Augmented Mechanical Response of Muscle Thin-Fiber Sensory Receptors Recorded from Rat Muscle–Nerve Preparations In Vitro After Eccentric Contraction

Toru Taguchi, Jun Sato, and Kazue Mizumura
Department of Neural Regulation, Division of Regulation of Organ Function, Research Institute of Environmental Medicine, Nagoya University, Nagoya, Japan

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Taguchi, Toru, Jun Sato, and Kazue Mizumura. Augmented mechanical response of muscle thin-fiber sensory receptors recorded from rat muscle–nerve preparations in vitro after eccentric contraction. J Neurophysiol 94: 2822–2831, 2005; doi:10.1152/jn.00470.2005. Unaccustomed strenuous exercise, especially that from eccentric muscular work, often causes muscle tenderness, which is a kind of mechanical hyperalgesia. We developed an animal model of delayed-onset muscle soreness (DOMS) from eccentric muscular contraction (ECC) in rats and demonstrated the existence of muscle tenderness by means of behavioral pain tests and c-Fos protein expression in the spinal dorsal horn. The purpose of the present study was to examine whether the sensitivities of muscle thin-fiber sensory receptors to mechanical, chemical, and thermal stimuli were altered after repetitive ECC in a rat model of DOMS. ECC was caused in the animals by electrical stimulation of the common peroneal nerve innervating the extensor digitorum longus muscle (EDL) while the muscle was being stretched. Activities of single thin-fiber receptors (sensitive to pressure but insensitive to stretch, with conduction velocity slower than 2.0 m/s) were recorded from muscle (EDL)–nerve preparations in vitro 2 days after ECC when mechanical hyperalgesia was at its peak. The mechanical threshold of thin-fiber receptors was found to be very much lower in the ECC preparations than in the nontreated control (CTR) [median 65.4 mN (interquartile range [IQR] 46.6–122.0 mN) in the CTR preparation vs. 38.2 mN (IQR 26.8–55.8 mN) in the ECC, P < 0.001]. In addition, the total number of evoked discharges during a ramp mechanical stimulus, taken as an index of the magnitude of the mechanical response, nearly doubled in the ECC preparations compared with the CTR [24.7 spikes (IQR 14.2–37.1 spikes) in the CTR preparation vs. 54.2 spikes (IQR 24.3–89.0 spikes) in the ECC, P < 0.001]. In contrast, the numbers of discharges induced by chemical (pH 5.5, lactic acid, adenosine triphosphate, and bradykinin) and thermal (cold and heat) stimuli were not different between the two preparations. These results suggest that augmentation of the mechanical response in muscle thin-fiber sensory receptors might be related to the muscle tenderness in DOMS after ECC.

INTRODUCTION

Muscle pain is quite common, with an especially high incidence in the elderly. Its typical characteristics are muscle tenderness and movement-related pain. It often refers to remote areas and is accompanied by changed physical properties of the muscle (palpated as a firm nodule). However, its neural mechanism has not been intensively studied. So far, several animal models of muscle pain have been reported: Intramuscular injection of algogenic substances such as carrageenan (Berberich et al. 1988; Radhakrishnan et al. 2003), acidic saline (Issberner et al. 1996; Sluka et al. 2001), and tumor necrosis factor-alpha (Schafer et al. 2003) have been used to induce muscle hyperalgesia or muscle-induced cutaneous hyperalgesia. Delayed-onset muscle soreness (DOMS) may be another model.

DOMS is a quite common sequel of unaccustomed strenuous exercise, especially eccentric exercise. It usually peaks at 24–72 h and disappears within 7 days after exercise (Armstrong 1984; Graven-Nielsen and Arendt-Nielsen 2003; Newham 1988). The most characteristic symptoms of DOMS are muscle tenderness and movement-induced pain in the exercised muscle; both are types of mechanical hyperalgesia. There is usually no pain at rest (Graven-Nielsen and Arendt-Nielsen 2003). In spite of its high incidence, DOMS is usually subclinical because subjects recover from the soreness without any medical treatment. However, DOMS interferes with the motor performance of athletes, and there is a possibility that DOMS leads to more debilitating and chronic injury and results in chronic pain/hyperalgesia with plastic changes in the CNS.

Although the mechanism underlying DOMS remains unclear, eccentric muscular work (contraction while being stretched) is known to cause it more effectively than concentric work (Armstrong et al. 1983; Newham 1988; Pyne 1994). Eccentric exercise has been widely used in human and animal studies of DOMS, many of which have found histological (Armstrong et al. 1983; Friden and Lieber 1998; McCurry and Faulkner 1985), ultrastructural (Newham et al. 1983; Ogilvie et al. 1988), biochemical (Armstrong et al. 1983; Blais et al. 1999; Ostrowski et al. 1998), and physical (see Proske and Morgan 2001 for review) changes. Lactic acid was long considered to be responsible for DOMS, but this belief has been largely rejected because of the failure of higher levels of lactate to induce soreness in concentric exercise (Armstrong 1984; Schwane et al. 1983) or in McArdle’s disease (Kazemi-Esfarjani et al. 2002), a different time course for soreness and the blood lactate increase after exercise (Schwane et al. 1983), and other findings. Although there has been dispute as to whether an inflammatory process is involved in the underlying mechanisms of DOMS (see Smith 1991 for review), the possibility cannot be excluded that some inflammatory mediators or cytoplasmic components, released from muscle cells as a result of...
the micro-injury after eccentric contraction, may sensitize the nociceptors to mechanical stimulation. There have been reports of elevated muscle interstitial adenosine 5'-triphosphate (ATP) (Li et al. 2003) and venous bradykinin (BK) (Blais et al. 1999; Stebbins et al. 1990) during contraction, but these elevated ATP/BK levels did not last long after cessation of the exercise and thus are unlikely to have been sensitizing agents in DOMS. Higher local temperature was found in muscle exercised eccentrically than in muscle undergoing concentric contractions (Nadel et al. 1972). It is possible that this higher temperature could sensitize muscle nociceptors, but again this change did not last long after exercise.

To date, mechanical hyperalgesia in exercised muscle has not been shown in animal models. In a previous paper, we demonstrated the existence of muscle tenderness by behavioral pain tests and c-Fos protein expression in the spinal dorsal horn after ECC in rats (Taguchi et al. 2005): The mechanical threshold in muscle measured by Randall–Selitto test decreased after ECC, whereas that in the skin measured by von Frey hair remained constant throughout the experimental period. Moreover, compression of the muscle 2 days after ECC caused a significant increase in the number of c-Fos-ir neurons in the superficial dorsal horn of the spinal cord at L4.

It has been generally accepted that muscle pain is mediated by activation of thin-fiber (group III (A-δ) and IV (C)) receptors, although a possible contribution of large fiber afferents to soreness after ECC has recently been suggested in human experiments (Weerakkody et al. 2001, 2003). In past studies, a majority of muscle thin-fiber receptors responded in vivo to mechanical stimulation (Franz and Mense 1975; Kumazawa and Mizumura 1976, 1977) and also to a variety of algic chemicals, such as low pH (Hoheisel et al. 2004), ATP (Hanna et al. 2004; Reinohl et al. 2003), BK (Franz and Mense 1975; Kaufman et al. 1982; Kumazawa and Mizumura 1977; Mense and Meyer 1988), and capsaicin (Hoheisel et al. 2004; Kaufman et al. 1982). Some thin-fiber receptors responded to noxious thermal stimuli (Hertel et al. 1976), and in the case of canine muscle thin-fiber afferents >90% responded to noxious heat as well as mechanical and algic chemical stimuli, thus identified as polymodal receptors (Kumazawa and Mizumura 1977).

Because little is known about changes in thin-fiber nociceptors in DOMS, we examined in this study whether the sensitivities of muscle thin-fiber sensory receptors to mechanical, chemical, and thermal stimuli were altered in muscle that was rendered hyperalgesic to mechanical stimulus by eccentric contraction. Single nerve fiber activities were recorded in vitro in rat muscle–nerve preparations, in which various kinds of noxious stimuli could be applied to the receptive fields without interfering with the integrity of the muscle, and the temperature and concentrations of test solutions could be well controlled.

METHODS

Animals

Fifty male Sprague–Dawley rats (SLC, Shizuoka Ken, Japan) weighing 335–440 g (10–13 wk) were used in this study. Twenty-five of them received eccentric contraction (ECC, described below) 2 days before single-fiber recording (ECC group), and the rest served as the control (CTR group). The animals were kept one to three per cage under a 12-h light/dark cycle (light between 0600 and 1800 h or 0700 and 1900 h) in an air-conditioned room (22–24°C). Food and water were available without restriction. All experimental procedures were approved by the Animal Care Committee, Nagoya University.

Exercise protocol

The animals receiving ECC (ECC group) were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally [ip] administered). Rectal temperature was kept in the physiological range (37–38°C) with a heating pad during the exercise period. The exercise protocol was the same as described previously (Taguchi et al. 2005). Briefly, a pair of needle electrodes, which were insulated except for the tips, were transcutaneously inserted near the common peroneal nerve that innervates the extensor digitorum longus (EDL) muscle, and correct location of the needles was ensured by the dorsiflexion of ankle joint and extension of toes on electrical stimulation of the common peroneal nerve. Repetitive contraction of the EDL muscle was induced by electrical stimuli applied to the common peroneal nerve. Parameters of electrical stimulation were as follows: Current magnitude of three times the twitch threshold, frequency of 50 Hz with pulse width of 1 ms, and stimulus period of 1 s. The foot of the same side was plantar-flexed with a servomotor to stretch the EDL muscle in synchrony with electrical stimulation of the nerve over a 1-s period and then returned to the starting position over a 3-s period. This pattern was repeated every 4 s for a total of 500 repetitions (thus overall exercise period was about 33 min).

Electrophysiology

The EDL muscle, nearly 40 mm in length, about 3–4 mm in thickness, and about 200 mg in weight, was carefully excised with the common peroneal nerve attached under pentobarbital anesthesia (50 mg/kg, ip) 2 days after ECC. The EDL muscle obtained from animals with no treatment served as the control (CTR). In the CTR group (n = 25 rats), the EDL–peroneal nerve preparations were taken from the right (n = 13), left (n = 4), and both hindpaws (n = 8). In the ECC group (n = 25 rats), the preparations were taken from the exercised side (right side) only, because the contralateral side might potentially have some influence of ECC. One nerve fiber was recorded from each preparation. Animals were killed with an overdose of the anesthetic after dissection of the preparation. The isolated preparation was then placed in an organ bath [similar to the one used for the skin–nerve preparation reported by Reeh (1986)] with the proximal and distal ends of the EDL muscle pinned in the test chamber. A schematic drawing of the experimental setup is shown in Fig. 1. The preparation was maintained at 34.0 ± 0.5°C (pH 7.4) under superfusion with Krebs–Henseleit solution (Krebs solution), which contained (in mM) 110.9 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25.0 NaHCO₃, and 20.0 glucose, and the perfusate was continuously bubbled and equilibrated with a gas mixture of 95% O₂ and 5% CO₂. The common peroneal nerve was drawn through a hole to the recording chamber filled with paraffin oil and small filaments of the nerve were repeatedly split with sharpened watchmaker forceps and a thin needle until a single-unit activity could be recorded. Action potentials were amplified, filtered, and displayed on an oscilloscope and continuously recorded on magnetic tapes (for off-line analysis). They were analyzed on a computer with an analog–digital converter and a SPIKE/SPIDI software package (C. Forster, University of Erlangen-Nurnberg, Germany).

Muscle thin-fiber sensory receptors that fulfilled the following criteria were used in this study: 1) sensitive to mechanical stimulation from probing with a glass rod, 2) no intensity-dependent increase in the discharge rate while the muscle was stretched by a length of a few millimeters, 3) conduction velocity slower than 2.0 m/s. The conduction velocity of the fibers was calculated from the distance and conduction latency between the recording and the stimulating elec-
solution (pH 5.5), 4 mechanical stimulator, stronger than the threshold and that consistently induced a response. was marked with the von Frey hair that was one or two grades a mechanical response was taken as the threshold. The receptive field of the fiber. The strength of the weakest filament that caused in diameter) for a detailed search. A series of the hand-made von Frey was investigated with a large (6 mm in diameter) and a small (1 mm in

...trodes, the latter being placed on the receptive field. Electrical stimulation ±50 V with pulse duration of 100–500 μs was used.

The distribution (the size and location) of the receptive fields of a fiber was investigated with a large (6 mm in diameter) and a small (1 mm in diameter) glass rod, then followed by von Frey hairs (VFHs, 0.5 mm in diameter) for a detailed search. A series of the hand-made von Frey filaments (1.8–66.8 mN) was used to determine the mechanical threshold of the fiber. The strength of the weakest filament that caused a mechanical response was taken as the threshold. The receptive field was marked with the von Frey hair that was one or two grades stronger than the threshold and that consistently induced a response.

Once a fiber was identified, mechanical, chemical, and thermal stimulations were applied to the receptive field mostly in the following order: 1) a ramp mechanical stimulation with a servo-controlled mechanical stimulator, 2) Krebs solution (pH 7.4), 3) low pH Krebs solution (pH 5.5), 4) 25 mM lactic acid (pH 7.4), 5) 25 mM lactic acid (pH 5.5), 6) ATP 1 or 10 mM, 7) BK 1 or 10 μM, 8) cold (cooled from 34 to 10°C), 9) heat (heated from 34 to 50°C). Intervals between stimuli varied: when a stimulus induced no excitation, then the interval before the next stimulus was set about 5 min, and when the previous stimulus induced an excitation, more than 10 min elapsed after the end of the response before a new stimulus was started.

Spontaneous activity of a fiber was calculated during the control period for 60 s immediately before the ramp mechanical stimulation with the mechanical stimulator described below.

Mechanical stimulation

For quantitative analysis of the mechanical response of muscle thin-fiber receptors, mechanical stimulus was applied by a mechanical stimulator with feedback regulation of the force (manufactured by S. Aizawa, Goto College of Medical Arts and Science, Tokyo, Japan). The stimulator had a plastic, cylindrical probe with a flat circular tip (tip size 2.28 mm²). A ramp mechanical stimulus, linearly increasing from 0 to 196 mN in 10 s, was applied to the most sensitive point of the identified receptive field. The mechanical threshold was defined as the intensity that induced a discharge that exceeded the mean frequency + 2 SD of spontaneous discharges during the control period (60 s, Fig. 2), when there were two or more consecutive discharges exceeding this level.

Chemical and thermal stimulations

After the mechanical stimulation, chemical solutions and hot/cold Krebs solutions (described below) were locally superfused to the mechanically sensitive receptive fields through a metal tube (3 mm in diameter, Fig. 1), the opening of which was placed as near to the receptive fields as possible to minimize the dilution of the solution while leaving a space for the solution to flow out. The speed of superfusion was about 5 ml/30 s. A thermocouple was positioned at the tip of the metal tube so that the temperature on the receptive field could be continuously monitored. With this method all fibers examined showed an increased discharge rate in response to hypertonic Krebs solution (containing 734 mM Na; data not shown), suggesting that chemical stimulants had access to the sensory receptors.

To examine the effect of acid (low pH) or lactic acid on the response of thin-fiber sensory receptors, three kinds of solution were applied. Krebs solution with low pH (pH 5.5) was made by adding phosphate buffer (mixing ratio, 0.5:10.0 0.1 M NaHPO₄ · 2H₂O:0.1 M NaH₂PO₄ · 12H₂O) to Krebs solution without NaHCO₃ (mixing ratio, 1:9 phosphate buffer:Krebs solution without NaHCO₃). Lactic acid solutions (25 mM) with different pHs (pH 7.4 and pH 5.5, respectively) were also used. Lactic acid (L-6661, Sigma, St. Louis, MO) was dissolved in Krebs solution, and its pH was finally adjusted by adding a small amount of either 1 N NaOH or HCl solution. Low pH Krebs and lactic acid solutions were applied on the receptive fields for 30 and 60 s, respectively, without bubbling with a gas mixture of 95% O₂-5% CO₂. The application period was set longer for lactic acid because preliminary experiments with 30-s application did not give a clear result.

ATP (Sigma, 1 or 10 mM, pH 7.35 and 6.3, respectively) and BK (Peptide Institute, Osaka, Japan, 1 or 10 μM) were dissolved in Krebs solution, and applied for 30 and 60 s, respectively. The application period was set longer for BK because a longer latency was expected based on previous results (Kumazawa and Mizumura 1977). All the chemical solutions were prewarmed with a heat exchanger set around the tubing (Fig. 1) so that the temperature on the surface of the muscle could be maintained at 34.0 ± 0.5°C when they were applied.

FIG. 1. Experimental setup. Isolated EDL–peroneal nerve preparation was placed in the test chamber with the proximal and distal ends of the muscle pinned to the base of the chamber covered with silicon. Preparation was maintained at 34 ± 0.5°C (pH 7.4) under superfusion with warmed Krebs–Henseleit solution. Single-fiber activities were recorded in the recording chamber with the dissection method. Mechanical stimulus was applied through a servo-controlled mechanical stimulator; chemical and thermal stimuli were applied by a superfusion of chemicals or hot/cold Krebs solution through a tube. Temperatures of chemicals were maintained by a heat exchanger set around the tubing.

FIG. 2. Sample recording of the mechanical response of the muscle thin-fiber sensory receptor. Abscissas (top and bottom): time in seconds. Ordinates (top and bottom): instantaneous frequency of discharges (Hz) and force recording applied by a servo-controlled mechanical stimulator to the receptive field of the fiber, respectively. Each action potential is shown as one dot. Spike shape is shown as an inset in the graph. This fiber was taken from the eccentric muscular contraction (ECC) preparation and had a low rate of spontaneous activity. When a ramp mechanical stimulus, linearly increasing from 0 to 196 mN in 10 s, was applied, an intensity-dependent mechanical response was observed. Mechanical threshold was defined as the intensity that induced the first discharge (open dot) of 2 consecutive discharges exceeding the mean of spontaneous discharges during the control period (marked with a fine broken line) by 2 SDs (marked with a coarse broken line). In this case the mechanical threshold thus measured was 57.8 mN.
Thermal stimulation was performed by superfusing cold or hot Krebs solutions to the receptive field through the same tube that was used to apply chemical solutions. Krebs solution was precooled or prewarmed so that the temperature of the receptive field of the muscle was cooled or warmed to about 10 or 50°C after superfusion for 60 or 30 s, respectively. The temperature of the solution was measured with the thermocouple that was positioned at the tip of the metal tube for superfusion.

At the beginning of the chemical stimulation, Krebs solution at normal temperature (34.0°C and pH 7.4), was applied for 30 s to check whether local superfusion of any fluid itself acted as mechanical stimulus because the mechanical threshold of the fibers was relatively low in most cases. The thin-fiber receptors that showed excitation to local application of Krebs solution showed much larger, clear responses to other chemical stimuli; there were no cases in which it was difficult to judge the existence of a response.

When a fiber fulfilled the following criteria, it was defined to be sensitive to a stimulus: 1) net increase of discharge rate during the stimulus period or after 60 s (because of the late appearance of the increased discharge in some fibers (mainly with ATP and BK) ≥0.1 impulses (imp)/s, 2) instantaneous discharge rate of two consecutive discharges exceeded the mean + 2 SD of the spontaneous discharge rate observed during the control period before stimulus (60 s).

**Statistical analyses**

Results were expressed as median and interquartile range (IQR). Electrophysiological data were compared by Mann–Whitney U test (comparison between CTR and ECC). The incidence of responding fibers was compared between groups with the use of Fisher’s exact probability test. Correlation was analyzed with either Pearson’s or Spearman’s correlation coefficient, as appropriate. P < 0.05 was considered significant.

**RESULTS**

**General**

A total of 58 fibers were identified and recorded (33 from CTR and 25 from ECC). Samples of spike forms and response patterns to mechanical, chemical, and thermal stimuli are shown in Fig. 3. Because the same thin-fiber receptors could not be studied both in the control condition and 2 days after ECC, and we could not predict which receptor subtypes have changed their sensitivity after ECC, recorded fibers are not classified in the following sections.

Conduction velocities of the fibers ranged between 0.22 and 1.86 m/s, and the majority of them were slower than 1 m/s; it is thus highly likely that they were C-fibers (Fig. 4A). Median conduction velocities were 0.51 m/s (IQR; 0.42–0.85 m/s) in CTR and 0.56 m/s (IQR; 0.46–1.01 m/s) in ECC group, which was not statistically different (P = 0.551, Mann–Whitney U test).

As seen in the sample recordings from CTR and ECC preparations (Fig. 3), spontaneous activities were low: there were no spontaneous discharges in eight fibers in the CTR group and 13 in the ECC group, and spontaneous discharges of ≤0.1 imp/s in eight fibers in the CTR group and four in the ECC group. Spontaneous activities of the fibers were not different between the two groups [median 0.12 imp/s (IQR; 0.01–0.17 imp/s) in CTR and 0 imp/s (IQR; 0–0.20 imp/s) in ECC group; Fig. 4B]. Ongoing activities sometimes appeared...
after some stimulus and disappeared after another stimulus: in the sample shown in Fig. 3B, ongoing discharge appeared after a vigorous response to a mechanical stimulus, continued for a while although there was a rather long intervening resting period (about 3 h), and then disappeared after heat stimulus.

Most of the receptive fields of the thin-fiber sensory receptors we recorded were distributed on the front side (the anterior side) (Fig. 5). This was because we usually started to look for fibers on the front side. The difficulty of sparing thin nerve branches innervating the proximal and distal parts of the muscle meant that fewer receptive fields were found in these areas. The fields varied in size and shape: the receptive fields were round or oval in shape with a diameter ranging between 0.5 and a few millimeters, and sometimes were elongated to about 5 mm in length like a rope. More receptive fields tended to be found near the musculotendinous junction than in other parts.

The incidence of fibers responding to various stimuli are listed in Table 1 (for criteria of response, see METHODS). There were no differences between the CTR and ECC groups in the incidence of fibers sensitive to each stimulus. A detailed description of the response to each stimulus is given below.

### Mechanical response

Mechanical threshold measured with VFHs varied between 3.2 and 52.3 mN (median; 17.6 mN) in the thin-fiber receptors of the CTR group, and between 1.8 and 22.7 mN (median; 7.1 mN) in those of the ECC group. VFH mechanical threshold of thin-fiber receptors was significantly lower in the ECC group than in the CTR group (Mann–Whitney U test, \( P < 0.0001 \)).

To analyze the mechanical sensitivity more quantitatively, we used ramp mechanical stimulation (196 mN in 10 s). Many thin-fiber receptors in the control preparation started to respond with variable delays after initiation of ramp mechanical stimulus (sample recording in Fig. 3A) and showed a roughly intensity dependent increase in discharge rate thereafter (Fig. 6). On the other hand, the buildup of the response in the ECC preparation was steeper than that in the CTR preparation (sample in Fig. 3B, summary in Fig. 6). Some fibers reached a peak discharge rate before the stimulus intensity reached its peak and showed an adapting response while the stimulus intensity was still increasing. The majority of sensory receptors stopped firing immediately after the mechanical stimulus ended, but some showed afterdischarges that lasted 1 or 2 min. The sample fiber in Fig. 3B had exceptionally long afterdischarges. The numbers of sensory receptors with afterdischarges were four out of 33 fibers in the CTR group and five out of 25 fibers in the ECC group, which were not statistically different. Spontaneous activities of some fibers were suppressed for a while after the end of mechanical stimulus.

Mechanical threshold (see METHODS) measured by ramp mechanical stimulus of the muscle thin-fiber sensory receptors in the CTR preparations varied considerably, ranging fairly continuously between 22.3 and 183.5 mN (Fig. 7), so we did not attempt to separate them into low- and high-threshold groups. The threshold in the ECC preparations, on the other hand, was lower, <60 mN, except for five fibers that had relatively high thresholds. The average mechanical threshold in the ECC preparations was only slightly more than half that in the CTR, that is, 38.2 mN (IQR; 26.8–55.8 mN, \( n = 25 \)) in the ECC versus 65.4 mN (IQR; 46.6–122.0 mN, \( n = 33 \)) in the CTR preparation (Fig. 7A), a significant difference (Mann–Whitney \( U \) test, \( P < 0.001 \)). This low threshold in the ECC group is in accordance with the result obtained with von Frey hairs. In fact, there was a significant correlation between the thresholds

![FIG. 5. Size and distribution of the receptive fields of the muscle thin-fiber sensory receptors. Front: side facing the anterior tibial muscle. Back: opposite side. Shading shows the tendinous area. Each spot represents the receptive field of a sensory receptor. Numbers of fibers studied are shown in parentheses.](http://jn.physiology.org/)

**TABLE 1. Responders and response magnitude to various stimuli**

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>CTR (n=33)</th>
<th>ECC (n=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. units examined</td>
<td>No. units responded, %</td>
<td>Response magnitude, imp/s</td>
</tr>
<tr>
<td>No. units examined</td>
<td>No. units responded, %</td>
<td>Response magnitude, imp/s</td>
</tr>
<tr>
<td>pH 5.5 Krebs solution</td>
<td>30 (23)</td>
<td>0.12 (0.08–0.82)</td>
</tr>
<tr>
<td>Lactic acid (pH 7.4)</td>
<td>16 (19)</td>
<td>0.20 (0.15, 0.20, 0.55)</td>
</tr>
<tr>
<td>Lactic acid (pH 5.5)</td>
<td>14 (21)</td>
<td>0.22 (0.12, 0.22, 0.47)</td>
</tr>
<tr>
<td>ATP 1 mM</td>
<td>10 (10)</td>
<td>0.22 (0.12–0.47)</td>
</tr>
<tr>
<td>ATP 10 mM</td>
<td>19 (47)</td>
<td>0.32 (0.12–0.69)</td>
</tr>
<tr>
<td>Bradykinin 1 µM</td>
<td>10 (50)</td>
<td>0.43 (0.11–0.80)</td>
</tr>
<tr>
<td>Bradykinin 10 µM</td>
<td>19 (74)</td>
<td>0.82 (0.30–2.86)</td>
</tr>
<tr>
<td>Cold</td>
<td>28 (18)</td>
<td>0.97 (0.15–7.45)</td>
</tr>
<tr>
<td>Heat</td>
<td>27 (41)</td>
<td>0.97 (0.50–1.13)</td>
</tr>
<tr>
<td>No. units examined</td>
<td>No. units responded, %</td>
<td>Response magnitude, imp/s</td>
</tr>
<tr>
<td>No. units examined</td>
<td>No. units responded, %</td>
<td>Response magnitude, imp/s</td>
</tr>
<tr>
<td>pH 5.5 Krebs solution</td>
<td>22 (14)</td>
<td>0.13 (0, 0.13, 1.23)</td>
</tr>
<tr>
<td>Lactic acid (pH 7.4)</td>
<td>21 (10)</td>
<td>0.10 (0.05, 0.15)</td>
</tr>
<tr>
<td>Lactic acid (pH 5.5)</td>
<td>21 (24)</td>
<td>0.10 (−0.01–0.38)</td>
</tr>
<tr>
<td>ATP 1 mM</td>
<td>20 (50)</td>
<td>0.07 (−0.03–0.76)</td>
</tr>
<tr>
<td>ATP 10 mM</td>
<td>20 (70)</td>
<td>0.45 (0.09–2.99)</td>
</tr>
<tr>
<td>Bradykinin 1 µM</td>
<td>18 (22)</td>
<td>0.35 (0.29–0.52)</td>
</tr>
<tr>
<td>Bradykinin 10 µM</td>
<td>17 (65)</td>
<td>0.50 (0.35–0.77)</td>
</tr>
</tbody>
</table>

Values in parentheses represent percentage of fibers that responded with an increase in discharges. Response magnitude was given as median and interquartile range (IQR) of the net increase of discharge rate during a stimulation period. Zero or negative values in the response magnitude were those of receptors that responded after the end of stimulus application. In case there were fewer than four fibers, individual values instead of IQR are listed. There were no significant differences in the incidence of response or response magnitude induced by any stimulus between the CTR and ECC groups.
measured with von Frey hairs and ramp mechanical stimulus in both groups [CTR \((n = 33)\); Spearman \(r = 0.469, P = 0.006\), ECC \((n = 25)\); Spearman \(r = 0.747, P < 0.0001\)].

As would be expected from the lowered mechanical threshold, the total number of evoked discharges during a ramp mechanical stimulus (the magnitude of the mechanical response) in the ECC preparations was twice as great as in the CTR, that is, 54.2 spikes (IQR; 24.3–89.0 spikes, \(n = 25\)) in the ECC preparation versus 24.7 spikes (IQR; 14.2–37.1 spikes, \(n = 33\)) in the CTR preparation (Fig. 7B), the difference being statistically significant (Mann–Whitney \(U\) test, \(P < 0.001\)). The mechanical threshold and the total number of evoked discharges were negatively correlated both in the CTR (Pearson \(r = -0.391, P < 0.05\)) and in the ECC preparation (Pearson \(r = -0.492, P < 0.05\)).

Response to thermal applications

Heat stimulus was applied to 27 and 17 fibers in the CTR and ECC groups, respectively. Discharge usually appeared above 40°C in a typically transient response pattern (Fig. 3A), sometimes with several clusters of burst discharges (Fig. 3B). No difference was seen in the incidence of fibers sensitive to heat stimulus \(\leq 50^\circ\text{C} (P = 0.215, \text{Fisher’s exact probability test, Table 1}),\) threshold temperatures, or heat response magnitude between CTR and ECC (Fig. 8, A and B).

Cold Krebs application in 28 CTR fibers and 18 ECC fibers induced a small increase in discharge rate in a small percentage of fibers (Table 1). One exceptional fiber showed a vigorous increase in discharge rate \(>13\text{ imp/s}\). The response magnitude during cold application was not different between the two groups (Table 1), nor was the threshold temperature to cold stimulus \([10.0^\circ\text{C} (\text{IQR}; 8.4–21.6^\circ\text{C}, n = 5)\) in CTR vs. \(10.1^\circ\text{C} (\text{IQR}; 9.7–14.5^\circ\text{C}, n = 4)\) in ECC, \(P = 0.730, \text{Mann–Whitney} \text{ U test}\). Spontaneous activities of some fibers (4/28 \(= 14.3\%\) in the CTR; 4/18 \(= 22.2\%\) in the ECC group) were clearly suppressed by cold stimulus (completely or partially).

Responses to low pH and lactic acid

Acidic Krebs (pH 5.5) and lactic acid (pH 5.5) solutions excited 23 and 21\% of CTR fibers, respectively (Table 1). Half of responding thin-fiber receptors showed a transient increase in discharge rate after latencies from a few seconds to 1 min, but some showed a gradual response without a transient burst of discharges. Incidences of responding fibers in ECC group...
were similarly low (Table 1). Lactic acid (25 mM) at pH 7.4 induced activation in somewhat lower percentage of thin-fiber receptors than at pH 5.5. Response magnitude to acidic Krebs and lactic acid solutions (pH 5.5 and pH 7.4) were also similar between the CTR and ECC groups (Table 1).

Responses to ATP and BK

ATP 1 mM was used only in CTR, and only one in ten thin-fiber receptors responded. ATP 10 mM activated more fibers than ATP 1 mM (Table 1). Half of responding fibers in CTR (five out of nine fibers) and ECC (six out of ten fibers) were activated with long-lasting discharges for 5–20 min after initial burst discharges that appeared with latencies from a few seconds to 1 min; the rest responded weakly with relatively long latency (samples in Fig. 3, A and B). Response latency of fibers responding to ATP was 10.6 s (IQR; 5.3–32.6 s, n = 9) in CTR and 34.0 s (IQR; 7.5–52.3 s, n = 10) in ECC. Response duration of fibers responding to ATP was 479.1 s (IQR; 242.3–740.5 s, n = 9) in CTR and 577.7 s (IQR; 108.7–1,197 s, n = 10) in ECC. There was no difference in either parameter between the groups. Despite the high concentration used, the response magnitude to ATP 10 mM in both discharge rate during ATP application and net evoked discharge numbers during application and the subsequent 60 s, were rather small except for one fiber and were not statistically different between CTR and ECC (Fig. 9, A and B).

BK 1 μM was used only in CTR, and half (five out of ten) of thin-fiber receptors responded. BK 10 μM activated similar percentages of fibers in CTR and ECC groups (Table 1). In CTR, the response magnitude (mean discharge rate during BK application period) to BK 10 μM was significantly larger than that to BK 1 μM (Table 1, P < 0.05, Mann–Whitney U test). In both groups a variety of response patterns to BK were observed. Some fibers showed slowly rising and slowly decaying response patterns (Fig. 3, A and B), whereas others increased their discharge rate only transiently and some responded vigorously with intermittent burst discharges. All fibers that were sensitive to ATP (n = 9 in CTR, n = 10 in ECC) responded to BK. The response latency of the BK-sensitive fibers was 10.0 s (IQR; 4.2–35.1 s, n = 14) in CTR and 15.9 s (IQR; 7.8–52.3 s, n = 14) in ECC (no significant difference). Response duration of these fibers was 289.8 s (IQR; 90.4–553.3 s, n = 14) in CTR and 201.7 s (IQR; 126.3–466.6 s, n = 14) in ECC (again, no significant difference). Response magnitude to BK 10 μM varied among fibers both in CTR and ECC groups (Fig. 9, C and D), but the distribution patterns were similar. They were not different between CTR and ECC.

DISCUSSION

Thin-fiber receptor recording in muscle–nerve preparation in vitro

The present experiments were done in vitro on excised EDL muscle–nerve preparations. C-fiber recording in vitro was previously done by Ge and Khalsa (2003) in the gracilis muscle, which has a thickness about one third that of the EDL muscle. They reported that all afferents were initially silent, but they all began to have a low-level spontaneous discharge that averaged about 0.3 Hz during interstimulus intervals. These spontaneous activities were similar to other reports with in vivo preparations (Berberich et al. 1988; Kumazawa and Mizumura 1977). In our preparation spontaneous activities in the CTR group were also low, mostly within a range of 0–0.2 imp/s. In addition, electrical stimulation of the peroneal nerve induced contraction of the EDL muscle even at the end of the experiment. These observations indicate that the preparations used in the present experiment were in good condition.

The mechanical threshold of muscle thin-fiber receptors in CTR group was 17.6 mN (median) with VFH and 65.4 mN (median) with ramp mechanical stimulus. These values were about the half those of the cutaneous thin fiber receptors measured by our group with the same instruments in vivo and in vitro (Suzuki et al. 2002; Takahashi et al. 2003). The
existence of thin-fiber receptors with low and high mechanical threshold in the muscle has been reported (Mense and Meyer 1985). However, the distribution of the mechanical thresholds was continuous in the present study and no natural segregation into subgroups was observed. Thus it was impossible for us to classify receptors into low- and high-threshold groups. One of the reasons we saw no segregation into subgroups while Mense’s group did, might be that we examined mechanical sensitivity in vitro with a solid backing. Alternatively, usage of ramp mechanical stimulation with a servo-controlled mechanical stimulator might have induced adaptation of the response that modified the threshold. However, this is unlikely because there was good correlation between the thresholds measured with VFH and ramp mechanical stimuli. Five times lower threshold measured with VFH than that measured with servo-controlled mechanical stimulator is considered to be a result of the small size of its tip.

All chemical and thermal stimuli in this experiment were done by superfusing the chemical or preheated/precooled Krebs solutions onto the receptive field. BK 10 μM induced excitation in >70% fibers. This percentage was higher than that of group IV muscle afferents that was obtained by intraarterial injection of BK (Franz and Mense 1975). Together with our preliminary result that hypertonic Na+ solution excited all thin-fibers examined, this result further demonstrated that this application method can also be used for stimulating thin-fiber receptor terminals in EDL muscle, and thus this preparation is useful to investigate the response of muscle afferent fibers to chemical stimuli.

ATP 10 mM (pH 6.3) induced excitation in 47% fibers (9/19 fibers examined). We used this high concentration because mM level of ATP is released from damaged cells, and cutaneous nociceptor excitation has been reported with millimolar level of ATP (Cook and McCleskey 2002; Hamilton et al. 2001; Yajima et al. 2005). Although we examined ATP only at pH 6.3, a slightly higher sensitivity might be expected with pH 7.4 because Reinohl et al. (2003) reported that acidic ATP had weaker excitatory effects on group IV muscle afferent fibers (i.e., 7.6 mM ATP excited 60% C-fibers at pH 5.5 vs. about 80% at pH 7.4). Hanna and Kaufman (2004) reported that three out of 18 group III and seven out of nine group IV afferents in cats were excited by a P2X3 agonist, α,β-methylene ATP (intraarterial injection), suggesting an involvement of P2X3 receptor in thin-fiber excitation by ATP.

The percentage of fibers that responded to acid and lactic acid was smaller (nearly 10–20%) than we expected (Table 1) based on previous reports: Acidic phosphate buffer (pH 6) directly injected into receptive fields of rat gastrocnemius muscle excited 56.0% of group IV units (Hoheisel et al. 2004), and 24 mM lactic acid (1–4 ml) administered intradermally caused activation of 13 out of 20 group III afferents of cat gastrocnemius muscle (Sinoway et al. 1993). The discrepancy between our results and that of others’ may have arisen from the application method for the chemicals and from the muscle used. The magnitude of the response to acid and lactic acids, given by a net increase in mean discharge rate during the application period, was not different between CTR and ECC. It has been shown that elevated lactic acid levels return to preexercise levels within hours after exercise. In addition, blood lactic acid was not elevated by downhill running (eccentrically biased exercise), although this kind of exercise caused much more soreness than concentric exercise (Schwane et al. 1983). These past findings together with the present results suggest that low pH and lactic acid are not factors causing DOMS. However, we have not examined the effects of acidic or lactic acid solution to mechanical sensitivity, and thus the possibility remains that these solutions sensitize thin-fiber receptors to mechanical stimulus as reported in the skin (Steen et al. 1992).

About 40% of the fibers in the CTR group responded to heat stimulus ≥50°C, and were thus considered to be of the polymodal receptor type. This percentage of polymodal receptors is low in comparison with a previous report on C- and A-delta fibers in canine gastrocnemius-soleus muscle (Kumazawa and Mizumura 1977). This difference might be a result of the different species, muscles, or stimulation methods used.

**Effects of ECC**

Spontaneous activities of the thin-fibers in our present experiment were low in the ECC group during the 60-s control period just before the mechanical stimulus, and were not different from those of the CTR group. This observation is compatible with our everyday experience that spontaneous pain does not usually exist in DOMS (Graven-Nielsen and Arendt-Nielsen 2003).

The major finding of this study is that ECC in rats significantly lowered the mechanical threshold of the muscle thin-fiber sensory receptors and significantly increased the magnitude of the response during a ramp mechanical stimulus. This agrees well with our previous finding that rats became hyperalgesic to mechanical stimulus 2 days after ECC (Taguchi et al. 2005). Notably, other sensitivities were not modified after ECC. The absence of difference in sensitivities to ATP and BK between the two groups is not because the response had already reached its maximum in the CTR condition with high concentrations of these substances: Even with these high concentrations, the response magnitude was still not high (0.5–0.6 imp/s for ATP and 1.2–1.3 imp/s for BK). Thus we conclude that the responses to chemical stimuli were not influenced by ECC. Several possible mechanisms for the increased sensitivity to mechanical stimulus are considered.

First, mechanical sensitivities could have been facilitated by some chemical mediators existing in the muscle after ECC. Treatment with low pH was found to cause a significant and lasting decrease of the mechanical thresholds in almost all C-fibers in rat skin–nerve preparations in vitro (Steen et al. 1992). BK also lowered the mechanical threshold of muscle nociceptors in vivo (Mense and Meyer 1988) and sensitized visceral nociceptors to mechanical stimulus in vitro (Koda and Mizumura 2002). In addition, prostaglandin E2, histamine (Koda and Mizumura 2002), and ATP (Page et al. 2000) are known to sensitize nociceptor responses to mechanical stimulus. All these substances also sensitize nociceptors to heat (Koda et al. 1996; Kress and Guenther 1999; Mizumura et al. 1993; Petho et al. 2001; Yajima et al. 2005), although the concentration needed to sensitize sensory receptors to mechanical stimulus is higher than that needed to sensitize them to heat (Koda et al. 2002). This result might suggest that the above substances are unlikely to be responsible for the sensitization to mechanical stimulus in the ECC group because the heat sensitivities were not altered in the present experiment. In addi-
tion, elevation of ATP (Li et al. 2003) and BK (Blais et al. 1999) concentrations after exercise reaches a peak within a half day; therefore the possibility would seem to be low that these substances are sensitizers involved in DOMS. There might be another or yet unknown substance(s) that sensitize(s) nociceptors specifically to mechanical stimulus.

Second, changes in mechanical properties of the exercised muscle might be involved in the mechanical hyperalgesia after eccentric contraction: Exercised legs are usually kept in a slightly flexed position (Jones et al. 1987), increased passive tension was reported in humans and cats (Whitehead et al. 2001), and increased stiffness of the muscle was frequently observed after ECC in rabbits (Itoh and Kawakita 2002). In addition, swelling of the muscles after ECC was found in humans (Friden et al. 1988; Whitehead et al. 2001) and rats (preliminary results from our laboratory). Changes in the muscle stiffness and edema of the muscle may affect the response properties of muscle thin-fiber sensory receptors to mechanical stimulus. This point needs to be studied further.

Finally, the augmented mechanical response might have resulted from an increased number of mechano-transducer/ion channels, which have not yet been identified, at the terminal of muscle thin-fiber receptors after eccentric exercise. Clarification of this point must await identification of mechano-transduction channels.

In conclusion, only the mechanical sensitivity (response threshold and magnitude) of the muscle thin-fiber sensory receptors was facilitated after eccentric contraction. No other sensitivities were facilitated. These results suggest that augmentation of the mechanical response in muscle thin-fiber sensory receptors might be related to the tenderness in DOMS after ECC. Further experiments to elucidate the mechanisms of mechanical hyperalgesia in DOMS are needed.

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