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Effect of protons on the mechanical response of rat muscle nociceptive fibers and neurons in vitro

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ABSTRACT

Strong exercise makes muscle acidic, and painful. The stimulus that activates muscle nociceptors in such instance may be protons. Reportedly, however, not many afferents are excited by protons alone. We, therefore, posited that protons sensitize muscular nociceptors to mechanical stimuli. We examined effects of protons on mechanical sensitivity of muscle nociceptors by single-fiber recording from rat muscle-nerve preparations in vitro and by whole cell patch-clamp recording of mechanically activated (MA) currents from cultured rat dorsal root ganglion neurons. We recorded 38 Aδ- and C-fibers. Their response magnitude was increased by both pH 6.2 and pH 6.8; in addition the mechanical threshold was lowered by pH 6.2. Decrease in the threshold by pH6.2 was also observed in MA currents. Presently observed sensitization by protons could be involved in several types of ischemic muscle pain, and may also be involved in cardiovascular and respiratory controls during exercise.

1. Introduction

Muscle pain can be induced not only by inflammation (Ambalavanar et al., 2006; Dina et al., 2008) or neuropathic conditions (Kirillova et al., 2011), but also by conditions related to exercise in non-pathological and non-injured conditions. These are: (1) pain that occurs during exercise; (2) delayed onset muscle soreness; (3) pain from rigorous involuntary contraction, known as muscle cramps, that suddenly starts and lasts for several seconds or longer (see ref. Miles and Clarkson (1994) for review). Pain during exercise can be experienced when the oxygen demand of the tissue exceeds its supply and the muscles exercised become acidic (Miles and Clarkson, 1994). Therefore, this type of pain can be classified as ischemic pain similar to angina pectoris and pain in sickle cell anemia (Fu and Longhurst, 2009).

Noxious information from the muscle is transmitted by muscular thin-fiber afferents (Aδ- and C-fibers). Acid sensing ion channels (ASICs) (Naves and McCleskey, 2005) or transient receptor potential vanilloid 1 (TRPV1) (Tominaga et al., 1998) expressed on these afferent terminals are considered to be molecular substrates that sense pH changes in the tissue. Specifically, ASIC3 is indicated to play an important role in detecting low pH condition that occurs in muscle ischemia (Immke and McCleskey, 2001a,b; Naves and McCleskey, 2005; Xing et al., 2012). On the other hand, ischemia produced by occluding the blood supply to a limb alone does not induce pain (Macwilliam and Webster, 1923; Smith et al., 1966). This observation is supported by reports that showed a low number of muscle C-fibers excited by superfusion of acid (pH 5.5) only (Taguchi et al., 2005), and little intracellular calcium increase in cultured dorsal root ganglion (DRG) neurons with application of acid (down to pH 6.6) only (Light et al., 2008).

Recent investigation on cultured DRG neurons in vitro suggests that combination of ATP and acid, and/or lactate in the physiological range is more potent in activating DRG neurons than any of them alone (Light et al., 2008; Birdsong et al., 2010). Plenty of lactate is produced through the glycolytic pathway in a painful ischemic condition. ATP is also released from the muscle by mechanical stimulation of the muscle or contraction (Li et al., 2003; Taguchi et al., 2008). These observations suggest that excitation of muscle nociceptors, and thus pain, could be induced by the combined

Abbreviations: ASICs, acid sensing ion channels; BK, bradykinin; CAP, capsaicin; DRG, dorsal root ganglion; EDL, extensor digitorum longus; IA currents, intermediary adapting currents; IB4, isolectin B4; MA currents, mechanically activated currents; RA currents, rapidly adapting currents; SA currents, slowly adapting currents; TRPV1, transient receptor potential vanilloid 1.

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effects of substances produced by mechanical events in ischemic conditions.

It must be noted that, striking and persistent pain occurs when the occluded limb is exercised lightly (Macwilliam and Webster, 1923). Therefore, it is reasonable to speculate that protons produced during exercise alone are too weak to induce nociceptor excitation, but would "sensitize" muscular nociceptors to mechanical stimuli such as muscle contraction and movement. In a human study, the degree of local pain was not different between the injection of acidic solution at pH 5.2 infused at a speed of 20 ml/h and normal phosphate buffer set at pH 7.3 infused at a speed of 40 ml/h, significantly more pain was evoked by the injection of the acidic solution in the higher rate infusion (Frey Law et al., 2008). These results suggest that the higher rate infusion had a mechanical effect. This also support the concept that acid would sensitize muscular nociceptors to mechanical stimuli.

It is known that acid and inflammatory mediators sensitize sensory fibers to mechanical stimuli in the skin and internal organs (Steen et al., 1992; Koda and Mizumura, 2002). Inflammatory mediators are known to also sensitize muscle thin-fibers to mechanical stimuli (Rotto et al., 1990; Murase et al., 2010). Our previous patch clamp experiments showed that mechanically activated (MA) currents of small DRG neurons were facilitated by low pH (Kubo et al., 2012). However, it is not known how muscle nociceptors behave. For this it is necessary to record muscle afferent nerve activities.

In this experiment, we recorded single fiber activities from rat muscle-nerve preparations and examined whether low pH at a level that could occur during exercise sensitized nociceptors to mechanical stimulation. We also examined the activation threshold of MA currents is decreased by low pH in DRG neurons by patch clamp method to gain more insight.

Preliminary results have appeared in abstract form (Hotta et al., 2010).

2. Methods

2.1. General

The adult animals were kept one to four per cage and pups were kept with their mothers under a 12-h light/dark cycle in an air-conditioned room (22–24 °C) until experiments. Food and water were available without restriction. All experimental procedures were approved by the Animal Care Committee, Nagoya University.

2.2. Single fiber recording experiment

2.2.1. Animal

Twenty-five male Sprague-Dawley rats (SLC, Shizuoka, Japan) weighing 375–546 g were used in this study.

Methods making preparations, recording single fibers and stimulation methods are basically similar as previously reported (Taguchi et al., 2005). The extensor digitorum longus (EDL) muscle was excised with the common peroneal nerve attached under anesthesia with pentobarbital (50 mg/kg, i.p.). Animals were killed with an overdose of anesthetic after dissection of the preparation.

2.2.2. Electorophysiology

The isolated preparation was then placed in a test chamber and maintained at 34 ± 0.5 °C (pH 7.4) with superfusion of modified Krebs-Henseleit solution, which contained (in mM) NaCl 110.9, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, and glucose 20.0, and was bubbled with a 5% CO_2 + 95% O_2 gas mixture. 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 single-fiber activity could be recorded. Action potentials were amplified, filtered, and displayed on an oscilloscope and continuously recorded on a computer via an A/D converter and magnetic tapes for off-line analysis. These data were analyzed on a computer using SPIKE/SPIDI (C. Foster, University of Erlangen-Nurnberg) and Chart Pro, Spike Histogram (AD Instruments) software.

Muscle thin-fiber afferents that fulfilled the following criteria were used: (1) sensitive to mechanical stimulation from gentle probing with a glass rod; (2) no intensity-dependent increase in the discharge rate in response to muscle stretch; and (3) conduction velocity slower than 15.0 m/s. The conduction velocity of the fibers was calculated from the distance and conduction latency between the recording and stimulating electrodes, the latter being placed on the receptive field or axon. We defined fibers with conduction velocity of less than 2.0 m/s as C and less than 15.0 m/s as Aδ.

2.2.3. Mechanical stimulation

After identifying a single thin afferent fiber, mechanical stimulus (linearly increasing from 0 to 196 mN in 10 s) was applied to the receptive field utilizing a servo-controlled mechanical stimulator equipped with a rounded probe (2.3 mm^2) in the same way as in our previous study (Taguchi et al., 2005). After an interval of at least 10 min, pH 6.2 or 6.8 Krebs buffer solution (made by adding phosphate buffer without bubbling O_2 and CO_2 gas mixture) was applied to the receptive field through a tube placed near the receptive field, and the mechanical response was examined again (n = 21 and 14, respectively). The reason why we used the pH values between 7.4 and 6.2 was that these pH values have been found in skeletal muscle and cardiac muscles during exercise or ischemia in mammals without any injury or disease (i.e., physiological range) (Sinoway et al., 1989; Bangsbo et al., 1993, 1996; Pan et al., 1999; Street et al., 2001; Yagi et al., 2006; Liu et al., 2007). The speed of superfusion was about 5 ml/30 s and superfusion was stopped 30 s before application of the mechanical stimulus. The rationale for setting an interval of at least 10 min was based on a previous study which showed that an inter-trial interval of 3 min is necessary before neurons produce reproducible responses to a given load (Ge and Khalsa, 2003). The effect of pH 7.4 phosphate buffer solution was similarly examined as a control (n = 26).

2.2.4. Chemical and thermal stimulations

After examining the mechanical response, we also examined sensitivity to chemicals [10 μM bradykinin (BK), 10 mM ATP, 1 μM capsaicin (CAP)] and heat stimulus to identify receptor types. The chemical and heated Krebs solutions were locally superfused to the receptive field through a 3-mm φ metal tube. The opening was placed as close to the receptive field as possible to minimize dilution of the solution while leaving space for the solution to flow out. The speed of superfusion was about 5 ml/30 s. The fiber was defined as being sensitive to these stimuli when the change in the discharge rate induced by them fulfilled the following criteria, used in our previous study: (1) net increase in discharge rate of >0.1 Hz during the stimulus period or after 60 s; (2) instantaneous discharge rate of two consecutive discharges exceeded the mean + 2 SD of the background activity (Ge and Khalsa, 2003).

2.3. Experiment using cultured neurons

2.3.1. DRG culture preparation and mechanical stimulation

The DRG culture preparation and mechanical stimulation method were exactly the same as in our previous report (Kubo et al., 2012). Briefly, DRG neurons were removed from P2-13 rats, digested using collagenase (Sigma, St. Louis, MO) and trypsin (Invitrogen, Carlsbad, CA), mechanically triturated and plated on coverslips precoated with poly-L-lysine (Sigma) and laminin (Invitrogen). Cultures were used for patch-clamp experiments within