

Laboratory Investigation

Dong Su Shin, M.D.¹Eun Hyun Kim, M.D.²Kwan Young Song, M.D.¹Hyun Jong Hong, M.D.¹Min Ho Kong, M.D.¹Se Jin Hwang, M.D.²

Department of Neurosurgery¹
Seoul Medical Center
Seoul, Korea

Department of Anatomy and
Cell Biology²
College of Medicine
Hanyang University
Seoul, Korea

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-peptidergic 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 μ l 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 P2X₃. 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 muscle afferents also expressed CGRP. In contrast, expression of P2X₃ immunoreaction 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 fields²⁴. 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 skin⁴⁶, 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 horn¹⁵. 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

- Received : October 29, 2007
- Accepted : January 14, 2008
- Address for reprints :
Se Jin Hwang, M.D.
Department of Anatomy and
Cell Biology, College of Medicine
Hanyang University
17 Haengdang-dong
Seongdong-gu
Seoul 133-791, Korea
Tel : +82-2-2290-8201
Fax : +82-2-2281-7841
E-mail : hwangsj@hanyang.ac.kr

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 (calcitonin gene related peptide) while non-peptidergic fibers are positive for isolectin B4 (IB4), and a purinergic receptor, P2X₃. 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 pain⁴².

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 capsaicin⁸. 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 capsaicin¹⁶ and intraarterial injection of capsazepine, an antagonist of TRPV1, attenuated the cardiovascular response evoked by injection of acid into the hind limb muscle¹¹. 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 P2X₃, 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-anesthetized 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 μ m, 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- P2X₃ 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 μ m.

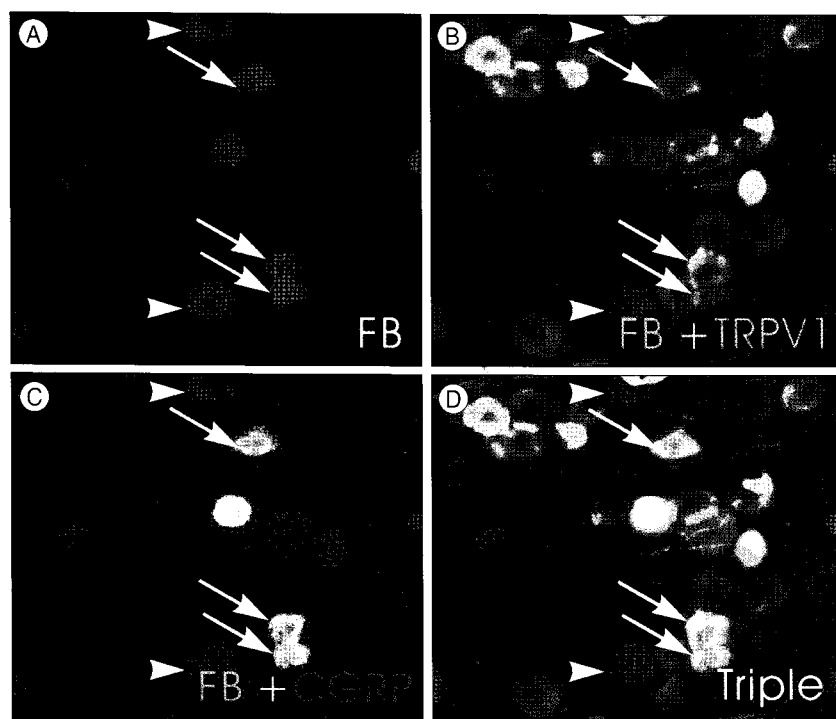


Fig. 2. Higher magnification of triple labeling for FB (blue, A), TRPV1 (red, B) and CGRP (green, C) and merged image (D) in the L5 DRG from an animal injected with FB into gastrocnemius muscle. The arrowheads indicate the FB-positive cells do not expressing TRPV1. The arrows indicate the FB-positive 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 P2X₃ were acquired and 4 DRG sections of each of three rats per experimental group were chosen. In each section, one image (430 \times 330 μ m) were

acquired with the same shutter speed and digital gain. The number of double- and triple-labeled cells was counted and averaged.

RESULTS

FB labeled 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 P2X₃

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 P2X₃-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).

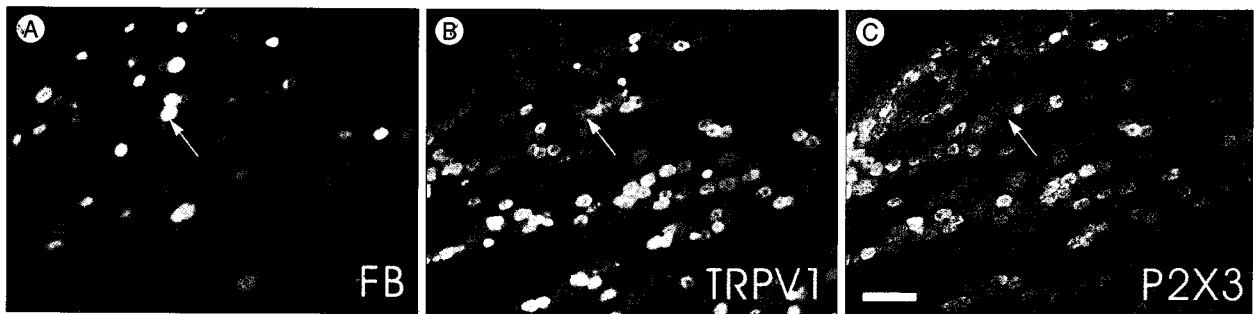


Fig. 3. Triple immunofluorescence for L5 DRG neurons traced from the gastrocnemius muscle labeled with FB (A) and stained for TRPV1 (B) and P2X₃ (C). Most of DRG neurons labeled for FB and TRPV1 were not stained for P2X₃ (arrows). Scale bar=100 μm.

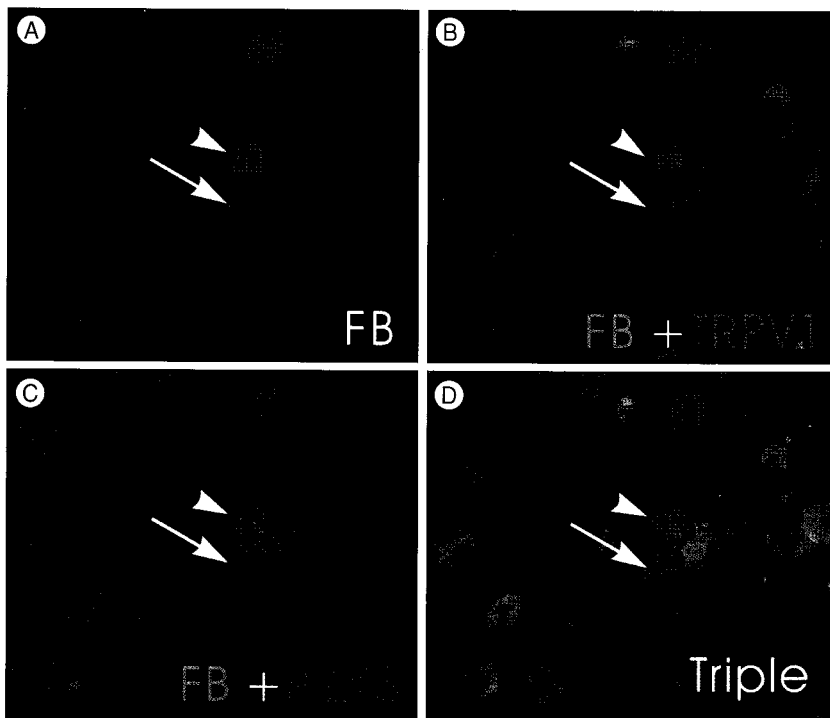


Fig. 4. Higher magnification of triple labeling for FB (A), TRPV1 (B) and P2X₃ (C) and merged image (D) in the L5 DRG from an animal injected with FB into gastrocnemius muscle. The arrowheads indicate the FB-positive cells do not expressing TRPV1. The arrows indicate the FB-positive cells also expressing TRPV1, but not P2X₃.

Table 1. Summary of the expression of TRPV1, CGRP, P2X₃, TRPV1+CGRP, TRPV1+P2X₃ among the FB-labeled DRG neurons traced from erector spinae and gastrocnemius 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 gastrocnemius 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 P2X₃

About 13% of cells traced from both gastrocnemius and erector spinae expressed P2X₃ (Fig. 3C, 4C). Seven percent of DRG neurons labeled from gastrocnemius and 8% from erector spinae were labeled with both TRPV1 and P2X₃ (Fig. 4D, 5), (Table 1). This means that 14% and 20% of TRPV1-positive neurons from gastrocnemius and erector spinae, respectively, also expressed P2X₃, implying that the expression of P2X₃ in the TRPV1-positive skeletal muscle afferents are much lower than that of CGRP.

Double immunofluorescence 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).

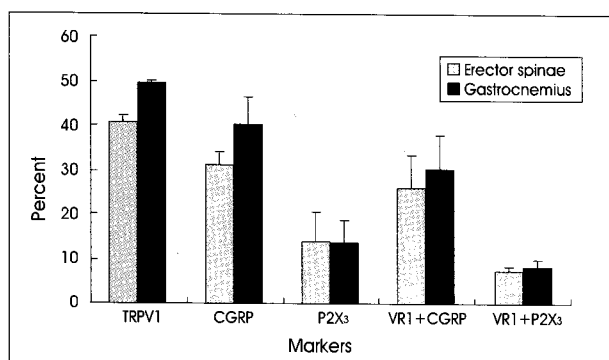


Fig. 5. Expression of TRPV1, CGRP, P2X₃, TRPV1+CGRP, TRPV1+P2X₃ among the FB-labeled DRG neurons traced from erector spinae and gastrocnemius muscles. The values are expressed as mean \pm S.D.

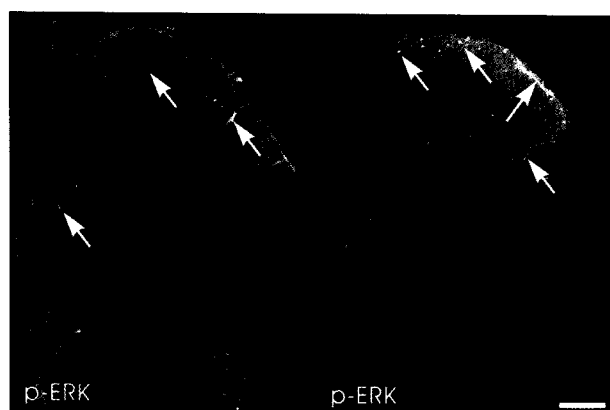


Fig. 6. Expression of pERK in the spinal cord after injection of 10% acetic acid into the gastrocnemius and erector spinae muscles. The animals were perfused 20 minutes after injections. In the animal injected into the gastrocnemius, pERK-positive cells (arrows) are observed in the lateral part of lamina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

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 capsaicin⁸. 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 IB4³¹. TRPV1 is transported peripherally to both skin and viscera⁴³, and centrally to the spinal and trigeminal dorsal horn¹³. *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 capsazepine⁴⁴.

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 afferents¹⁷, 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 unmyelinated 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 muscles¹. 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

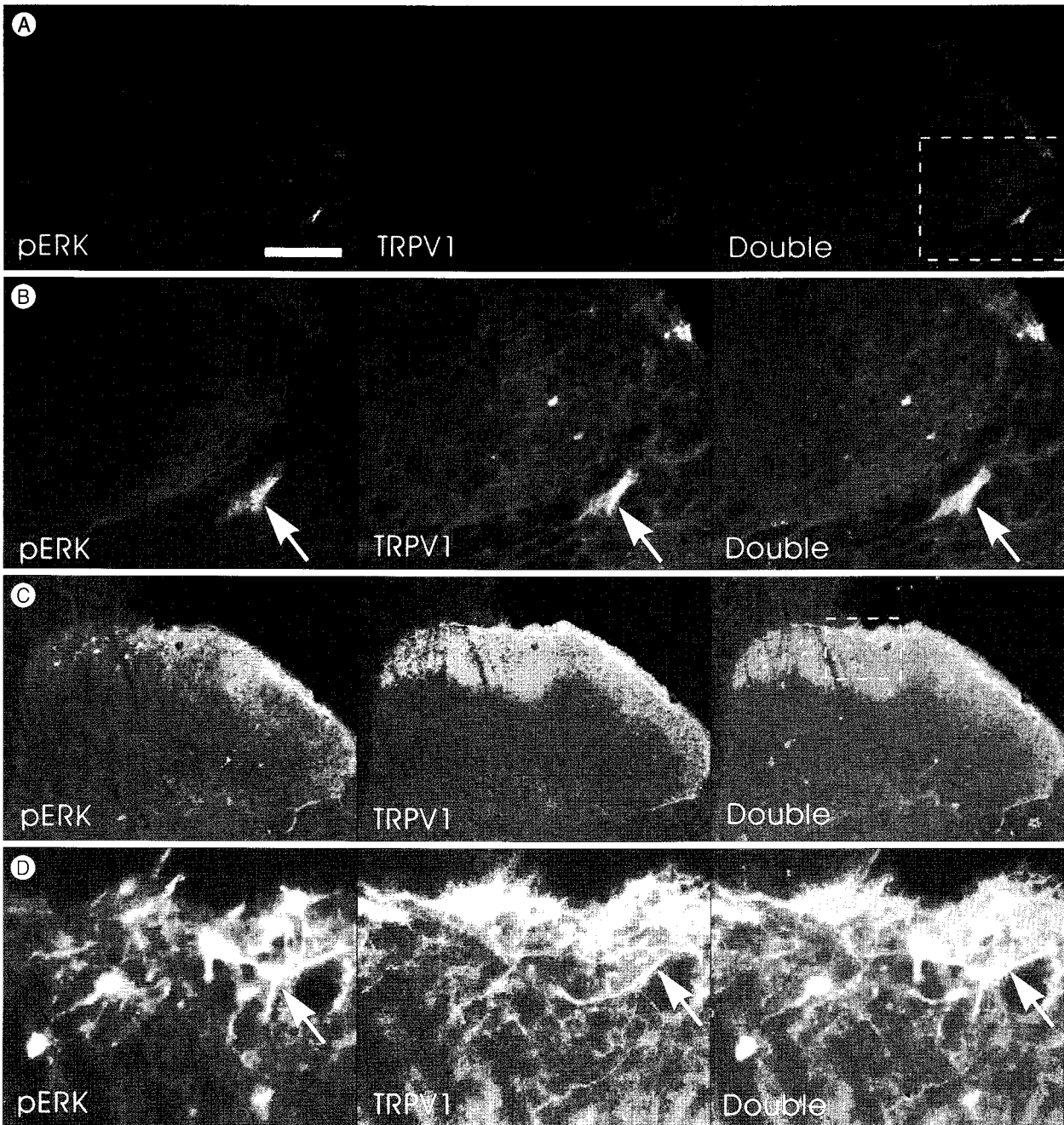


Fig. 7. Double immunofluorescence for pERK (red) and TRPV1 (green) in spinal dorsal horn of the animals injected with 10% acetic acid into the gastrocnemius and erector spinae muscles. Dotted area in A and C are magnified in B and D, respectively. In the dorsal horn of the animal stimulated on gastrocnemius 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 μ m.

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 afferents³⁴. 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%²¹. This may indicate that the differential expression of peptide between trigeminal and spinal sensory systems.

In our results, the expression of P2X₃ 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 P2X₃^{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 allodynia³⁸. 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 (P2X₁₋₇) can assemble to form homomeric and heteromeric receptors. The P2X₂ and P2X₃ receptors are expressed in the autonomic and sensory ganglia and thought to play a role in the nociception¹⁰. It was expected that P2X₃ 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 pH¹⁶. However, the expressions of P2X₃ 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 P2X₃ 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 II³⁵. 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 III²⁹, and also in the rat³⁶. 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.

• Acknowledgement

This work was supported by research grant from Seoul Medical Center Research Institute.

References

1. Abrahams VC : Group III and IV receptors of skeletal muscle. *Can J Physiol Pharmacol* 64 : 509-514, 1986
2. Ambalavanar R, Moritani M, Haines A, Hilton T, Dessem D : Chemical phenotypes of muscle and cutaneous afferent neurons in the rat trigeminal ganglion. *J Comp Neurol* 460 : 167-179, 2003
3. Ambalavanar R, Moritani M, Moutanni A, Gangula P, Yallampalli C, Dessem D : Deep tissue inflammation upregulates neuropeptides and evokes nociceptive behaviors which are modulated by a neuropeptide antagonist. *Pain* 120 : 53-68, 2006
4. Aoki Y, Ohtori S, Takahashi K, Ino H, Ozawa T, Douya H, et al : P2X₃-immunoreactive primary sensory neurons innervating lumbar intervertebral disc in rats. *Brain Res* 989 : 214-220, 2003
5. Avelino A, Cruz C, Nagy I, Cruz F : Vanilloid receptor 1 expression in the rat urinary tract. *Neuroscience* 109 : 787-798, 2002
6. Bjorkman R, Hallman KM, Hedner J, Hedner T, Henning M : Acetaminophen blocks spinal hyperalgesia induced by NMDA and substance P. *Pain* 57 : 259-264, 1994
7. Caterina MJ, Leffler A, Malmberg AB, Rosen TA, Levine JD, Julius D : Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* 288 : 306-313, 2000
8. Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D : The capsaicin receptor : a heat-activated ion channel in the pain pathway. *Nature* 389 : 816-824, 1997
9. De Koninck Y, Ribeiro-da-Silva A, Henry JL, Cuello AC : Spinal neurons exhibiting a specific nociceptive response receive abundant substance P-containing synaptic contacts. *Proc Natl Acad Sci USA* 89 : 5073-5077, 1992
10. Dunn PM, Zhong Y, Burnstock G : P2X receptors in peripheral neurons. *Prog Neurobiol* 65 : 107-134, 2001
11. Gao Z, Henig O, Kehoe V, Sinoway LL, Li J : Vanilloid type 1 receptor and the acid-sensing ion channel mediate acid phosphate activation of muscle afferent nerves in rats. *J Appl Physiol* 100 : 421-426, 2006
12. Gunthorpe MJ, Harries MH, Prinija RK, Davis JB, Randall A : Voltage- and time-dependent properties of the recombinant rat vanilloid receptor (rVR1). *J Physiol* 525 Pt 3 : 747-759, 2000
13. Guo A, Vulchanova L, Wang J, Li X, Elde R : Immunocytochemical localization of the vanilloid receptor 1 (VR1) : relationship to neuropeptides, the P2X₃ purinoceptor and IB4 binding sites. *Eur J Neurosci* 11 : 946-958, 1999
14. Hoheisel U, Lehmann-Willenbrock E, Mense S : Termination patterns of identified group II and III afferent fibres from deep tissues in the spinal cord of the cat. *Neuroscience* 28 : 495-507, 1989
15. Hoheisel U, Mense S : Long-term changes in discharge behaviour of cat dorsal horn neurones following noxious stimulation of deep tissues. *Pain* 36 : 239-247, 1989
16. Hoheisel U, Reinohl J, Unger T, Mense S : Acidic pH and capsaicin activate mechanosensitive group IV muscle receptors in the rat. *Pain* 110 : 149-157, 2004
17. Hwang SJ, Min Oh J, Valtchanoff JG : Expression of the vanilloid receptor TRPV1 in rat dorsal root ganglion neurons supports different roles of the receptor in visceral and cutaneous afferents. *Brain Res* 1047 : 261-266, 2005
18. Hwang SJ, Oh JM, Valtchanoff JG : The majority of bladder sensory afferents to the rat lumbosacral spinal cord are both IB4- and CGRP-positive. *Brain Res* 1062 : 86-91, 2005
19. Hwang SJ, Valtchanoff JG : Vanilloid receptor VR1-positive afferents are distributed differently at different levels of the rat lumbar spinal cord. *Neurosci Lett* 349 : 41-44, 2003
20. Ishizuka N, Mannen H, Hongo T, Sasaki S : Trajectory of group Ia afferent fibers stained with horseradish peroxidase in the lumbosacral

- spinal cord of the cat : three dimensional reconstructions from serial sections. *J Comp Neurol* 186 : 189-211, 1979
21. Jami L : Golgi tendon organs in mammalian skeletal muscle : functional properties and central actions. *Physiol Rev* 72 : 623-666, 1992
 22. Ji RR, Baba H, Brenner GJ, Woolf CJ : Nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity. *Nat Neurosci* 2 : 1114-1119, 1999
 23. Jin SX, Zhuang ZY, Woolf CJ, Ji RR : p38 mitogen-activated protein kinase is activated after a spinal nerve ligation in spinal cord microglia and dorsal root ganglion neurons and contributes to the generation of neuropathic pain. *J Neurosci* 23 : 4017-4022, 2003
 24. Kantor PF, Coetzee WA, Carmeliet EE, Dennis SC, Opie LH : Reduction of ischemic K⁺ loss and arrhythmias in rat hearts. Effect of glibenclamide, a sulfonylurea. *Circ Res* 66 : 478-485, 1990
 25. Karai L, Brown DC, Mannes AJ, Connelly ST, Brown J, Gandal M, et al : Deletion of vanilloid receptor 1-expressing primary afferent neurons for pain control. *J Clin Invest* 113 : 1344-1352, 2004
 26. Kim SJ, Koh KS, Chung IH : The effect of sciatic nerve transection on the somatostatin and substance P cells in dorsal root ganglia of rats. *J Korean Neurosurgical Soc* 19 : 21-30, 1990
 27. Kiss S, Yoshiyama M, Cao YQ, Basbaum AI, de Groat WC, Lecci A, et al : Impaired response to chemical irritation of the urinary tract in mice with disruption of the preprotachykinin gene. *Neurosci Lett* 313 : 57-60, 2001
 28. Lawson SN, Crepps BA, Perl ER : Relationship of substance P to afferent characteristics of dorsal root ganglion neurones in guinea-pig. *J Physiol* 505 Pt 1 : 177-191, 1997
 29. Ling LJ, Honda T, Shimada Y, Ozaki N, Shiraishi Y, Sugiura Y : Central projection of unmyelinated (C) primary afferent fibers from gastrocnemius muscle in the guinea pig. *J Comp Neurol* 461 : 140-150, 2003
 30. Mense S : Nociception from skeletal muscle in relation to clinical muscle pain. *Pain* 54 : 241-289, 1993
 31. Michael GJ, Priestley JV : Differential expression of the mRNA for the vanilloid receptor subtype 1 in cells of the adult rat dorsal root and nodose ganglia and its downregulation by axotomy. *J Neurosci* 19 : 1844-1854, 1999
 32. Nichols ML, Allen BJ, Rogers SD, Ghilardi JR, Honore P, Luger NM, et al : Transmission of chronic nociception by spinal neurons expressing the substance P receptor. *Science* 286 : 1558-1561, 1999
 33. Nishiguchi J, Sasaki K, Seki S, Chancellor MB, Erickson KA, de Groat WC, et al : Effects of isolectin B4-conjugated saporin, a targeting cytotoxin, on bladder overactivity induced by bladder irritation. *Eur J Neurosci* 20 : 474-482, 2004
 34. O'Brien C, Woolf CJ, Fitzgerald M, Lindsay RM, Molander C : Differences in the chemical expression of rat primary afferent neurons which innervate skin, muscle or joint. *Neuroscience* 32 : 493-502, 1989
 35. Ohtori S, Takahashi K, Chiba T, Takahashi Y, Yamagata M, Sameda H, et al : Fos expression in the rat brain and spinal cord evoked by noxious stimulation to low back muscle and skin. *Spine* 25 : 2425-2430, 2000
 36. Panneton WM, Gan Q, Juric R : The central termination of sensory fibers from nerves to the gastrocnemius muscle of the rat. *Neuroscience* 134 : 175-187, 2005
 37. Petruska JC, Cooper BY, Gu JG, Rau KK, Johnson RD : Distribution of P2X₁, P2X₂, and P2X₃ receptor subunits in rat primary afferents : relation to population markers and specific cell types. *J Chem Neuroanat* 20 : 141-162, 2000
 38. Riedel W, Neeck G : Nociception, pain, and antinociception : current concepts. *Z Rheumatol* 60 : 404-415, 2001
 39. Seger R, Krebs EG : The MAPK signaling cascade. *Faseb J* 9 : 726-735, 1995
 40. Shordand PJ, Wang HF, Molander C : Transganglionic transport of the lectin soybean agglutinin (Glycine max) following injection into the sciatic nerve of the adult rat. *J Neurocytol* 27 : 233-245, 1998
 41. Silverman JD, Kruger L : Selective neuronal glycoconjugate expression in sensory and autonomic ganglia : relation of lectin reactivity to peptide and enzyme markers. *J Neurocytol* 19 : 789-801, 1990
 42. Stucky CL, Lewin GR : Isolectin B(4)-positive and -negative nociceptors are functionally distinct. *J Neurosci* 19 : 6497-6505, 1999
 43. Tominaga M, Caterina MJ, Malmberg AB, Rosen TA, Gilbert H, Skinner K, et al : The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron* 21 : 531-543, 1998
 44. Walker KM, Urban L, Medhurst SJ, Patel S, Panesar M, Fox AJ, et al : The VR1 antagonist capsazepine reverses mechanical hyperalgesia in models of inflammatory and neuropathic pain. *J Pharmacol Exp Ther* 304 : 56-62, 2003
 45. Widmann C, Gibson S, Jarpe MB, Johnson GL : Mitogen-activated protein kinase : conservation of a three-kinase module from yeast to human. *Physiol Rev* 79 : 143-180, 1999
 46. Willis WD, Trevino DL, Coulter JD, Maunz RA : Responses of primate spinothalamic tract neurons to natural stimulation of hindlimb. *J Neurophysiol* 37 : 358-372, 1974
 47. Zimmer A, Zimmer AM, Baffi J, Usdin T, Reynolds K, Konig M : Hypoalgesia in mice with a targeted deletion of the tachykinin 1 gene. *Proc Natl Acad Sci U S A* 95 : 2630-2635, 1998