Enhancement of Ectopic Discharge in Regenerating A- and C-Fibers by Inflammatory Mediators

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Grossmann L, Gorodetskaya N, Baron R, Jänig W. Enhancement of ectopic discharge in regenerating A- and C-fibers by inflammatory mediators. J Neurophysiol 101: 2762–2774, 2009. First published March 11, 2009; doi:10.1152/jn.91091.2008. Afferent A- and C-fibers regenerating into a nerve following peripheral nerve injury are exposed to inflammatory mediators released by Schwann cells, resident and invading macrophages, and other inflammatory cells. Here we tested the hypothesis that ongoing and evoked activity in these afferent fibers are enhanced by a mixture of inflammatory mediators [inflammatory soup (IS)] applied to the injured nerve. Using in vivo electrophysiology, regenerating afferent nerve fibers were studied 7–14 d after sural nerve crush lesion. The ectopic activity was studied before and 1.5 h after topical application of IS to the nerve in 73 C-fibers and 22 A-fibers that were either ectopically active before application of IS (61 C-fibers, 17 A-fibers) or recruited by IS (12 C-fibers, 5 A-fibers). More than one half of the C-fibers were activated by IS for ≤90 min after its removal. The majority of mecano- (23/38) and heat-sensitive (29/35) C-fibers as well as mechano-sensitive A-fibers (12/17) decreased their activation thresholds and/or increased the response magnitude to mechanical and/or heat stimulation of the nerve. Noxious cold sensitivity, but not nonnoxious cold sensitivity, was weakly influenced by IS. Some initially nonresponsive C- and A-fibers developed new ectopic properties, i.e., were recruited, and exhibited ongoing activity and/or could be activated by physiological stimuli after application of IS. The results suggest that inflammatory mediators may be critical to enhance ectopic excitability of regenerating afferent nerve fibers. These peripheral mechanisms may be important triggering and maintaining neuropathic pain.

INTRODUCTION

Following a nerve injury, many lesioned afferent nerve fibers develop ongoing activity and ectopic sensitivity to mechanical and/or thermal stimuli applied along the affected nerve. Starting within 4–6 h after the lesion, this ectopic activity can persist for weeks and months (Blenk et al. 1996; Blumberg and Jänig 1984; Gorodetskaya et al. 2003; Grossmann et al. 2009; Jänig et al. 2009). Experiments conducted within 2–33 h after nerve lesion have shown that a combination of inflammatory mediators can sensitize these lesioned afferent nerve fibers to mechanical stimuli (Michaelis et al. 1998). The same effect was reported for nerve fibers trapped in a long-standing neuroma (Riverra et al. 2000).

Inflammatory mediators can sensitize intact cutaneous afferent nerve fibers to heat stimuli and induce ongoing activity when applied to their receptive fields (Chen et al. 1999; Lang et al. 1990; Linhart et al. 2003). The same applies to cultured sensory cells of dorsal root ganglia of newborn rats (Vyklicky et al. 1998). Similarly, afferents innervating skeletal muscle or joints can be sensitized to mechanical stimuli by components of inflammatory soup (IS) such as bradykinin, prostaglandin E2 (PGE2), or histamine (Mense 1993; Schaible and Grubb 1993). The sensitization of nociceptors is caused by the interaction of the inflammatory mediators with distinct receptors in the membrane of the afferent nerve fibers that are directly or indirectly (via intracellular pathways) coupled to the transduction mechanisms for mechanical or thermal stimuli or to voltage-gated cation channels (Hu et al. 2006; Hucho and Levine 2007; Julius and McCleskey 2006; Lewin and Moshourab 2004; McMahon et al. 2006).

Following nerve lesion, receptor molecules and ion channels follow the anterograde axonal transport to the sprouts of the regenerating nerve fibers. Here they accumulate and are incorporated into the cell membrane, thus causing ectopic excitability as reported recently (Gorodetskaya et al. 2003; Grossmann et al. 2009; Jänig et al. 2009). These molecules are probably also present in the axon membrane, as they are in the cell body membrane, contributing potentially to the excitability of the sensory axons (Lawson 2005; Moalem et al. 2005). The ectopic excitability is believed to play a major role in the process of central sensitization and the generation of neuropathic pain states. Throughout the time of regeneration, nerve fibers are exposed to the inflammatory processes accompanying the Wallerian degeneration distal to the lesion. Schwann cells, macrophages, mast cells, and fibroblasts release inflammatory cytokines (TNFα, IL-1, IL-6) (Watkins and Maier 2002). Via distinct pathways, these lead to an increased level of inflammatory mediators like PGE2, bradykinin, serotonin, histamine, etc., in the tissue surrounding the nerve trunk (Jänig and Levine 2006; Lawson 2005; McMahon et al. 2006; Richardson and Vasko 2002). Do inflammatory mediators influence and/or induce ectopic excitability of regenerating nerve fibers and could this process be a crucial component in triggering neuropathic pain?

This study was undertaken to find out whether inflammatory mediators can affect the ectopic excitability of lesioned and regenerating afferent nerve fibers. We studied the effect of IS on the ectopic ongoing activity, mechanosensitivity, and thermosensitivity of myelinated as well as unmyelinated nerve fibers under regeneration.

METHODS

Surgical procedures

Sural nerve crush lesion was performed on 32 male Wistar rats (body weight, 280–350 g) 7–14 d before the final electrophysiological experiments. The animals were anesthetized with pentobarbital...
sodium (Narcoren, 60 mg/kg, ip). Under aseptic conditions, the left sural nerve was exposed in 30 rats 18–25 mm proximal to the ankle and crushed with fine watch maker’s forceps three times for 10 s each (width of lesion, 1–1.5 mm). The wound was closed in layers, and recovery was uneventful. In four rats, no sural nerve lesion was performed. These rats served as controls.

On the day of the final experiment, the rats were again anesthetized with pentobarbital sodium (Narcoren, 60 mg/kg, ip). Catheters were inserted into the jugular vein for regular fluid and drug administration and into the tail artery for blood pressure recording and blood gas analysis. Throughout the experiments, the mean arterial blood pressure exceeded 70 mmHg. The trachea was cannulated, and the animals breathed spontaneously during the initial surgery. Sufficient anesthesia was maintained by injecting 10–20 mg/kg/h pentobarbital sodium intravenously. The animals were paralyzed with pancuronium (Organon, initial dose 1 mg/kg, iv; maintenance with 0.4 mg/kg/h, iv) and artificially ventilated at 70 strokes/min with O₂-enriched room air. Blood gases were analyzed regularly throughout the experiment (ABL5, Radiometer), and the arterial pO₂ always exceeded 90 mmHg. Rectal temperature was kept constant at ~37°C using a servo-controlled heating blanket. At the end of the experiments the animals were killed under deep anesthesia by an intravenous injection of a saturated potassium chloride solution. All experiments had been approved by the local animal care committee of the state administration and were conducted in accordance with German Federal Law.

The left sural nerve was exposed from 10 mm proximal to the ankle to its junction with the sciatic nerve where it was cut, isolated from its connective tissue, and placed on a rigidly fixed black perspex platform. The exposed nerve and surrounding tissue were covered with warm (30°C) paraffin oil in a pool made from skin flaps.

**Recording and electrical stimulation**

Fine filaments were teased out of the proximal cut end of the sural nerve and placed on a platinum wire electrode for recording. The indifferent recording electrode was connected to the nearby tissue. The signals were differentially amplified (input resistance 10 MΩ), filtered with a bandwidth of 120 Hz to 1.0–1.2 kHz (unmyelinated fibers) or with a bandwidth of 120 Hz to 40 kHz (myelinated fibers), and fed through window discriminators.

For electrical stimulation, the nerve was isolated from the surrounding tissue 5–7 mm proximal to the lesion site and put on a pair of platinum electrodes. The nerve was stimulated with square wave impulses of 0.1 (A-fibers) or 0.5 ms (C-fibers) duration and with variable intensities ≤40 V. According to their conduction velocities, fibers were classified as A- (conduction velocity > 2 m/s) or C-fibers (conduction velocity ≤ 2 m/s).

**Experimental protocol**

First we tested if a filament contained at least two to three fibers exhibiting ongoing activity and/or either mechanical or thermal ectopic sensitivity. Mechano- and thermosensitivity were tested qualitatively by applying a fine-tipped blunt glass rod or fine-tipped copper rods of 50 or 3–5°C, respectively, to the sural nerve. Per animal, only one strand of nerve fibers isolated from the sural nerve proximal to the lesion site was chosen and further analyzed. Figure 1 shows the experimental protocols. In 20 rats with nerve lesions, IS was applied after testing period 1 (experimental protocol A in Fig. 1). In 10 rats with nerve lesions, Tyrode’s solution was applied to the nerve (control experiments; experimental protocol B in Fig. 1). The experimental steps were as follows.

1) Ongoing activity was recorded for 3–5 min at a temperature of the nerve surface in the oil pool of about 28°C.

2) A series of mechanical, cold, and heat stimuli was applied to the nerve from 5 mm proximal to 18 mm distal to the lesion site to determine the number of ectopically activated fibers, their receptive fields, and stimulus-response curves to these stimuli. This testing period 1 lasted for 30–60 min.

3) Either a combination of inflammatory mediators (called here IS) was applied to the nerve for 2 min followed by a washout with Tyrode’s solution for 30 s (experimental group A) or Tyrode’s solution was applied to the nerve for 20 min (experimental group B, control group).

![FIG. 1. Experimental protocol. The experimental procedures were identical in protocol A [application of inflammatory soup (IS) to the nerve] and protocol B (application of Tyrode’s solution to the nerve, control). Strong mechanical stimuli were applied by a fine-tipped blunt glass rod; stimuli of 3–5 and about 50°C applied by the thermode were strong cold and strong heat stimuli, respectively.](http://jn.physiology.org/doi/10.1152/jn.00582.2009)
4) In testing period 2 starting ~10 min after application of IS and lasting for 30–60 min, the series of mechanical and thermal stimuli was repeated as in testing period 1.

5) Ongoing discharge was measured over 3–5 min. In four rats without nerve lesions, IS was applied to the intact or acutely transected sural nerve distal to the recording site and 10–15 mm proximal to the transection site while recording from afferent nerve fibers. In these experiments, it was tested whether ongoing activity and responses to mechanical or heat stimuli (45°C) applied to the intact nerve can be induced by IS. In two of these rats, the IS contained 0.2% dimethylsulfoxide (DMSO) to increase the permeability of the perineurium.

Physiological stimulation

The interval between the physiological stimuli was 2–3 min. The duration of the stimuli was 20–25 s. For quantitative analysis of the responses, we measured the afferent activity within the first 20 s after the beginning of the stimuli.

MECHANICAL STIMULI Mechanical sensitivity was tested first with a fine-tipped blunt glass rod (strong stimulus) at about five sites of the nerve (lesion site and distal to the lesion site) and, in case of a response, the strength of the finest filament able to evoke activation was considered to be the activation threshold. The strength of the finest filament able to evoke activation was considered to be the activation threshold. The next stronger von Frey filament was tested in case of an activation (i.e., triggering of an action potential) or the next stronger filament if there was no response. The strength of the finest filament able to evoke activation was considered to be the activation threshold.

THERMAL STIMULI Thermal sensitivity was tested using a water-perfused thermode that was positioned on the nerve at the lesion site and distal to the lesion site. The length of the contact site with the nerve was 3 mm. The temperature was measured at this contact site (see Gorodetskaya et al. 2003). To identify cold-sensitive fibers, we first perfused the thermode with ice-cold water, decreasing the temperature to 3–5°C (strong stimulus) at the contact site with the nerve. If there was a response, the nerve fibers were stimulated with a series of cold stimuli of 10–21°C in ascending order. By analogy, we initially used temperatures of 47–50°C (strong stimulus) to test the heat sensitivity of the nerve fibers, followed by a series of consecutively lower temperatures until no more response was observed. All thermal stimuli lasted for ~20 s starting from a baseline temperature of 28°C. A fiber was considered to be cold/heat activated either if at least three action potentials were evoked during a stimulus in a silent fiber or in fibers with rates of ongoing activity ≤0.5 imp/s or if the ongoing activity increased by 50% in fibers with ongoing activity >0.5 imp/s. The lowest/highest temperature which evoked a response that met these criteria of activation was considered to be the activation threshold for a heat/cold-sensitive fiber, respectively.

Data analysis

Neural activity, temperature of the stimulation thermode, and arterial blood pressure were simultaneously fed into a computer (IBM-compatible) with ADC and counter interface (Burr-Brown PCI-20000, data acquisition software CARDS by S. Tiedemann) and stored on a digital tape recorder (DTR-2602, Biologic, Claix, France) for further off-line analysis (custom data acquisition software and template-matching program; Forster and Handwerker 1990). Data are expressed as means ± SE. Statistical analysis is based on ANOVA, the Wilcoxon matched pairs signed-rank test, or the paired t-test. Paired values missing for the ANOVA analysis were replaced by group means.

RESULTS

Overall description

In experimental group A (application of IS to the nerve), a total of 138 C- and 51 A-fibers were electrically identified. The

| TABLE 1. Ectopic properties of regenerating A- and C-fibers before and after the application |
|------------------------------------------|----------|----------|----------|----------|
|                                       | A-fibers | C-fibers |          |          |
|                                       | Before IS | After IS | Before IS | After IS |
| Total number of fibers with ectopic properties | 17   | 22   | 61   | 73   |
| **Individual Discharge Properties**  |          |          |          |          |
| OA                                      | 0   | 4   | 32   | 45   |
| Mehanosensitivity                      | 17  | 22  | 38   | 48   |
| Thermosensitivity                      | 6   | 10  | 43   | 53   |
| Cold activated                         | 4   | 8   | 27   | 34   |
| Heat activated                         | 4   | 9   | 35   | 44   |
| **Combination of Discharge Properties**|          |          |          |          |
| Mehanosensitive/OA                     | 11/0 | 12/2 | 14/3 | 13/3 |
| Cold-sensitive/OA                      | 0/0 | 0/0 | 3/3 (3/3 cold type 1) | 3/3 (2/2 cold type 1) |
| Heat-sensitive/OA                      | 0/0 | 0/0 | 7/3 | 6/2 |
| Cold and heat sensitive/OA             | 0/0 | 0/0 | 9/6 (5/4 cold type 1) | 9/6 (5/4 cold type 1) |
| Mehanos- and cold-sensitive/OA        | 2/0 | 1/0 | 5/4 | 6/2 |
| Mehanos- and heat-sensitive/OA        | 2/0 | 2/0 | 9/6 | 13/9 |
| Mehanos- and cold- and heat-sensitive/OA | 2/0 | 7/2 | 10/3 | 16/13 (1/1 cold type 1) |
| OA only                                 | 0   | 0   | 4   | 7   |

Fibers with OA are indicated by the numbers behind the slash. These numbers are included in the numbers before the slash. IS, inflammatory soup; OA, ongoing activity.
Conduction velocities of the A- and C-fibers ranged from 6 to 40 (26 ± 1.9 m/s) and 0.24 to 1.8 m/s (0.78 ± 0.04 m/s), respectively. No clear distinction into Aβ- and Aδ-fibers was possible, probably because of shrinkage of the fibers after crush lesion (Hu and McLachlan 2003; Jänig and McLachlan 1984). A total of 61 C- and 17 A-fibers could be ectopically activated by either mechanical and/or thermal stimuli applied to the nerve and/or exhibited ongoing activity before application of IS. A total of 73 C- and 22 A-fibers could be activated ectopically and/or exhibited ongoing activity 10–20 min after application of IS (Table 1). In experimental group B (application of Tyrode’s solution to the nerve, control group,) 20 C- and 9 A-fibers were studied.

The individual response characteristics of the fibers with ectopic activity and their patterns of discharge correspond to our earlier results obtained for this time period after sural nerve crush lesion (Gorodetskaya et al. 2003; Table 1). C-fibers with cold sensitivity were separated into two types (Grossmann 30 s

**FIG. 2.** Activation of 2 afferent C-fibers by IS applied to the nerve. A: activation of a silent C-fiber. This fiber became active 80 s after application of IS and became silent again 9 min after removal of IS. It could not be activated by mechanical or thermal stimuli applied to the nerve before or after application of IS. B: activation of a C-fiber with ongoing activity. This fiber was immediately activated by IS, its activity remained increased for ~30 min. It could also be activated by heat and mechanical stimuli (activation thresholds 35°C and 2 mN, respectively).
et al. 2009; Jänig et al. 2009): C-fibers with type 1 cold sensitivity usually had a high rate of ongoing activity at 28°C nerve temperature, exhibited low thresholds and graded responses to cooling, had high rates of activity to maximal cooling, and their ongoing activity was inhibited by heat stimuli. These fibers were, with one exception, not mechanosensitive; some were excited by heat stimuli following inhibition. C-fibers with type 2 cold sensitivity had no or a low rate of

**TABLE 2.** Numbers of regenerating C- and A-fibers studied in experimental protocol A (see Fig. 1) before and after application of IS to the nerve

<table>
<thead>
<tr>
<th>Combination of Ectopic Response Characteristics</th>
<th>a (Mech Only)</th>
<th>b (Heat Only)</th>
<th>c (Cold 1 Only)</th>
<th>d (Cold 1 and Heat)</th>
<th>e (Cold 2, Heat, and/or Mech)</th>
<th>f (Tested Before and After IS)</th>
<th>g (Recruited by IS)</th>
<th>h (Sensitized/Total)</th>
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<tbody>
<tr>
<td>1 Mechano-sensitivity</td>
<td>9/14</td>
<td>7/11</td>
<td></td>
<td>14/24</td>
<td>5/6</td>
<td>17/23</td>
<td>5</td>
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<td>C</td>
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<td>C</td>
<td>6/7</td>
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<td>3 Type 1 cold sensitivity</td>
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<td>A</td>
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<td>19 (Fig. 9)</td>
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<td>15/26</td>
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<td>4 Type 2 cold sensitivity</td>
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Columns a to e show the numbers of afferents separated by their functional characteristics (number of sensitized afferents/total number of afferents). Afferents having combinations of sensitivity (mechano, type 2 cold and/or heat) were put together in one group (column e). Column f, total number of fibers tested before and after application of IS. Column g, total number of afferents recruited after IS. Column h, number of sensitized afferents/total number of afferents (this includes the recruited afferents). Rows 1 to 4: type of sensitivity tested before and after application of IS. The figures containing the quantitative data (thresholds, stimulus-response curves) for mechano-, heat, and cold sensitivity are indicated in columns f and g. IS, inflammatory soup.
ongoing activity and exhibited high thresholds and low rates of activity to cold stimuli. They were additionally mechano- and/or heat-sensitive.

In four rats without sural nerve lesion, we tested whether application of IS (2 rats) or IS plus DMSO (2 rats) to the intact nerve distal to the recording electrode induced ongoing activity, mechanosensitivity (to mechanical stimuli applied to the nerve with a fine-tipped glass rod), or heat sensitivity (to a stimulus of 45°C applied to the nerve). Both types of stimuli were applied at two sites of the nerve 7–10 mm apart. In two rats, 26 A-fibers and 55 C-fibers tested were unresponsive to the mechanical and heat stimuli before and after application of IS and did not develop ongoing activity after application of IS. In two rats, 117 C-fibers were tested to IS plus DMSO. In these rats, two C-fibers showed weak responses to (4–8 impulses in 2 min) to the heat stimuli and to IS, but the responses to heat were not enhanced by IS; the other 115 C-fibers were unresponsive to heat stimuli. All 117 C-fibers were unresponsive to mechanical stimuli before and after application to IS.

**Ongoing activity**

C-FIBERS. Before application of IS 32/61, C-fibers in experimental group A exhibited ongoing activity. Seven of these fibers were type 1 cold-sensitive afferents and discharged at a rate of 4.8 ± 1.4 imp/s. The remaining 25 C-fibers discharged at a rate of 0.23 ± 0.04 imp/s. Twenty-one of them were mechano-, high-threshold (type 2) cold-, and/or heat-sensitive, and four had ongoing activity only (Table 1). Ten to 20 min after application of IS to the nerve (and after *testing period 1*; see Fig. 1), 45/73 C-fibers exhibited ongoing activity. Seventeen of these C-fibers were silent before application of IS, and four of the initially spontaneously active fibers lost their ongoing activity. The rate of ongoing activity in seven of eight type 1 cold-sensitive C-fibers was 4.11 ± 1.25 imp/s 10–20 min after application of IS and not significantly different from the rate before application of IS. The ongoing discharge rate of

![Graph](image-url)
the remaining 38 C-fibers was 0.47 ± 0.08 imp/s 10–20 min after application of IS and significantly higher than before application of IS (P < 0.005, Wilcoxon matched pairs signed-rank test).

Figure 2 shows typical examples of activity in a silent C-fiber (Fig. 2A) and a C-fiber with ongoing activity (Fig. 2B) that were not cold sensitive during and after application of IS to the nerve. The latency of activation onset ranged from 7 to 188 s but occurred in almost all fibers within 120 s of the IS application and was not different between the groups of C-fibers with and without initial ongoing activity. Figure 3 shows the activity of the four groups of C-fibers before, during, and after application of IS or Tyrode’s solution to the nerve. The activity significantly increased in the first two groups of C-afferents for 20–25 min (Fig. 3A) but did not change in the type 1 cold-sensitive C-afferents (Fig. 3B). In the control population of C-afferents that also went through testing period 1 and in which Tyrode’s solution was applied to the nerve throughout 20 min (experimental group B; Fig. 1), the ongoing activity did not change significantly (Fig. 3B). Thus the mechanical and thermal stimuli applied to the nerve in the first testing period did not enhance the ongoing activity in these control C-fibers.

In summary, short-lasting application of IS to the nerve activates injured afferent C-fibers with nociceptive function for 20 min or longer.

A-FIBERS. Seventeen ectopically activated A-fibers were silent initially. Application of IS acutely excited 4/17 A-fibers (response latency 14.8 ± 4.8 s; median, 16.5 s). In three A-fibers, the activation stopped within 1 min after removal of IS; in one fiber, the activation was for >60 min. The maximal discharge frequency during the activation ranged from 0.02 to 3.7 imp/s.

Sensitization for mechanical and thermal stimuli

Table 2 shows the numbers of C- and A-fibers that were studied for sensitization by IS using protocol A as described in methods (Fig. 1). The afferent fibers were classified according to their mechano-, cold (type 1 or type 2), or heat sensitivity or combinations of these functional characteristics. In total, 57 C-afferents and 17 A-afferents that were activated by one of the physiological stimuli were studied. Twelve C-afferents and five A-afferents were recruited after application of IS for one or more types of physiological stimuli.

Mechanosensitivity

C-FIBERS. Thirty-eight of 57 C-fibers (67%) could be excited by repetitive mechanical stimuli applied to the nerve. Their activation thresholds ranged from 0.03 to 70 mN (median, 7.5 mN); one fiber responded to stimulation of the nerve with a glass rod only and therefore had an activation threshold >100 mN. After application of IS to the nerve, 23 (60%) of these fibers were sensitized to mechanical stimulation. Figure 4 illustrates the example of a C-fiber showing an increase of the response magnitude to stimuli of 0.45, 1, and 7.5 mN after application of IS. Within the population of C-fibers, the decrease of mechanical threshold was significant (Fig. 5A; median, 7.5 vs. 2 mN; P < 0.001). Similarly, the response magnitude within the population of C-fibers significantly increased (P < 0.001; Fig. 5B). There was no significant difference in sensitization between the group of high- (threshold ≥ 20 mN) and low-threshold (threshold ≤ 10 mN) mechanosensitive C-fibers (10/16 vs. 13/22 fibers were sensitized, respectively). Ten C-fibers that could not be activated by mechanical stimulation of the nerve initially were recruited after application of

FIG. 6. Responses of A-fibers to mechanical stimulation of the nerve. A: mechanical thresholds before and after application of IS (medians indicated by and *) and after Tyrode’s solution to the nerve (B) or application of Tyrode’s solution to the nerve (C). The nerve was stimulated repetitively (70 stimuli/min) with von Frey filaments of 0.45 or 0.7 mN to 70-mN strength for 20 s before and after application of IS or Tyrode’s solution. Ordinate scales as in Fig. 5. Response increased significantly after application of IS (P < 0.05, ANOVA) but not after application of Tyrode’s solution. Mean ± SE.
IS. Their activation thresholds were 7.5–70 mN (median, 15 mN). The stimulus-response function of these recruited C-fibers to mechanical stimulation is shown in Fig. 5B (○).

Nine mechanosensitive C-fibers that were studied with the same testing protocol but with application of Tyrode’s solution to the nerve showed no change of their stimulus-response curve (Fig. 5C; protocol B in Fig. 1), arguing that the application of mechanical, cold, and heat stimuli in the first testing period did not sensitize these afferents to mechanical stimulation.

A-FIBERS. Seventeen A-fibers were ectopically activated by repetitive mechanical stimuli applied to the nerve (activation thresholds, 1–70 mN; median, 7.5 mN). After application of IS, 12/17 fibers were sensitized to mechanical stimulation. The decrease of activation threshold in the population of A-fibers was significant (median, 7.5 vs. 1 mN; range, 1–70 vs. 0.07–45 mN; P < 0.01; Fig. 6A). The response magnitude in the population of A-fibers tested significantly increased after application of IS at stimulus strengths of 2–70 mN (P < 0.05; Fig. 6B). Five A-fibers that did not respond to mechanical stimuli initially became mechanosensitive after application of IS. Their activation thresholds ranged from 0.45 to >100 mN (median, 45 mN).

In the control population of nine A-fibers (application of Tyrode’s solution to the nerve), the response magnitude to mechanical stimulation did not change (Fig. 6C).

In summary, IS applied to the nerve sensitizes regenerating afferent C- and A-fibers to mechanical stimulation.

Heat sensitivity

C-FIBERS. In total, 35/57 C-fibers were activated by heat stimuli applied to the nerve before application of IS. Their activation thresholds ranged from 35 to 49°C (42 ± 0.79°C; median, 42.5°C) and were evenly distributed. After application of IS, 29 (81%) of these C-fibers were sensitized to heat stimuli. Within the population of heat-sensitive C-fibers, the heat threshold decreased significantly from 41 to 38°C (median; range, 35–48 vs 30–48°C; P < 0.005; Fig. 7A), and the magnitude of response increased significantly (P < 0.001; Fig. 7B). There was no significant difference in sensitization between the group of low-threshold (≤42°C; median, 39°C) and high-threshold (≥43°C; median, 47.5°C) heat-sensitive fibers (15/18 vs. 14/17 were sensitized, respectively). The activity of ~0.82 imp/s at 28°C nerve temperature in the population of heat-sensitive C-fibers was generated by four of five C-fibers, which were additionally type 1 cold-sensitive (Table 2, row 2; Fig. 9A).

Nine C-fibers that could not be activated by heat stimuli ≥50°C before application of IS became heat sensitive after application of IS. Figure 8 shows the recording from a filament with four C-fibers, two being heat-sensitive; one C-fiber was heat-sensitive before and sensitized by application of IS (fiber 3) and one C-fiber was recruited by IS for heat stimuli (fiber 4). The recruited fibers had activation thresholds of 40–50°C (median, 47°C) and exhibited a stimulus-response function that

FIG. 7. Responses of regenerating afferent C-fibers to heat stimuli before (●) and after (▲) application of IS to their receptive fields in the nerve (A and B) or application of Tyrode’s solution to the nerve (C). A: activation thresholds (medians indicated by ● and ▲; *P < 0.005, Wilcoxon matched pairs signed-rank test). B and C: stimulus-response functions. The open diamonds in B show the activity in C-fibers recruited for heat stimulation after application of IS. The ongoing activity (at 28°C) was not subtracted from the responses generated by the heat stimuli. It was 0.82 ± 0.4 imp/s before application of IS and 1.0 ± 0.37 imp/s about 10 min after application of IS and before the 2nd testing procedure. This ongoing activity was largely dependent on the activity in 4/5 afferent fibers with type 1 cold sensitivity that was completely depressed during heating (see Fig. 9A). Ordinate scale in A: stimulus strength in °C. Ordinate scales in B and C: mean impulse rate during 20 s of heat stimulation. Response increased significantly after IS (P < 0.001, ANOVA) but not after application of Tyrode’s solution. Mean ± SE.
was shifted to the right compared with the initially heat-sensitive C-fibers (Fig. 7B, C).

Nine heat-sensitive C-fibers that were studied with the same testing protocol but with application of Tyrode’s solution to the nerve showed no change of their stimulus-response curve (Fig. 7C), arguing that the application of mechanical, cold, and heat stimuli in testing period 1 did not sensitize these afferents for heat stimuli.

In summary, IS applied to the nerve sensitized >50% of regenerating heat-sensitive C-fibers to heat stimuli (Table 2).

A-fibers. Four initially heat-sensitive A-fibers (activation thresholds, 36–41°C) were not sensitized by IS. Five A-fibers initially unresponsive to heat stimuli were recruited by heat stimuli after application of IS. Their activation thresholds were 40–50°C.

Cold sensitivity

C-fibers. A total of 27 of 57 C-fibers were cold-sensitive before application of IS. Eight of these fibers exhibited type 1 cold sensitivity (activation threshold, 19 ± 1.6°C; median, 18.5°C). Their responses to cooling were not significantly changed after application of IS to the nerve (Fig. 9A). Five of the fibers with type 1 cold sensitivity were also excited by heating. These responses were enhanced; however, this enhancement was not significant.

Nineteen cold-sensitive C-fibers exhibited type 2 cold sensitivity (activation threshold, 8.4 ± 1.5°C; median, 5°C). The responses to cooling were slightly enhanced by IS; however, this enhancement was only significant for 5°C (Fig. 9B). The responses to heating of the C-fibers with type 2 cold sensitivity were significantly enhanced (P < 0.01). Seven C-fibers were recruited by cold stimulation after application of IS to the nerve. The recruitment occurred at stimuli of 5°C. These recruited C-fibers also showed heat sensitivity (Fig. 9B).

In summary, responses to cold stimuli were not enhanced by IS applied to the nerve in regenerating type 1 cold-sensitive C-fibers but weakly enhanced in type 2 cold-sensitive C-fibers.

A-fibers. Four A-fibers were activated by cold stimuli before application of IS to the nerve. One of these fibers was sensitized by IS to cold stimuli. Additionally, four A-fibers were recruited by IS to cold stimuli. Their activation thresholds were 4–5°C.

Discussion

Ectopic excitability of myelinated and unmyelinated regenerating afferent nerve fibers 1–2 wk following a sural nerve crush lesion was studied before and after application of a combination of inflammatory mediators (IS) to the nerve for 2 min. The overall results are 1) the ongoing activity is enhanced or induced for 20–25 min or longer in ~50% of the unmyelinated fibers with ectopic activity that are not type 1 cold sensitive; 2) most mechanosensitive A- and C-fibers are sensitized to mechanical stimuli; 3) most heat-sensitive C-fibers are sensitized to heat stimuli; 4) responses to noxious cold stimuli are enhanced in some C-fibers with type 2 cold sensitivity; 5) C-fibers with type 1 cold sensitivity are not influenced in their responses to cooling by IS; 6) some C- and A-fibers are recruited to mechanical or thermal stimuli by IS.

Nonlesioned cutaneous and deep somatic afferent neurons can be sensitized for mechanical and/or heat stimuli by inflammatory mediators applied to their receptive fields (Chen et al. 1999; Lang et al. 1990; Linhart et al. 2003; Mense 1993; Meyer et al. 2006; Schaible and Grubb 1993). This sensitization is caused by action of inflammatory mediators on distinct receptors in the membrane of the afferent nerve fibers that are coupled via intracellular pathways (or directly) to the transduction mechanisms for mechanical or thermal stimuli or to voltage-gated sodium channels (Hucho and Levine 2007; Julius and McCleskey 2006; Lewin and Moshourab 2004; McMahon et al. 2006; Woolf and Ma 2007). Within 30 h after axotomy, unmyelinated afferent nerve fibers are excited and can be sensitized to mechanical stimuli by inflammatory mediators (Michaelis et al. 1998). The same is true for nerve fibers ending in a neuroma of the saphenous nerve (Rivera et al. 2000). Our results substantially extend these findings showing that the application of inflammatory mediators also cause an acute sensitization of regenerating A- and C-fibers by increasing or inducing ongoing activity, by increasing the sensitivity
to heat and/or cold stimuli, and by recruiting responses in injured afferent C- and A-fibers for mechanical or thermal stimuli.

Application of IS on the sural nerve, that was either acutely transected distally to the application site or left intact, neither induced ongoing activity nor responses in the afferent A- or C-fibers to mechanical or heat stimuli. These negative results may be explained by the low permeability of the perineurium of a normal nerve. However, we obtained the same results with IS plus DMSO. Furthermore, the perineurial permeability in a nerve increases only twofold after injury and by 20% in the time period 7–14 days after injury (Weerasuriya 2005; Weerasuriya and Hockman 1992), in which we tested most of our time period 7–14 days after injury (Weerasuriya 2005; Weerasuriya and Hockman 1992), in which we tested most of our studies, neurophysiological studies) showed that this channel is essential for cold sensation and the equivalent behavior over a wide range of cold temperatures including sensing of unpleasant cold stimuli and cold-induced nociception (Bautista et al. 2007; Colburn et al. 2007; Dhaka et al. 2007). The transduction channels TRPV3 and TRPV4 are mainly, although not exclusively, expressed on keratinocytes (Lumpkin and Caterina 2007). Type 1 cold sensitivity in our afferent C-fibers may involve the TRPM8 ion channel (Bautista et al. 2007; Dhaka et al. 2007; McKemy et al. 2002). Our type 1 cold-sensitive afferents are rather similar, in their response to cooling, to trigeminal neurons innervating the cornea (de la Pena et al. 2005; Madrid et al. 2006). Experiments on mice with a deficient TRPM8 receptor (behavioral studies, neurophysiological studies) showed that this channel is essential for cold sensation and the equivalent behavior over a wide range of cold temperatures including sensing of unpleasant cold stimuli and cold-induced nociception (Bautista et al. 2007; Colburn et al. 2007; Dhaka et al. 2007). The transduction channels underlying the responses of the injured afferent fibers to noxious cold stimuli may not involve one specific channel only such as TPPA1 (Bandell et al. 2004; Bautista et al. 2006; Reid 2005; Story et al. 2003; Zurborg et al. 2007). In fact, transduction of noxious cold stimuli may depend on several mechanisms including activation of specific TRP channels,
inhibition of potassium channels, and activation of other channels (for discussion, see Foulkes and Wood 2007). The mechanisms underlying mechanical transduction of the afferent fibers are also not well understood. They probably involve several ionic channels being responsible for low- or high-threshold mechanical transduction (for review, see Hu et al. 2006; Lewin and Moshourab 2004; Lumpkin and Caterina 2007; O’Neil and Heller 2005).

Inflammatory substances like PGE2 can interact with TTX-R Na+ channels (England et al. 1996; Gold 1999; Gold et al. 1996) and may in this way enhance the excitability of the lesioned nerve fibers, increasing or inducing ongoing activity. However, this effect seems to be small and has been questioned (Zheng et al. 2007). Inflammatory mediators also affect the membrane potential via other molecular pathways as shown in isolated rat sensory neurons: PGE2 suppresses an outward K+ current (Nicol et al. 1997) and bradykinin, PGE2, and serotonin increase the intracellular Ca2+ concentration (Linhart et al. 2003). These effects may lead to a shift of the membrane potential, giving possible explanations for the acute reactions we observed during the IS application.

That most regenerating heat-sensitive C-fibers (29/35) were sensitized to heating stimuli after application of IS is a novel finding. It was shown that bradykinin and PGE2 can both excite and sensitize neurons expressing TRPV1 (Lang et al. 1990; Liang et al. 2001; Linhart et al. 2003; Moriyama et al. 2005) via the B2 and EP1 receptors, respectively. Because the vanilloid receptor channels are often a heteromeric combination of different vanilloid receptor subtypes (Smith et al. 2002), it is possible that inflammatory mediators also influence the transduction of heat stimuli involving the TRPV2, TRPV3, and TRPV4 channels.

Application of IS had a comparably weak influence on cold sensitivity. Type 1 cold sensitive neurons are not influenced in their responses to cold stimuli by IS. These results seem to be at variance with experiments of Linte et al. (2007), showing that cold- and menthol-sensitive rat dorsal root ganglion cells in primary culture are desensitized in their response to cold stimuli by the inflammatory mediators bradykinin and prostaglandin E2. A subpopulation of our afferent fibers with type 1 cold sensitivity was also excited by heating (see Tables 1 and 2; Grossmann et al. 2009). This agrees with findings showing that a subpopulation of dorsal root or trigeminal ganglion cells responding to cooling and menthol, i.e., expressing very likely the transduction channel TRPM8, also respond to capsaicin (Viana et al. 2002; Xing et al. 2006). C-fibers with type 1 cold sensitivity excited by heat stimuli could be enhanced by IS for the heat stimuli. C-fibers with type 2 cold sensitivity could be sensitized for cold stimuli, however, only at low temperatures. Furthermore, type 2 cold sensitivity was recruited in some C-fibers by IS but not type 1 cold sensitivity.

The mechanisms underlying the sensitization of regenerating afferent A- and C-fibers to mechanical stimulation by IS are not well understood. Some specific mechanically gated cation channels of the epithelial Na+ channel/acid sensing ion channel (ENaC/ASIC) family have been shown to be involved in the transduction of mechanical stimuli (Lumpkin and Caterina 2007; Price et al. 2000; Waldmann and Lazzunski 1998), although these channels do not seem to be involved in mechanonociception, and it is not yet known whether inflammatory mediators can directly interact with them. Furthermore, indirect evidence suggests that the heat-sensitive channels TRPV2 and TRPV4 are also mechanosensitive (Alessandri-Haber et al. 2006; Mutai and Heller 2003; O’Neil and Heller 2005; Suzuki et al. 2003). A sensitization to heat and mechanical stimuli may therefore be correlated. Indeed, most intact mechanosensitive afferents in monkey responsive to IS can be sensitized to both mechanical and heat stimuli (Davis et al. 1993), although in our experiments, this was the case in only 50% of the polymodal C-fibers (Table 2).

Bove et al. (2003), Dilley and Bove (2008), and Dilley et al. (2005) have shown that peripheral nerve inflammation by complete Freund’s adjuvant or disruption of axoplasmic transport can induce axonal mechanosensitivity of intact conducting afferent A- and C-fibers. The mid-axonal mechanosensitivity develops in ≥1 day after nerve inflammation in deep somatic afferent A- and C-fibers and is almost absent in cutaneous afferent nerve fibers. This inflammation-induced mid-axonal mechanosensitivity is different from the mechanosensitivity in the regenerating A- and C-fibers, which could be acutely enhanced by IS applied to the nerve as reported here.

Finally, inflammatory mediators can cause a shift in the membrane potential of afferent neurons (Linhart et al. 2003; Nicol et al. 1997), increasing their excitability with lowered activation threshold and increased response frequencies. This may have occurred in recruited afferent fibers expressing novel ectopic properties after the IS.

In conclusion, ongoing and evoked ectopic excitability of regenerating nociceptive afferent nerve fibers are enhanced by inflammatory mediators applied to the injured nerve. Endogenous inflammation in an injured nerve occurs during regeneration of nerve fibers involving Schwann cells, fibroblasts, resident and invading macrophages, and other inflammatory cells releasing inflammatory mediators. This ongoing endogenous inflammatory process increases the ectopic excitability of regenerating afferent nerve fibers. Early suppression of these inflammatory processes could be a promising therapeutic step to prevent or attenuate the development of neuropathic pain following nerve lesion.

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REFERENCES


ENHANCEMENT OF ACTIVITY IN REGENERATING AFFERENT FIBERS


