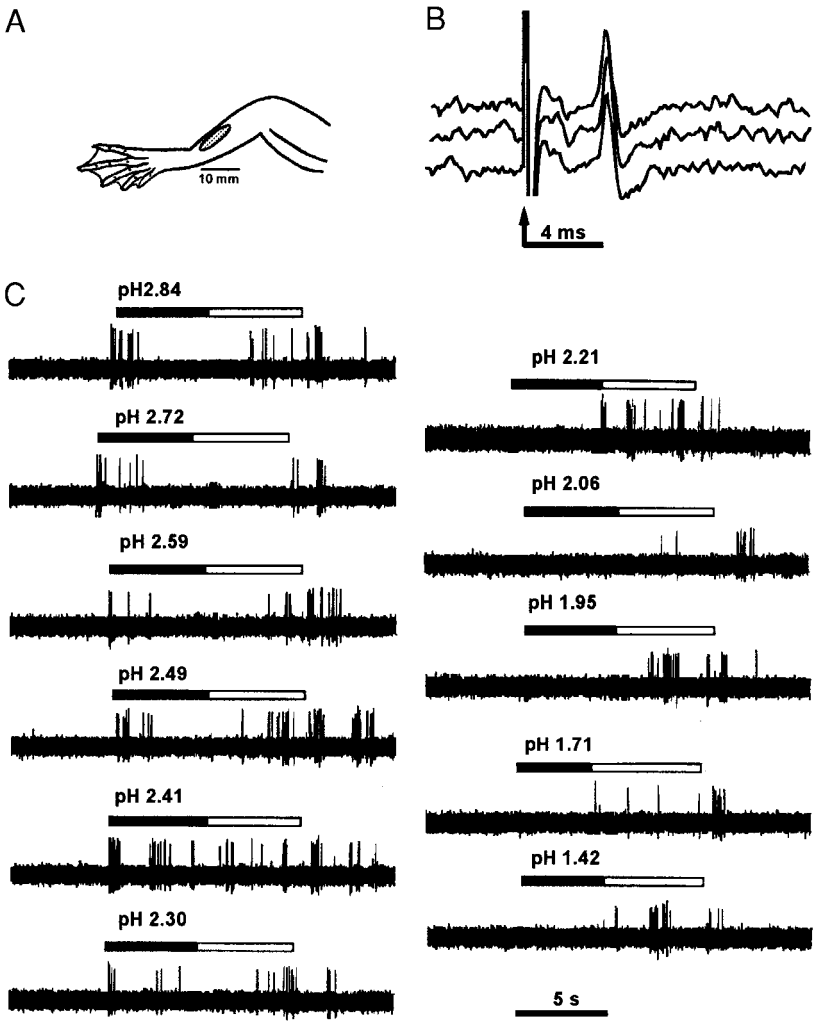

FIG. 2. Evoked responses of a representative example of a single Aδ mechanoreceptor. The response threshold of this fiber to mechanical stimuli was 0.05 mN, and a constant mechanical stimulus evoked a rapidly adapting response. Heat (≤47°C) or cold (down to −4°C) did not excite this fiber, and thus this fiber was functionally classified as a low-threshold mechanoreceptor. The format of this figure is similar to that of Fig. 1. *A*: the receptive field of this fiber was located on the lateral aspect of the hind limb. *B*: the conduction velocity of this fiber was 11.0 m/s. *C*: evoked responses of this fiber to solutions of acetic acid.

fiber was excited by the least acidic solution of acetic acid (pH 2.84) with 13 impulses. The number of impulses evoked by acetic acid at pH 2.72, 2.59, and 2.49 ranged between 8 and 13 but increased to 18 impulses after application of acetic acid at pH 2.41. In a previous study, the median pH of acetic acid that evoked the nocifensive wiping response in frogs was pH 2.41 (Hamamoto et al. 2000). Hence, this fiber may be an example of the fibers contributing to the wiping response in the acetic acid test. This fiber exhibited impulses that continued for a few seconds after the rinsing of the skin ended. Furthermore, subsequent application of acetic acid at more acidic pHs (2.30–1.42) evoked fewer impulses suggesting that this fiber became desensitized.

The C fiber illustrated in Fig. 3 had a small receptive field on the lateral surface of the lower leg (Fig. 3*A*) and was excited by pinching but not brushing. The conduction velocity of this fiber was 0.41 m/s (Fig. 3*B*). The mechanical threshold for this fiber was 8.05 mN, and this fiber exhibited a slowly adapting response to excitation with a suprathreshold von Frey monofilament. Responses to heat are shown in Fig. 3*C*. This fiber was unresponsive to cold stimuli down to −4°C. Responses of this fiber evoked by solutions of acetic acid are shown in Fig. 3*D*. Acetic acid at pH 2.06 evoked the greatest number of impulses and the impulses continued for 25 s. Application of acetic acid at pHs more acidic than 2.06 evoked only one impulse during the 5 s stimulus period. Thus this fiber became desensitized to more acidic solutions of acetic acid.

As illustrated in Fig. 4*A*, the percentage of fibers that were excited by acetic acid was greater for Aδ fibers (52%, 31/60) than for Aβ fibers (20%, 9/44; $P < 0.01$). Forty-four percent of C fibers (8/18) were excited by acetic acid, but this was not significantly greater than the percentage of Aβ fibers excited by acetic acid. Mean pH of the threshold solution of acetic acid was not different among Aβ (pH 2.74 ± 0.06), Aδ (pH 2.77 ± 0.02), and C (pH 2.60 ± 0.11) fibers. In contrast, as shown in Fig. 4*B*, the mean pH of the acetic acid solution that evoked the greatest number of impulses was significantly lower for C fibers (pH 2.00 ± 0.15) than for Aβ (pH 2.39 ± 0.10) and Aδ (2.42 ± 0.05) fibers ($P < 0.01$). However, the greatest number of evoked impulses did not differ among Aβ (18.4 ± 4.1), Aδ (13.5 ± 1.6), and C fibers (13.1 ± 3.4).

The stimulus-response relationships for Aβ (Fig. 4*C*), Aδ (Fig. 4*D*), and C fibers (Fig. 4*E*) demonstrate that the responses of Aβ and Aδ fibers peaked at less acidic pHs (2.42 for Aβ fibers and 2.59 for Aδ fibers) than did the responses of C fibers (pH 1.71). Moreover, responses of Aβ and Aδ fibers decreased to near zero as acetic acid at more acidic pHs were applied. In contrast, the responses of C fibers did not decrease to zero even after application of acetic acid at the most acidic pH (1.42).

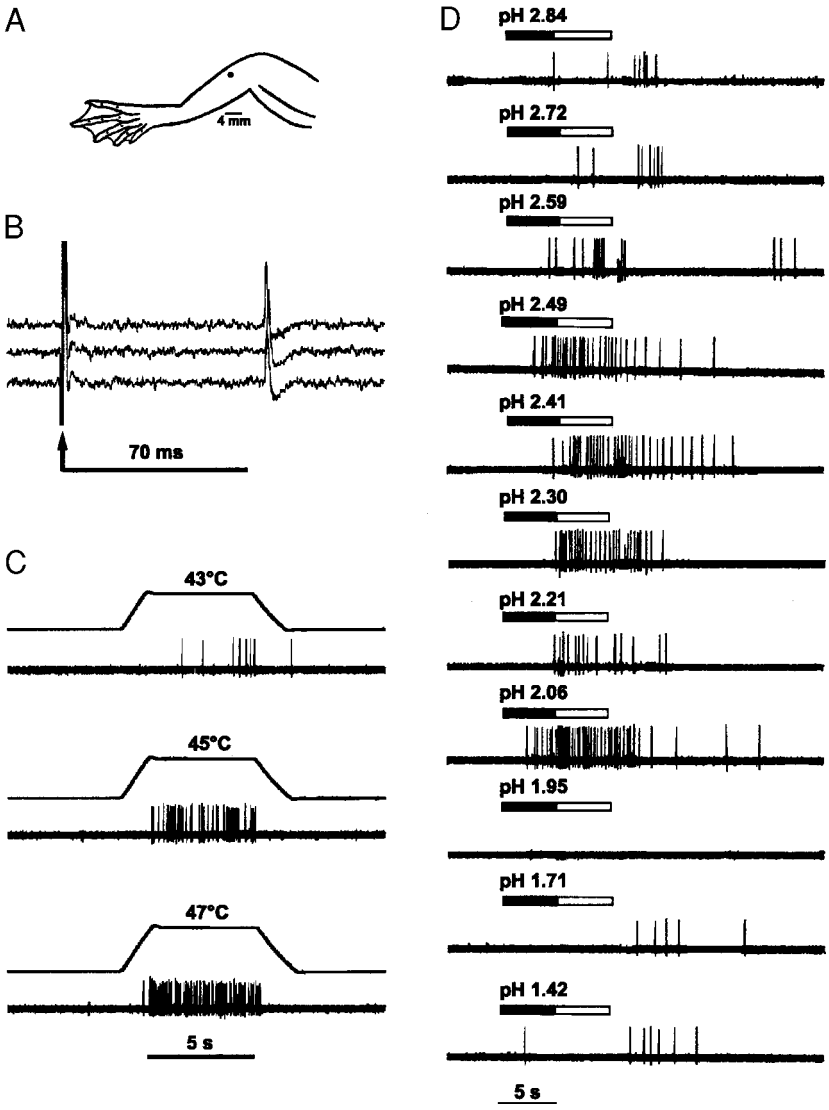

FIG. 3. Evoked responses of a representative example of a single C polymodal nociceptor. Pinching but not brushing excited this fiber, and thus this fiber was functionally classified as a nociceptor. The format of this figure is similar to that of Figs. 1 and 2. *A*: the receptive field of this fiber was located on the lateral aspect of the hind limb. *B*: the conduction velocity of this fiber was 0.41 m/s. *C*: evoked responses of this fiber to heat stimuli. This fiber had a response threshold of 43°C. *D*: evoked responses of this fiber to solutions of acetic acid. Acetic acid at pH 2.06 evoked the greatest number of impulses and the impulses continued for 25 s.

Figure 4*F* demonstrates that the number of impulses evoked by acetic acid was not significantly different among fiber types at pH 2.84; this pH evoked the wiping response in few (3%) frogs in our previous study (Hamamoto et al. 2000). The median pH of the solutions of acetic acid that evoked the wiping response was pH 2.41, but the number of impulses evoked by this solution was not significantly different between fiber types in the present study. In contrast, acetic acid at pH 1.71 evoked a significantly greater number of impulses from C fibers (8.6 ± 4.4 imp) than from Aβ (0.9 ± 0.7 imp) and Aδ (0.4 ± 0.2 imp) fibers ($P < 0.05$).

Response characteristics of nociceptors

GENERAL RESPONSE CHARACTERISTICS OF NOCICEPTORS. Nociceptors were defined as those fibers that were differentially excited by pinching. As shown in Table 2, nociceptors had slower conduction velocities ($P < 0.01$) and higher thresholds to mechanical stimulation ($P < 0.01$) than did low-threshold mechanoreceptors. The percentage of fibers that were excited by heat was greater for nociceptors than for low-threshold mechanoreceptors ($P < 0.01$). Although the mean response threshold for heat was higher for nociceptors than for low-threshold mechanoreceptors, this difference was not statistically significant. Similar percentages of nociceptors and low-threshold mechanoreceptors were excited by cold stimuli, but the mean response threshold temperature for cold stimuli was significantly lower for nociceptors than for low-threshold mechanoreceptors ($P < 0.01$). Thus nociceptors required more intense mechanical and cold stimuli to excite them and were more likely to be excited by heat stimuli than were low threshold mechanoreceptors.

RESPONSES OF NOCICEPTORS EVOKED BY ACETIC ACID. Similar percentages of nociceptors (46%, 16/35) and low-threshold mechanoreceptors (37%, 32/87) were activated by acetic acid, and there was no difference in the mean pH of the threshold solution of acetic acid between nociceptors (pH 2.68 ± 0.06) and low-threshold mechanoreceptors (pH 2.76 ± 0.03). Moreover, the greatest number of impulses evoked by acetic acid was not significantly different between nociceptors (13.1 ± 2.3 imp) and low-threshold mechanoreceptors (15.0 ± 1.8 impulses). However, the pH of the acetic acid that evoked the greatest number of impulses was lower for nociceptors (pH 2.19 ± 0.10) than for low-threshold mechanoreceptors (2.42 ± 0.06, $P < 0.05$).

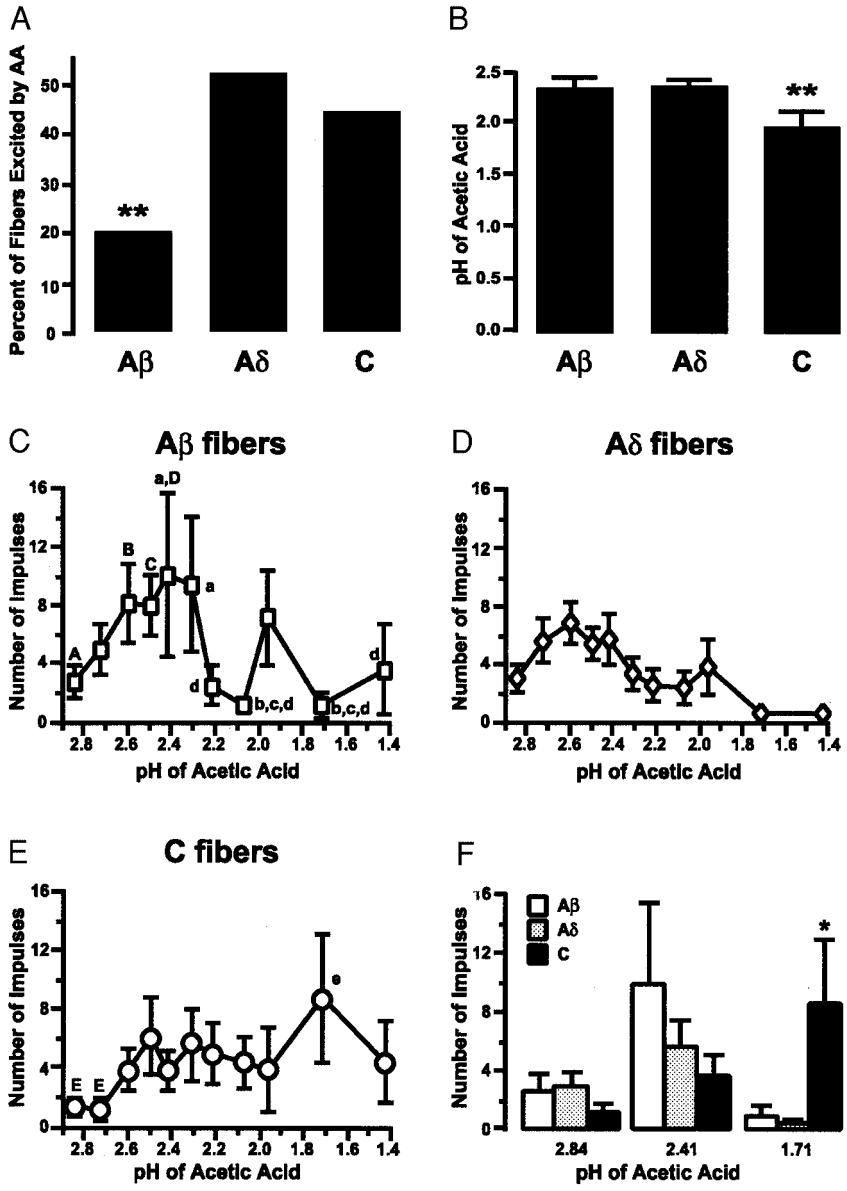

FIG. 4. Comparisons of responses of Aβ, Aδ, and C fibers evoked by acetic acid at various pHs. *A*: percentage of each class of fiber excited by acetic acid (AA). AA excited a greater percentage of Aδ than Aβ fibers (**$P < 0.01$). *B*: for each class of fiber, the pH of acetic acid that evoked the greatest mean (±SE) number of impulses. The greatest mean number of impulses was evoked by AA at a more acidic pH for C fibers than for Aδ and Aβ fibers (**$P < 0.01$). *C–E*: mean (±SE) number of impulses evoked by solutions of acetic acid for Aβ (*C*), Aδ (*D*), and C fibers (*E*). In *C* and *E*, the mean number of impulses evoked by acetic acid at one pH, designated by a capital letter (e.g., "A"), was significantly different from the mean number of impulses evoked by acetic acid at another pH, designated by the same letter in lowercase (e.g., "a;" $P < 0.05$). *F*: comparisons of responses evoked by AA among Aβ, Aδ, and C fibers. AA at pH 1.71 evoked a greater mean (±SE) number of impulses from C fibers than from Aβ and Aδ fibers (*$P < 0.05$).

Response characteristics of primary afferent fibers that were excited by acetic acid

To further elucidate the fibers that may contribute to the acetic acid-induced wiping response, the characteristics of fibers that were excited by acetic acid were compared with those of fibers that were not excited. Stimulus-response relationships for fibers that were excited by acetic acid and for fibers that did not are shown in Fig. 5*A*. Fibers that were excited by acetic acid exhibited an increasing number of impulses as solutions of acetic acid at pH 2.84–2.59 were applied. The number of impulses was greater when acetic acid at pH 2.59, 2.49, 2.41, and 2.30 were applied than when acetic acid at pH 2.84 was applied ($P < 0.05$). The number of impulses generally decreased as solutions of acetic acid at more acidic pHs were applied. However, the response to acetic acid at pH 1.95 was an exception to this observation. Thus fibers that responded to acetic acid exhibited the greatest response to the solutions of acetic acid (pH 2.59–2.41) that evoked the wiping response in most frogs in our previous study (Hamamoto et al. 2000).

As shown in Fig. 5*B*, conduction velocity was slower for fibers that were excited by acetic acid (9.0 ± 0.8 m/s) than for fibers that were not excited (14.0 ± 1.2 m/s, $P < 0.01$). A greater percentage of fibers that were excited by acetic acid also were excited by heat (22 vs. 4%, $P < 0.05$); this is illustrated in Fig. 5*C*. Although cold stimuli excited a similar percentage of acetic acid sensitive (29%) and insensitive (16%) fibers, Fig. 5*D* shows that fibers that were excited by acetic acid responded to cold stimuli that were less cold (17.5 ± 1.6°C) than fibers that were not excited by acetic acid (11.3 ± 1.3°C, $P < 0.05$). Therefore fibers that were excited by acetic acid exhibited their greatest responses at pHs that evoked the wiping response in a majority of frogs (Hamamoto et al. 2000). These fibers differed from fibers that were not excited by acetic acid by having slower conduction velocities, a greater likelihood of being excited by heat, and being more sensitive to cold stimuli.

Previous studies have found that response characteristics of fibers differ among classes of fibers (i.e., Aβ vs. Aδ vs. C

TABLE 2. *General response characteristics of nociceptors and low-threshold mechanoreceptors*

	Nociceptors	Low-Threshold Mechanoreceptors
No. of fibers	35	87
Conduction velocity, m/s*	4.72 ± 0.78	14.9 ± 0.8
Range, m	0.16–17.07	1.72–37.8
Percent of fibers excited		
Heat, %†	25 (8/32)	6 (5/84)
Cold, %	30 (9/30)	20 (16/81)
Response thresholds		
Mechanical‡		
Median, mN	25.27	0.50
Range	0.05–137	0.05–137
n	35	86
Heat, °C	40.0 ± 1.1	34.6 ± 3.2
n	6	5
Cold, °C§	7.1 ± 2.3	18.7 ± 0.7
n	9	16

Values are means ± SE. Parentheses enclose number of fibers. * Conduction velocity was significantly slower for nociceptors than for low-threshold mechanoreceptors ($P < 0.01$). † A significantly greater percentage of nociceptors was excited by heat than were low-threshold mechanoreceptors ($P < 0.01$). ‡ Median mechanical threshold for nociceptors was significantly higher than for low-threshold mechanoreceptors ($P < 0.01$). § Mean response threshold temperature for cold stimuli was significantly lower for nociceptors than for low-threshold mechanoreceptors ($P < 0.01$).

fibers) (Burgess and Perl 1979; Raja et al. 1999). Thus differences in the characteristics of fibers that were excited by acetic acid compared with those that were not excited, as illustrated in Fig. 5, could be due to differences in the percentage of Aδ and C fibers in each group (see Fig. 4*A*). Hence, the characteristics of fibers that were excited by acetic acid and fibers that were not excited were compared within each class of fiber.

Figure 6 demonstrates that Aβ fibers that were excited by acetic acid differed from Aβ fibers that were not excited by acetic acid in several characteristics. Mean conduction velocity for Aβ fibers that were excited by acetic acid (16.5 ± 0.6 m/s) was slower than that for fibers that were not excited (21.2 ± 0.9 m/s, $P < 0.05$); this is illustrated in Fig. 6*A*. Furthermore, the percentages of fibers that were excited by pinch (11 vs. 0%, Fig. 6*B*), heat (11 vs. 0%, Fig. 6*C*), and cold (71 vs. 6%, Fig. 6*D*) were greater for Aβ fibers that were excited by acetic acid than Aβ fibers that were not ($P < 0.05$). Thus Aβ fibers excited by acetic acid were more likely to also be excited by other noxious stimuli such as pinch, heat, and cold.

Response characteristics of Aδ fibers excited by acetic acid were generally similar to the characteristics of Aδ fibers that were not excited. There were no significant differences in conduction velocities or percentage of fibers that were excited by pinch, heat, or cold between these two groups of fibers. However, Aδ fibers that were excited by acetic acid responded to cold stimuli that were less cold (19.0 ± 0.8°C) than those that were not excited by acetic acid (11.1 ± 2.8°C, $P < 0.05$).

As illustrated in Fig. 7*A*, C fibers that were excited by acetic acid had slower mean conduction velocities (0.55 ± 0.17 m/s) than C fibers that were not (1.26 ± 0.23 m/s, $P < 0.05$). A greater percentage of C fibers that were excited by acetic acid were also excited by pinch (100 vs. 60%, Fig. 7*B*) and heat (86 vs. 25%, Fig. 7*C*) than C fibers that were not excited by acetic acid ($P < 0.05$). Thus C fibers excited by acetic acid were more likely to be excited by other noxious stimuli such as pinch and heat and hence were most likely to have nociceptor function.

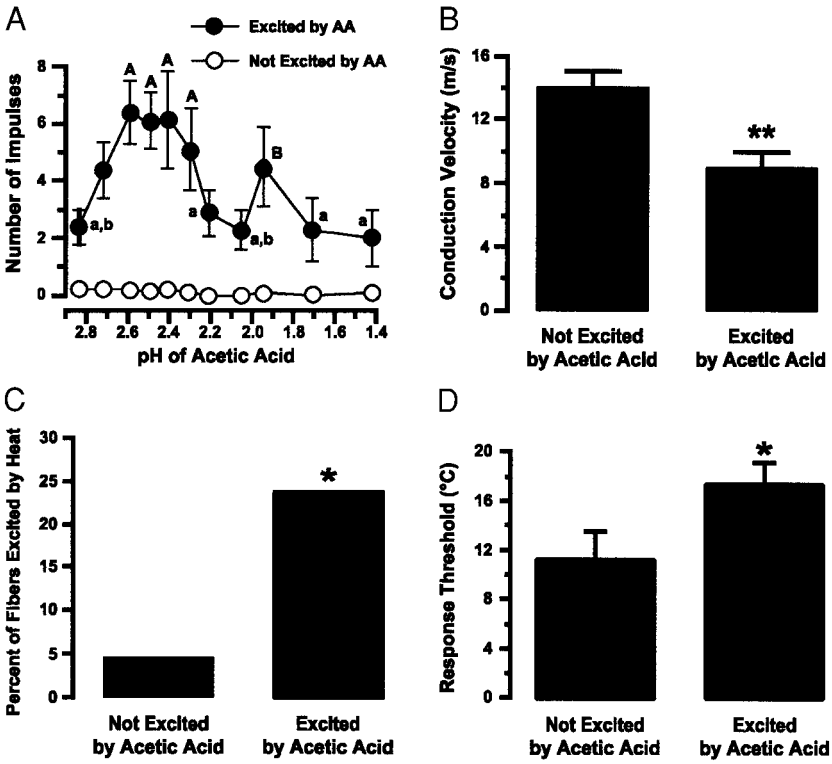

FIG. 5. Response characteristics of primary afferent fibers that were excited by AA compared with fibers that were not excited. *A*: mean (±SE) number of impulses evoked by solutions of AA for fibers that were excited by AA and fibers that were not excited. For fibers that were excited by AA, the mean number of impulses evoked by AA at one pH, designated by a capital letter (e.g., "A"), was significantly different from the mean number of impulses evoked by AA at another pH designated by the same letter in lowercase (e.g., "a;" $P < 0.05$). *B*: mean (±SE) conduction velocities were slower for fibers that were excited by acetic acid than for fibers that were not excited (**$P < 0.01$). *C*: percentage of fibers that were excited by heat stimuli was greater for fibers that were excited by AA than for fibers that were not excited (*$P < 0.05$). *D*: fibers that were excited by acetic acid were more sensitive to cold stimuli and had response thresholds (mean ± SE) that were less cold than fibers that were not excited by AA (*$P < 0.05$)

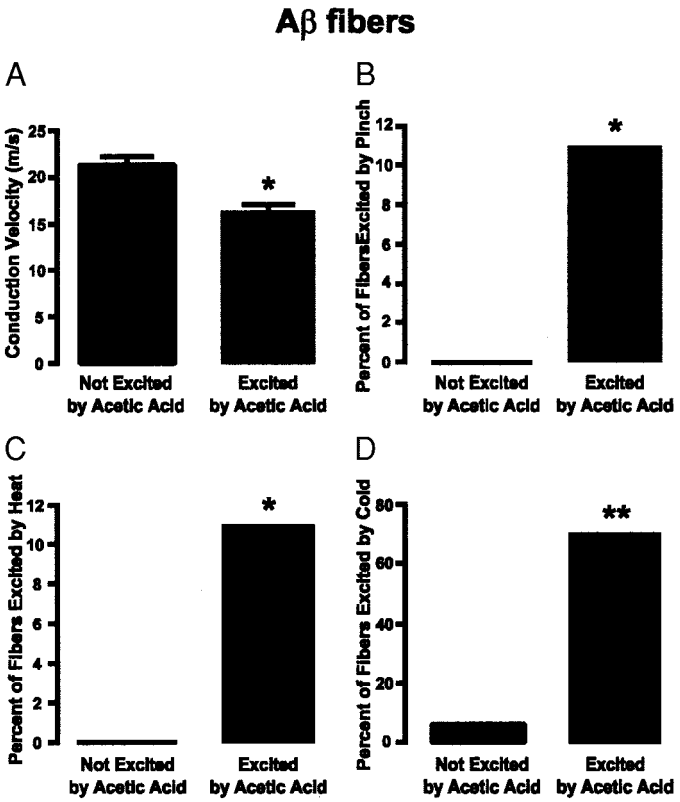

FIG. 6. Response characteristics of Aβ fibers that were excited by AA compared with Aβ fibers that were not excited. *A*: mean ($\pm$SE) conduction velocities were slower for Aβ fibers that were excited by AA than for Aβ fibers that were not excited ($^*P < 0.05$). *B*: percentage of Aβ fibers that were excited by pinching was greater for Aβ fibers that were excited by AA than for Aβ fibers that were not excited ($^*P < 0.05$). *C*: percentage of Aβ fibers that were excited by heat stimuli was greater for Aβ fibers that were excited by AA than for Aβ fibers that were not excited ($^*P < 0.05$). *D*: percentage of Aβ fibers that were excited by cold stimuli was greater for Aβ fibers that were excited by AA than for Aβ fibers that were not excited ($^{**}P < 0.01$).

DISCUSSION

Recent evidence suggests that tissue acidosis contributes to pain associated with inflammation. The acetic acid-induced wiping response in frogs may be a useful model of nociception for studying the mechanisms by which tissue acidosis produces pain because frogs have skin that is permeable to aqueous solutions. Chemical stimuli (e.g., acetic acid) can be topically applied to the skin of frogs, thus eliminating the need for an injection, which could mechanically injure the skin and sensitize nociceptors. Furthermore, this method of applying chemical stimuli topically does not require restraining frogs and thus avoids the potentially confounding effects of stress. However, a comprehensive investigation of the responses of cutaneous nociceptors in frogs to acetic acid has not been reported. The present study characterized responses of primary afferent fibers that were excited by acetic acid. The main finding of this study was that a significant proportion (39%) of primary afferent fibers, both nociceptors and low-threshold mechanoreceptors, were excited by acetic acid. Evoked responses of fibers increased as solutions of acetic acid at decreasing pHs (i.e., 2.84–2.59) were applied until excitation was greatest at pHs between 2.59 and 2.41. Evoked responses decreased with the subsequent application of more acidic solutions of acetic acid (pH 2.30–1.42); thus fibers exhibited desensitization to acetic acid at pHs <2.41.

Methodological considerations

There are few electrophysiological data collected from frogs that can be used to select the conduction velocities by which to classify fibers into Aβ, Aδ, and C fiber groups. In early studies, compound action currents or compound action potentials were recorded from the sciatic nerve of bullfrogs (Erlanger and Gasser 1930; Erlanger et al. 1924). From these studies, Aδ fibers conducted at ~14 m/s and C fibers conducted at velocities <1 m/s. In our own preliminary electrophysiological studies, 28 compound action potentials were recorded from the sciatic nerve of frogs (*R. pipiens*). The beginning of the Aδ wave had an average conduction velocity of 14.4 $\pm$ 1.0 m/s, and the fastest C fibers averaged 2.2 $\pm$ 0.2 m/s. Additional information can be obtained from studies of primary afferent fibers in frogs in which fibers were classified based on their functional characteristics (Adrian 1932; Catton 1958; Erlanger and Gasser 1930; Hogg 1935; Matthews 1929; Spray 1974). In these studies, the majority of the conduction velocities delineating Aδ from Aβ fibers were ~15 m/s (Adrian 1932; Catton 1958; Matthews 1929; Spray 1974). The average conduction velocity used to delineate C from Aδ fibers was 2 m/s (Adrian 1932; Catton 1958; Erlanger and Gasser 1930; Hogg 1935; Spray 1974). Thus in the present study, 15 m/s was selected as the conduction velocity cutoff by which to classify fibers as either Aβ or Aδ fibers and 2 m/s was selected as the conduction

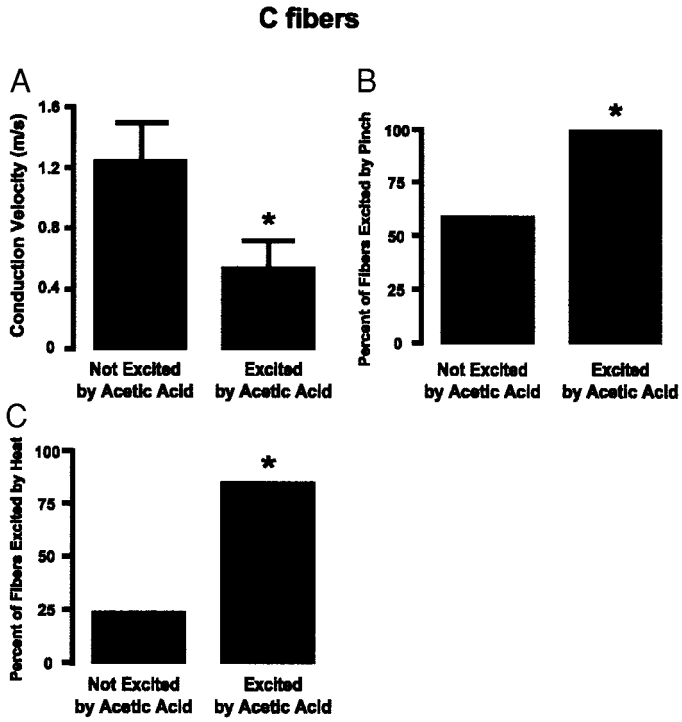

FIG. 7. Response characteristics of C fibers that were excited by AA compared with C fibers that were not excited. *A*: mean ($\pm$SE) conduction velocity was slower for C fibers that were excited by AA than for C fibers that were not excited ($^*P < 0.05$). *B*: percentage of C fibers that were excited by pinching was greater for C fibers that were excited by AA than for C fibers that were not excited ($^*P < 0.05$). *C*: percentage of C fibers that were excited by heat stimuli was greater for C fibers that were excited by AA than for C fibers that were not excited ($^*P < 0.05$).

velocity cutoff by which to classify fibers as either Aδ or C fibers.

Primary afferent fibers were classified as nociceptors based on their differential responses to innocuous (brush) and noxious (pinch) mechanical stimuli. Responses to thermal stimuli were not used to classify fibers as nociceptors because little is known about the stimulus temperatures that are nociceptive for frogs. In a review paper, Stevens and Willenbring reported that a thermal radiant heat source evoked the wiping response in frogs at a threshold temperature of 33.1 ± 2.3°C (mean ± SD) (Stevens and Willenbring 1997). In contrast, Kuffler and colleagues found that the threshold for evoking the wiping response was 38 ± 0.5°C when the feet of pithed frogs were placed in a water bath (Kuffler et al. 2002). In the present study, three fibers were excited by heat stimuli >33°C but were not classified as nociceptors based on their responses to pinching. However, reclassifying these fibers as nociceptors did not change the results from the statistical analyses of their responses to acetic acid. Fifteen fibers were excited by brushing and by cold stimuli. Relatively innocuous temperatures ranging from 18 to 22°C excited 12 of these brush- and cold-responsive fibers. The remaining three fibers had a slightly colder response threshold of 14°C. Holloway reported that "some" cutaneous nociceptors in frogs were excited by ice placed on their receptive fields (Holloway 1973). These nociceptors required noxious pinch or pinprick to excite them and thus were different from the brush- and cold-responsive fibers found in the present study. In mammals, nociceptors that were excited by noxious thermal stimuli were differentially excited by pinch (Burgess and Perl 1979; Leem et al. 1993; Simone and Kajander 1996, 1997) or were insensitive to mechanical stimulation (Handwerker et al. 1991; LaMotte and Thalhammer 1982). However, Leem and colleagues found that some low-threshold C mechanoreceptors were excited by noxious cold stimuli, but they did not classify these fibers as nociceptors (Leem et al. 1993). Thus thermally responsive nociceptors in mammals differ from brush- and cold-responsive fibers found in the present study because the latter responded to innocuous mechanical stimuli. In the present study, fibers that were excited by brushing and thermal stimuli but were not differentially excited by pinching were classified as low-threshold mechanoreceptors.

Responses of primary afferent fibers to application of acids

Few studies have examined responses of cutaneous primary afferent fibers in frogs to application of acidic stimuli. Adrian (1930) found that a 5% solution of acetic acid applied to the skin evoked both "rapid and slow impulses" in the dorsal cutaneous nerve of decerebrate frogs. Using an in vitro preparation consisting of the dorsal skin and attached nerve from frogs, Hogg (1935) found that lower concentrations of acetic acid (i.e., 2%) only excited fibers with slow conduction velocities (1.5–4.5 m/s). However, at higher concentrations (unspecified by Hogg), fibers with fast conduction velocities (again unspecified by Hogg) were excited. In toads, application of acetic acid (5–10%) to an excised nerve-skin preparation excited fibers with conduction velocities that ranged from 0.1 to 15 m/s (Maruhashi et al. 1952). The findings from the present study are in agreement with these previous observations in that fibers excited by acetic acid had a relatively wide range of conduction velocities (0.16–19.7 m/s). In contrast, fibers with faster conduction velocities (Aβ and Aδ fibers) were not excited by acidified buffers in a rat in vitro skin-nerve preparation (Steen et al. 1992). Thus in amphibians, acetic acid excites fibers with a wide range of conduction velocities, whereas in mammals, acids excite only fibers with slower conduction velocities.

In the present study, similar percentages of nociceptors (46%) and low-threshold mechanoreceptors (37%) were excited by acetic acid. Previous reports have also found that acetic acid excited "tactile" fibers in frogs (Adrian 1930; Hogg 1935). In contrast, all fibers recorded from toads that were excited by acetic acid were also excited by pinprick (Maruhashi et al. 1952). Similarly, in a rat in vitro skin-nerve preparation, low-threshold mechanoreceptors were not excited by acidified buffers; only C polymodal nociceptors showed stimulus-related responses that increased as the pH of the buffer was decreased (Steen et al. 1992). Thus primary afferent fibers in frogs appear to differ from those in mammals in that acids excite both nociceptors and low-threshold mechanoreceptors in frogs but only excite nociceptors in mammals.

Relationship between subepidermal pH and excitation of primary afferent fibers after application of solutions of acetic acid that evoke the wiping response in frogs

In the present study, the method of applying the solutions of acetic acid was identical to that used in our previous study (Hamamoto et al. 2000) so that comparisons could be made between behavioral responses, subepidermal pH, and electrophysiological responses of primary afferent fibers to the same solutions of acetic acid. In our previous study, solutions of acetic acid at pH 2.59–2.41 evoked the wiping response in the majority (58%) of frogs. These same solutions of acetic acid evoked the greatest number of impulses from primary afferent fibers, suggesting that excitation of these fibers contributes to the acetic acid-induced wiping response in frogs. Application of acetic acid at pH 2.41 decreased subepidermal pH to 6.69 ± 0.30. This subepidermal pH is similar to the pHs that have been shown to excite nociceptors (pH 6.9–6.1) (Steen et al. 1992) and to produce hyperalgesia to mechanical stimulation (pH 6.4–6.0) (Hamamoto et al. 1998) in rats. Moreover, subepidermal injection of acidic buffer in humans decreased tissue pH down to 6.2 and produced pH-dependent pain (Steen et al. 1995a). Thus in the present study, primary afferent fibers were excited by solutions of acetic acid that evoked the wiping response in frogs. These solutions of acetic acid decreased subepidermal pH to levels that excited nociceptors and produced hyperalgesia in rats and produced pain in humans. Hence, primary afferent fibers excited by acetic acid likely contribute to the acetic acid-induced wiping response in frogs.

The stimulus-response relationship of primary afferent fibers to acetic acid in frogs was similar to that observed in C polymodal nociceptors in rats. Using an in vitro skin-nerve preparation, Steen and colleagues found that C polymodal nociceptors in rats had response thresholds to acidified buffers ranging from pH 6.9 to 6.1 (Steen et al. 1992). The threshold solutions of acetic acid in the present study produced an average subepidermal pH of 7.14 ± 0.06 in our previous study (Hamamoto et al. 2000). Hence, the response threshold for cutaneous afferent fibers in frogs was at a slightly less acidic

pH than that for C polymodal nociceptors in rats. For the C polymodal nociceptors in rats and the primary afferent fibers in frogs, responses increased as more acidic stimuli were applied until a peak response was evoked and then responses decreased. In the rat preparation, buffer at pH 5.2 evoked the maximum discharge (Steen et al. 1992). In contrast, the solutions of acetic acid that evoked the greatest number of impulses in primary afferent fibers decreased subepidermal pH to 6.30 ± 0.15 (Hamamoto et al. 2000). Therefore acetic acid evoked the greatest excitation of primary afferent fibers in frogs at a less-acidic pH than the pH that evokes the greatest excitation in C polymodal nociceptors in rats.

Interestingly, when responses of only the C fibers in frogs were considered, the stimulus-response relationship was similar to that of C polymodal nociceptors in rats. The solutions of acetic acid that evoked the threshold response from the C fibers in frogs produced a subepidermal pH of 6.88 ± 0.30 in our previous study (Hamamoto et al. 2000). Moreover, the solutions of acetic acid that evoked the greatest number of impulses in C fibers in frogs were previously found to decrease subepidermal pH to 5.45 ± 0.42 (Hamamoto et al. 2000). Thus the stimulus-response relationship for C fibers in frogs was similar to that found in rats.

Conclusions

In summary, 39% of primary afferent fibers in frogs were excited by acetic acid. Primary afferent fibers were excited by solutions of acetic acid that evoked the wiping response in frogs and that decreased subepidermal pH to levels that have been found to excite nociceptors and produce hyperalgesia to mechanical stimuli in rats and to produce pain in humans. In rats, only nociceptors were excited by acidic stimuli, whereas similar proportions of nociceptors and low-threshold mechanoreceptors were excited in frogs. However, the stimulus-response relationship of C fibers in frogs was similar to that found in C polymodal nociceptors in rats. Thus the results of the present study suggest that the model of acetic acid-induced nociception in frogs may be useful for studying mechanisms by which tissue acidosis excites primary afferent fibers and produces pain. Further studies are needed to determine the relative roles of nociceptors and low-threshold mechanoreceptors in evoking the nocifensive wiping response in frogs.

DISCLOSURES

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