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Laboratory Investigation

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Neurochemical Characterization of the TRPV1-Positive Nociceptive Primary Afferents Innervating Skeletal Muscles in the Rats

Objective : Transient receptor potential vanilloid subfamily type 1 (TRPV1), a most specific marker of the nociceptive primary afferent, is expressed in peptidergic and non-pepetidergic primary afferents innervating skin and viscera. However, its expression in sensory fibers to skeletal muscle is not well known. In this study, we studied the neurochemical characteristics of TRPV1-positive primary afferents to skeletal muscles.

Methods : Sprague-Dawley rats were injected with total 20 μl of 1% fast blue (FB) into the gastrocnemius and erector spinae muscle and animals were perfused 4 days after injection. FB-positive cells were traced in the L4-L5 (for gastrocnemius muscle) and L2-L4 (for erector spinae muscle) dorsal root ganglia. The neurochemical characteristics of the muscle afferents were studied with multiple immunofluorescence with TRPV1, calcitonin gene-related peptide (CGRP) and $P2X_3$. To identify spinal neurons responding to noxious stimulus to the skeletal muscle, 10% acetic acids were injected into the gastrocnemius and erector spinae muscles and expression of phospho extracellular signal-regulated kinase (pERK) in spinal cords were identified with immunohistochemical method.

Results : TRPV1 was expressed in about 49% of muscle afferents traced from gastrocnemius and 40% of erector spinae. Sixty-five to 60% of TRPV1-positive muscles afferents also expressed CGRP. In contrast, expression of $P2X_3$ immnoreaction in TRPV1-positive muscle afferents were about 20%. TRPV1-positive primary afferents were contacted with spinal neurons expressing pERK after injection of acetic acid into the muscles.

Conclusion : It is consequently suggested that nociception from skeletal muscles are mediated by TRPV1-positive primary afferents and majority of them are also peptidergic.

KEY WORDS : Skeletal muscle · Pain · Primary afferents · TRPV1 · CGRP · Dorsal root ganglion.

INTRODUCTION

Chronic muscle pain and hyperalgesia are often related with disabilities and it is difficult to treat in clinical fields[24]. For the control of the clinical pain, understanding of the neurochemical characteristics of the primary afferents which conveys pain sense from the periphery to the spinal cord is the essential step. Until recently, however, basic studies on the pain mechanism was mostly focused on the afferents of the skin[46], and studies on afferents of the deep structures like skeletal muscle or visceral organs have been little despite of the importance in clinical applications.

Primary afferents to the skeletal muscles are responsible for the sensation and conduction of the sensory information for proprioception, nociception and also physiological and chemical changes in the skeletal muscle. Proprioception, a mechanical sensations from muscle spindle and Golgi tendon organs are conducted by large myelinated fibers, which belongs to group I and group II in anatomical classification[21,30], and their central terminals are projected to the lamina VI, V, IX and Clarke's nucleus of the spinal cord[14,20]. These sensory informations are utilized for the proprioception of the limbs and also for the motor control mechanisms. Thin myelinated fibers (group II and III) are high or low threshold mechanoreceptors and project to lamina I-II, and IV-V of the dorsal horn[15]. Nociceptive afferents are very thin myelinated (group III) and unmyelinated (group IV) fibers. The peripheral terminals of these fibers are distributed as thin free nerve endings in muscle and central terminals are projected to the lamina I-II of the dorsal horn.

In classical concept, nociceptive thin myelinated or unmyelinated fibers can be classified into peptidergic and non-peptidergic fibers in according to their excretion of peptides on

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central and peripheral terminals, and also dependence of neurotrophic factors on their survival during development. Neurochemically, peptidergic primary afferents are positive for substance P/CGRP (calcitonine gene related peptide) while non-peptidergic fibers are positive for isolectin B4 (IB4), and a purinergic receptor, $P2X_3$. Peptidergic fibers which are dependant on the nerve growth factor (NGF) in their development and plays an important role in the development of hyperalgesia induced by peripheral inflammation[6,9,26]. Non-peptidergic, IB4-positive cells also have their own physiological properties in conduction of pain[42].

TRPV1 (transient receptor potential vanilloid 1) or VR1 (vanilloid receptor 1) is the receptor of capsaicin, a pungent source of hot chili pepper. It is a nonspecific cation channel and respond to heat, hydrogen ion, and capsaicin[8]. TRPV1 is synthesized in small- and medium-sized neurons in the dorsal root ganglion (DRG) and trigeminal ganglion, transported to both centrally and peripherally and plays an essential role in the nociception in the skin and visceral organs[5,19].

The role of the TRPV1 in muscle afferents were supported by functional and morphological studies. Group IV muscle afferents were activated by either acid or capsaicin[16] and intraarterial injection of capsazepine, an antagonist of TRPV1, attenuated the cardiovascular response evoked by injection of acid into the hind limb muscle[11]. In this study, we studied the fraction of the TRPV1-positive primary afferents among the muscle afferents, and also their co-expression of CGRP, a marker of the peptidergic primary afferents and $P2X_3$, a marker for the nonpeptidergic primary afferents.

A type of intracellular transduction substance mitogen-activated protein kinase (MAPK) has been introduced into the studies on the pain mechanism. MAPK is a type of serine/threonine protein kinase, and it plays a role of converting external stimulation to intracellular reactions by the transcription and post-translational modification of target proteins[39,45]. The MAPK family consists of extracellular signal-regulated kinase (ERK), p38 MAPK, c-Jun N-terminal kinase/stress activate protein kinase (JNK/SAPK) and extracellular signal-regulated kinase 5 (ERK 5). ERK and other MAPK play a active role in the mechanism of hyperalgesia[22,23].

In this study, we also applied the pERK immunohistochemistry to label the spinal neurons activated by acute muscular pain. Spinal neurons activated with muscle afferents were demonstrated by c-Fos-immunohistochemistry but this method is not able to show the relation with primary afferents. We also tried double immunofluorescence to demonstrate the morphological relation of TRPV1-positive primary afferents and spinal neurons expressing pERK after noxious stimulation of skeletal muscles.

MATERIALS AND METHODS

Experimental animals

The animals used in these experiments were male Sprague-Dawley rats of 250-300 gm of body weight and purchased from SamTako Inc., Korea. A total of 16 rats were divided into Gastrocnemius tracing group (5), Erector spinae tracing group (5), and muscle pain by acetic acid injection group (6). The water and food were supplied to the animals ad libitum during experimental periods.

Retrograde tracing

To trace the muscle afferents to the gastrocnemius and erector spinae muscles, a retrograde tracer fast blue (FB) was injected into the muscles. Rats were anesthetized with intraperitoneal injection of the mixture of Ketamine (90 mg/Kg) and Xylazine (10 mg/Kg). After incision on the skin over gastrocnemius and deep back muscles, total 10 ml of 1% FB was divided by 10 injections for the individual muscles with a 26 Gauge needle attached to Hamilton syringe. Tracers escaped from the muscle were washed out to prevent leakage of tracers to the skin. The incision was sutured and animals were allowed to survive for 4 days.

Immunohistochemistry

At 4 days after tracer injections, the animals were re-anaesthetized with Ketamine and Xylazine and perfused with 100 ml of saline followed by 500 ml of 4% paraformaldehyde in phosphate buffer (PB, 0.1M, pH 7.4). L1- L5 dorsal root ganglions and spinal cords were removed, postfixed for 2 hrs in the fixative used for perfusion, cryoprotected in 30% sucrose in PB overnight, cut on a Cryostat at 40 μm, and the sections were collected in cold PB. For immunofluorescent staining, sections were blocked with 10% normal donkey serum (NDS, Jackson) in phosphate-buffered saline (0.01 M, pH 7.2) for 10 min and incubated overnight with the mixture of a goat anti-TRPV1 antibody (Neuromics; 1:1000 dilution) and guinea pig anti- $P2X_3$ antibody (Chemicon; 1:1000 dilution) and rabbit anti-CGRP antibody (Chemicon; 1:1000 dilution). Sections were rinsed and incubated in 2% NDS for 10 min and in fluorescein (FITC)-conjugated anti-guinea pig, Cy3-conjugated goat, and Cy5-conjugated anti-rabbit antibodies (Jackson; 1:200 dilution) for 3 hrs. After several rinses, sections were coverslipped with Vectashield (Vector) and observed on a fluorescent microscope. Gray scale images for each of Cy3,

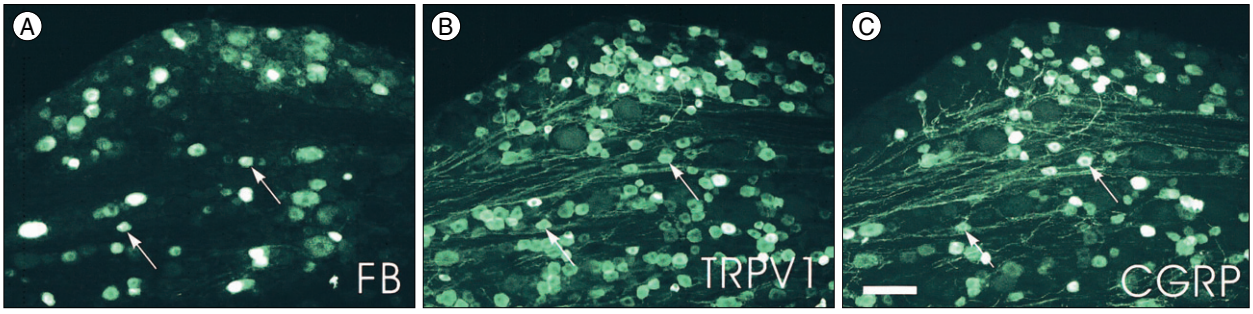

Fig. 1. Triple immunofluorescence for L5 DRG neurons traced from the gastrocnemius muscle labeled with FB (A), and stained for TRPV1 (B) and CGRP (C). FB-positive cells are variable in size, from small cells to large cells, while TRPV1 and CGRP positive cells are mostly small and medium sized. The arrows indicate the cells labeled for FB, TRPV1 and CGRP, Scale bar=100 μm.

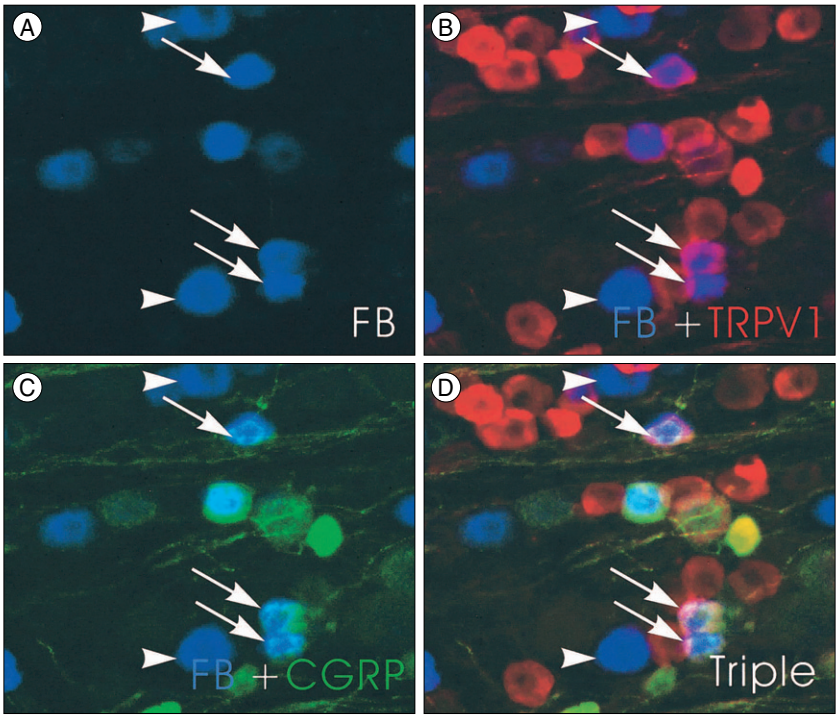

Fig. 2. Higher magnification of triple labeling for FB (blue, A), TRPV1 (red, B) and CGRP (green, C) and merged image (D) in the L5 DRG from an animal injected with FB into gastrocnemius muscle. The arrowheads indicate the FB-positive cells do not expressing TRPV1. The arrows indicate the FB-positive cells also expressing both TRPV1 and CGRP.

FITC, and Cy5 were acquired with a cooled charge-coupled device (CCD) camera (F-view, SIS Germany) and merged, and saved as TIFF files using Adobe Photoshop (version 7, Adobe). Contrast and brightness were adjusted with Adobe Photoshop and the final plate composed with CorelDraw (v. 11, Corel).

Data acquisition and analysis

For quantitative evaluation of colocalization patterns, double or triple fluorescent images immunostained for FB with TRPV1, CGRP and $P2X_3$ were acquired and 4 DRG sections of each of three rats per experimental group were chosen. In each section, one image (430×330 μm) were acquired with the same shutter speed and digital gain. The number of double- and triple-labeled cells was counted and averaged.

RESULTS

FB labled neurons from skeletal muscles

Most of FB-positive cells in the animal injected with FB in the gastrocnemius muscle were observed in L4 and L5 DRG, while those in the animal of injected into erector spinae were L1-L3 DRG. The size of the FB-labeled DRG cells was varied, from small sized cells to large cells (Fig. 1A, 2A, 3A, 4A).

Triple labeling with TRPV1 and $P2X_3$

TRPV1-labeled cells in DRG were both small and medium-sized cells. Those cells were evenly distributed in the DRG, and stronger immunoreaction was observed in the smaller cells (Fig. 1B, 2B). Among the whole FB labeled cells from the gastrocnemius muscle, 49% of DRG cells from gastrocnemius, and 40% of cells from erector spinae were TRPV1-positive (Fig. 2B, 4B), (Table 1). The ratio of the TRPV1-, CGRP-positive DRG cells and both TRPV1 and CGRP-, both TRPV1 and $P2X_3$-positive cells out of whole FB-labeled cells traced from gastrocnemius and erector spinae were summarized in Table 1 and Fig. 5.

CGRP-immunoreactive DRG cells were also varied in size. Most of CGRP-positive cells were small and medium sized, but also a few large cells were also labeled (Fig. 1C). Forty percent of cells traced from gastrocnemius, and 31% from cells from erector spinae expressed CGRP (Fig. 2C).

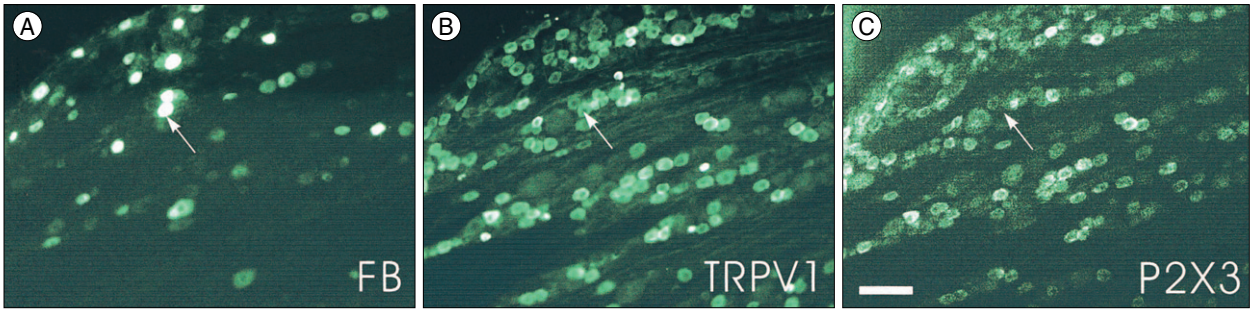

Fig. 3. Triple immunofluorescence for L5 DRG neurons traced from the gastrocnemius muscle labeled with FB (A) and stained for TRPV1 (B) and $P2X_3$ (C). Most of DRG neurons labeled for FB and TRPV1 were not stained for $P2X_3$ (arrows). Scale bar=100 μm.

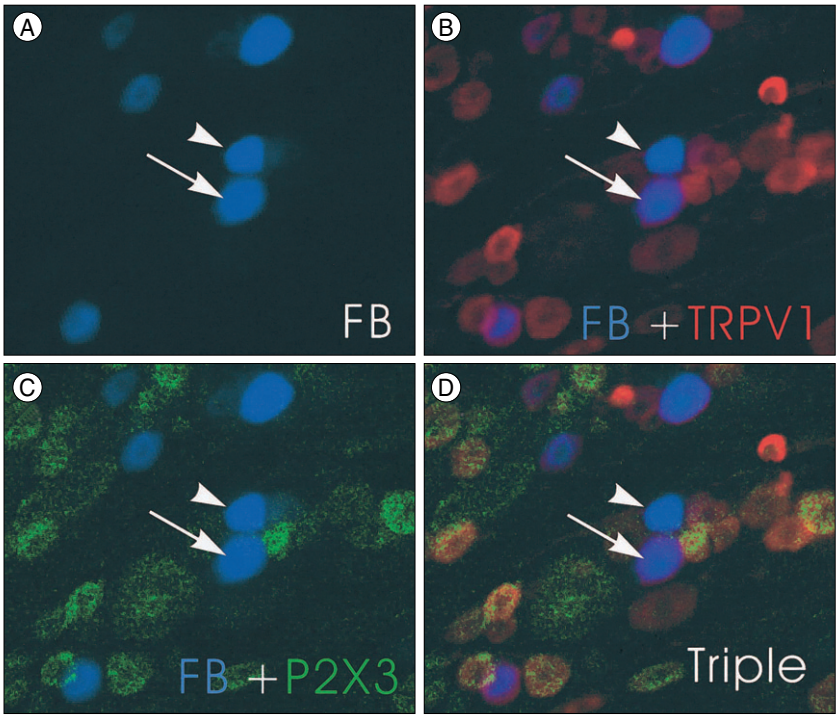

Fig. 4. Higher magnification of triple labeling for FB (A), TRPV1 (B) and $P2X_3$ (C) and merged image (D) in the L5 DRG from an animal injected with FB into gastrocnemius muscle. The arrowheads indicate the FB-positive cells do not expressing TRPV1. The arrows indicate the FB-positive cells also expressing TRPV1, but not $P2X_3$.

Table 1. Summary of the expression of TRPV1, CGRP, $P2X_3$, TRPV1+CGRP, TRPV1+$P2X_3$ among the FB-labeled DRG neurons traced from erector spinae and gastocnemius muscle. The values were expressed as mean±S.D.

Markers	Erector Spinae (%)	Gastrocnemius (%)
TRPV1	40.6±1.7	49.4±0.6
CGRP	31.4±2.7	40.3±6.3
$P2X_3$	13.8±6.9	13.7±5.2
TRPV1+CGRP	26.2±7.0	30.4±7.3
VR1+$P2X_3$	7.2±1.0	8.2±1.4

Note : TRPV1, Transient receptor potential vanilloid subfamily type 1; CGRP, calcitonin gene-related peptide, FB : fast blue

Thirty percent and 26% of DRG neurons labeled from gasrtrocnemius and erector spinae, respectively, were labeled with both FB and TRPV1 (Fig. 2D, 5), (Table 1). This means that 81% and 65% of TRPV1-positive neurons from gastrocnemius and erector spinae, respectively, were also positive for CGRP, implying that the majority of TRPV1- positive skeletal muscle afferents are peptidergic.

Triple labeling with TRPV1 and $P2X_3$

About 13% of cells traced from both gastrocnemius and erector spinae expressed $P2X_3$ (Fig. 3C, 4C). Seven percent of DRG neurons labeled from gasrtrocnemius and 8% from erector spinae were labeled with both TRPV1 and $P2X_3$ (Fig. 4D, 5), (Table 1). This means that 14% and 20% of TRPV1-positive neurons from gastrocnemius and erector spinae, respectively, also expressed $P2X_3$, implying that the expression of $P2X_3$ in the TRPV1-positive skeletal muscle afferents are much lower than that of CGRP.

Double immunoflourescnce for pERK and TRPV1 in spinal cord

In the animal injected into the gastrocnemius, pERK-positive cells were observed in the lateral part of lamina I, lateral collateral path and medial part of deep dorsal horn. In the animal injected into the gastrocnemius, pERK-positive cells were observed in the whole lamina I, medial part of lamina II and lateral collateral path (Fig. 6). In the spinal cord of the animal stimulated on gastrocnemius muscle, colocalization of TRPV1 and pERK was observed in the lateral collateral path. In double labeling with TRPV1 in the animal stimulated on erector spinae, TRPV1-positive primary afferent fibers are closely contacted with pERK-positive in the medial part of lamina I and lamina II (Fig. 7).

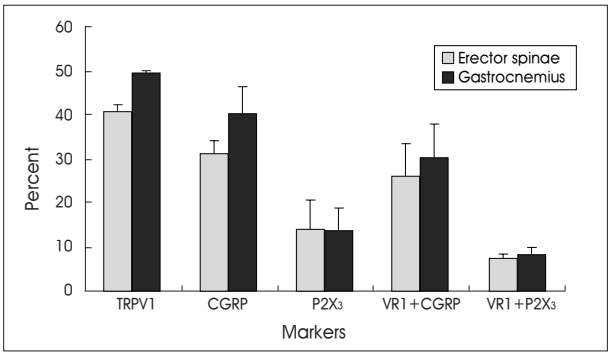

Fig. 5. Expression of TRPV1, CGRP, P2X3, TRPV1+CGRP, TRPV1+P2X3 among the FB-labeled DRG neurons traced from erector spinae and gastrocnemius muscles. The values are expressed as mean ± S.D.

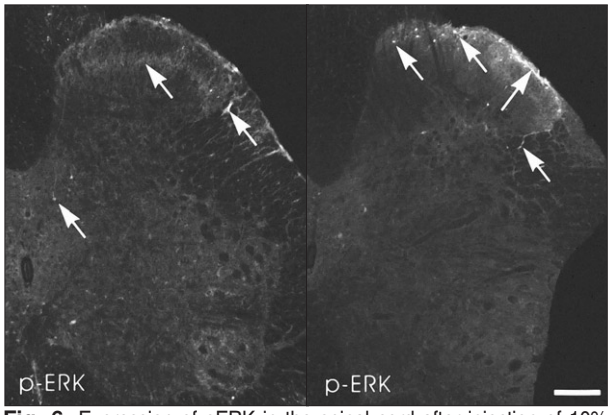

Fig. 6. Expression of pERK in the spinal cord after injection of 10% acetic acid into the gastrocnemius and erector spinae muscles. The animals were perfused 20 minutes after injections. In the animal injected into the gastrocnemius, pERK-positive cells (arrows) are observed in the lateral part of lamina I, lateral collateral path and medial part of deep dorsal horn. In the animal injected into the gastrocnemius, pERK-positive cells (arrows) are observed in the whole lamina I, medial part of lamina II and lateral collateral path. Scale bar=100 μm.

DISCUSSION

In this study, the TRPV1-positive primary afferents innervating gastrocnemius and erector spinae were traced in DRG and characterized in according to their expression of markers for the peptidergic and non-peptidergic marker. Chemical, thermal and mechanical stimuli to the skin or muscles can depolarize nociceptors. Recently, many of the proteins that transduce painful stimuli have been identified, and these proved to be receptors coupled to ionic channels in the plasma membrane. There is rapid progress in the understanding in the mechanism of the cutaneous nociceptors with recent studies on primary afferent neurons in DRG. However, the specific properties of nociceptors in deep tissues, for example, visceral organs, joints and skeletal muscles are poorly defined, and useful animal model for the muscular pain is not yet established.

An important step for the understanding of nociception was the cloning of the TRPV1, also called as vanilloid receptor 1(VR1), the receptor for capsaicin[8]. TRPV1 is a non-selective cation channel expressed by many DRG neurons, including the majority of those that contain Substance P (SP) and those that bind IB4[31]. TRPV1 is transported peripherally to both skin and viscera[43], and centrally to the spinal and trigeminal dorsal horn[13]. *In vitro,* TRPV1-expressing cells can be activated by protons (pH < 6) or heat (> 43°C) as well as by vanilloids, like capsaicin. Mice lacking the TRPV1 gene do not respond to vanilloids and have reduced sensitivity to noxious heat and low pH, indicating that TRPV1 contributes to heat and proton sensitivity *in vivo*[7,25]. TRPV1 in the skin is important for thermal nociception and inflammatory hyperalgesia and allodynia; the occurrence of thermal hyperalgesia in an experimental model of hindpaw inflammation could be prevented by knocking out TRPV1 gene[7,12] or by application of the selective TRPV1 antagonist capsazepine[44].

In our results, more than 40% of the muscle afferents expressed TRPV1 that were higher than in the skin. There are evidences of preferential expression of TRPV1 in the primary afferents innervating to the deep structures, e.g. visceral organs than to the skin afferents[17], about 60% of bladder afferents were TRPV1-positive, while 30% for the skin afferents. Our result indicates that the expression of TRPV1 in afferents to skeletal muscles is between skin and bladder afferents but it also indicates that majority of nociception from the muscles mediated by TRPV1 because visceral sensory fibers are mostly unmylelinated fibers, while considerable proportion of the muscle afferents are myelinated fibers which convey proprioceptive sense to Clarke's nucleus and dorsal column nuclei.

Higher proportion of primary afferents from gastrocnemius was TRPV1 positive than that of erector spinae. The proportion of the unmyelinated primary afferents are variable depending on the muscles[1]. In our results, however, the size distribution of the DRG neurons traced from both muscles were not different. Therefore, it is suggested that the differential expression of TRPV1 may be related with property of the pain and that the nociception from muscle of lower limb may be different in terms of the involvement of TRPV1 from that of deep back muscles which is related with low back pain.

Majority (about 60%) of the muscle TRPV1-positive muscle afferents expressed CGRP which is a marker of peptidergic primary afferents. Pain is conveyed via fine-caliber fibers originating from small neurons in the DRG, but not all small DRG neurons mediate pain. The study of pain pathways would be greatly facilitated if nociceptive afferents could be identified by a reliable anatomical marker. The most widely-accepted such marker is the neuropeptide SP, although its

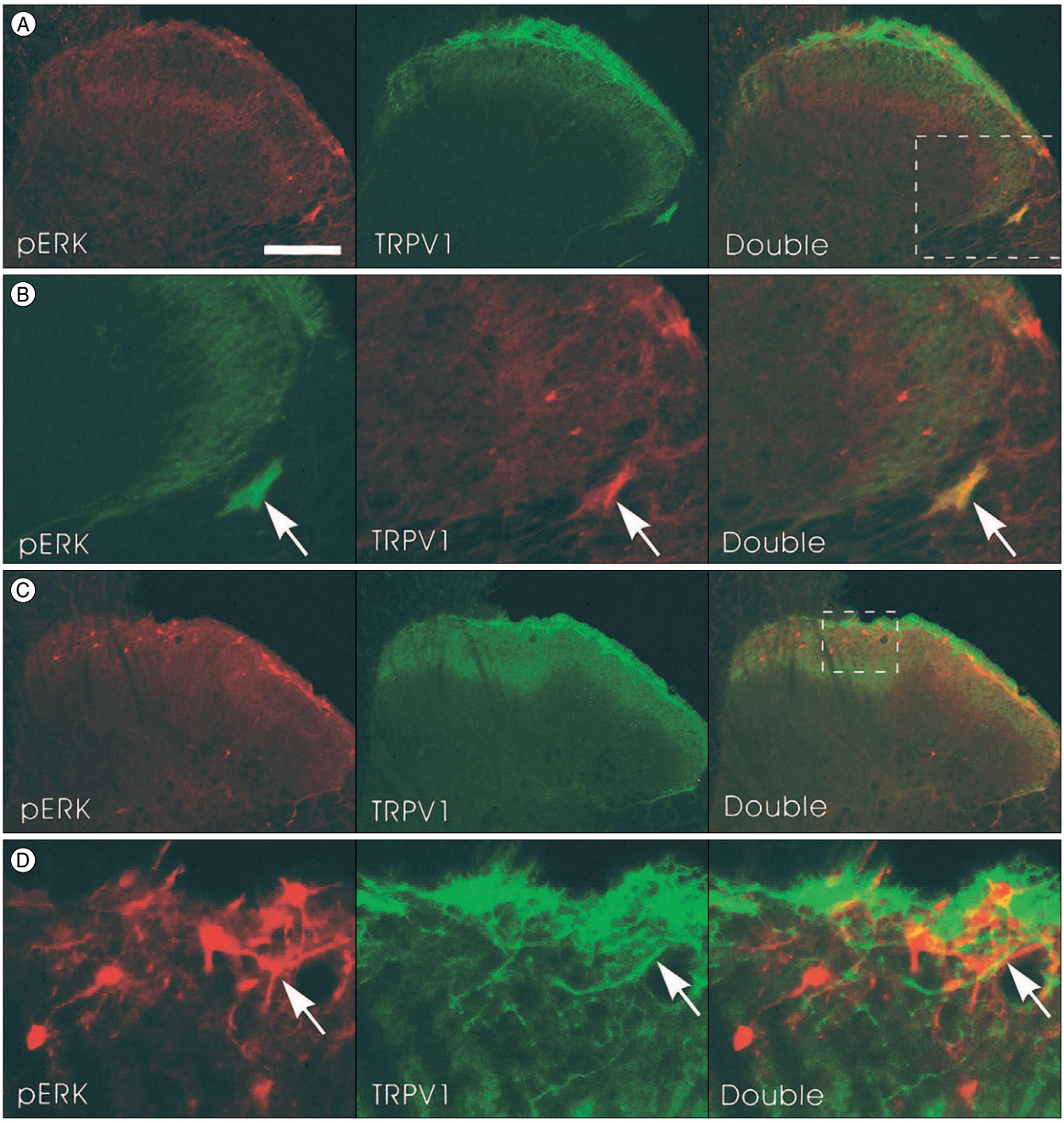

Fig. 7. Double immunofluorescence for pERK (red) and TRPV1 (green) in spinal dorsal horn of the animals injected with 10% acetic acid into the gastrocnemius and erector spinae muscles. Dotted area in A and C are magnified in B and D, respectively. In the dorsal horn of the animal stimulated on gastrocnemius muscle, colocalization of TRPV1 and pERK is observed in the lateral collateral path (arrows in B). In animal stimulated on erector spinae muscles, TRPV1-positive primary afferent fibers are closely contacted with pERK-positive neurons in the medial part of lamina I and lamina II (arrows in D). Scale bar=100 μm.

precise role in nociception remains controversial[27,28,32,47] and virtually all of the SP-positive primary afferents also express CGRP. It is well accepted that greater proportion of CGRP is expressed in the deep tissues such as muscle and joint when compared to the those of cutaneous afferents[34]. In contrary to our result, study on the trigeminal ganglion has shown that, the proportion of CGRP positive muscle afferents were 22% and those in cutaneous afferents were 26%[2]. This may indicate that the differential expression of peptide between trigeminal and spinal sensory systems.

In our results, the expression of $P2X_3$ in the TRPV1-positive muscle afferents was only 20 percents which is much less than that of CGRP. Some small DRG neurons are non-peptidergic, especially those binding the isolectin IB4 and

express $P2X_3$[37,41]. Although the distinction between SP-positive peptidergic and IB4-positive non peptidergic afferents may not be as clear as once thought[18,33,40], it is widely accepted that peptidergic primary afferents are involved in the mechanisms of central sensitization, leading to hyperalgesia and allodynia[38]. Central sensitization is thought to involve long-lasting biochemical changes, including both rapid (e.g., protein phosphorylation, including activation of the MAPK pathway) and slow processes (e.g., changes in protein expression, mediated at least in part via the transcription of immediate early genes like c-Fos).

The function of central sensitization in the skeletal muscle is still not clear and may need further studies. P2X receptors are a family of ligand-gated ion channels, activated by extracellular adenosine 5'-triphosphate (ATP). The seven subunits cloned ($P2X_{1-7}$) can assemble to form homomeric and heteromeric receptors. The $P2X_2$ and $P2X_3$ receptors are expressed in the autonomic and sensory gangllia and thought to be play a role in the nociception[10]. It was expected that $P2X_3$ might be expressed in the muscle afferent in a high proportion because in a functional study acid- and also capsaicin-sensitive muscle afferents can also be excited by ATP at neutral pH[16]. However, the expressions of $P2X_3$ in the afferents to the musculoskeletal tissues including intervertebral disc were very low, varying from 4-16%[3,4]. Our results also confirmed the low rate of expression of $P2X_3$ in both gastrocnemius and erector spinae muscles.

In this study, spinal neurons that responsive to noxious stimulus to the skeletal muscles were identified by immunohistochemical localization of pERK-positive neurons. In a study using c-Fos expression after stimulation of low back muscle, c-Fos-positive nuclei were increased in lamina I-V, but not in the lamina II[35]. It was also studied with anterograde labeling technique using Phaseolus vulgaris leucoagglutinin (PHA-L), the central terminals of the unmyelinated primary afferent fibers from gastrocnemius muscle of the guinea pig were identified in lamina I and II, sometimes in lamina III[29], and also in the rat[36]. However, pERK expression after muscle stimulation was not reported yet. With the use of pERK expression in the spinal cord, the general location of the spinal neurons responding to muscle stimulation was in support of previous studies, but it was also able to observe the close apposition of TRPV1-positive primary afferents and pERK positive neurons, indicating possible synaptic contact between two pre- and post-synaptic elements.

CONCLUSION

In this study, we studied the neurochemical characteristics of TRPV1-positive primary afferents to skeletal muscles. It is consequently suggested that nociception from skeletal muscles are mediated by TRPV1-positive primary afferents and majority of them are peptidergic.

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