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J Korean Neurosurg Soc 42 : 462-469, 2007

10.3340/jkns.2007.42.6.462

Laboratory Investigation

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The VR1-Positive Primary Afferent-Mediated Expression of pERK in the Lumbosacral Neurons in Response to Mechanical and Chemical Stimulation of the Urinary Bladder in Rats

Objective : This study characterized the neurons in the lumbosacral cord that express phospho ERK (pERK) after distension or irritation of the bladder, and their relation to the vanilloid receptor 1 (VR1) positive primary afferents.

Methods : Mechanical distension and chemical irritation of the bladder were induced by intravesical injection of the saline and mustard oil, respectively. Spinal neurons expressing pERK and the primary afferent fibers were characterized using multiple immunofluorescence for neurokinin 1 (NK1), neuronal nitric oxide synthetase (nNOS) and VR1.

Results : Neurons in lamina I, medial dorsal horn (MDH), dorsal gray commissure (DGC) and sacral parasympathetic nucleus (SPN) were immunoreactive for pERK after either mechanical or chemical stimulation. The majority of pERK positive cells were positive for NK1 in lamina I and SPN, but not in the DGC. Most of pERK positive cells are not stained for nNOS except in a small population of the cells in the SPN and DGC. Contacts between perikarya and dendrites of pERK-positive cells and terminals of primary afferents expressing VR1 were identified in lamina I, lateral collateral path (LCP) and SPN.

Conclusion : In this study, the lumbosacral neurons activated by mechanical and chemical stimulation of the urinary bladder were identified with expression of the pERK, and also provided the evidence that VR1-positive primary afferents may mediate the activation of these neurons.

KEY WORDS : Spinal cord · Visceral sense · Primary afferent · pERK · NK1 · VR1.

INTRODUCTION

The impairment of visceral sense of the bladder is an important mechanism for incontinence and other impairments of the control of bladder function because the sensation from the bladder is the main part of the reflex arc for control of the bladder. Besides the intrinsic sensory neurons in the bladder wall, dorsal root ganlion (DRG) cells of upper lumbar and lumbosacral level transmit sensation from the bladder to the spinal cord via hypogastric and pelvic nerve, respectively. The terminals of central processes of the DRG cells innervating bladder terminate in spinal cord to form synapses with the spinal sensory neurons.

Traditionally, immunohistochemical detection of cFos have been used for the morphological assessments of second sensory neurons responding to specific stimulations both in somatic and visceral sense[2,8]. Recently, immunohistochemical method for detection of a type of intracellular transduction substance mitogen-activated protein kinase (MAP kinase) has been introduced. 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 posttransalational modification of target proteins[30,33,40]. The MAPK family consists of ERK (extracellular singal-regulated protein kinase=p44/42 MAPK), p38 MAPK, c-Jun N-terminal kinase/stress activate protein kinase (JNK/SAPK) and ERK 5. Among them, ERK is activated by the depolarization of cell membrane and the calcium influx[29] and in the nervous system, it has been shown to be involved in the intracellular signal transduction pathway associated with the plasticity such as the long term potentiation, learning, memory, etc.[9,25,33].

According to recent studies, ERK and other MAP kinase have been recognized to play an

• Received : June 11, 2007
• Accepted : August 29, 2007
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active role in the mechanism of hyperlgesia[21,22]. However, most studies on ERK related with pain have been focused on the somatic sensation originated from the skin, and acitvation of ERK in association with visceral pain has been rarely reported. This may be due to the complexity in mechanism of the visceral sensation in contrast to the somatic sensation, which has relatively clear and specific anatomical pathway. In relation of ERK activation and visceral sense, the increased expression of ERK was induced by the pain in the colon, confirmed by immunoblotting method[11]. Xu et al.[41] and Gioia et al.[12] have reported that when acetic acid was injected to the peritoneal cavity, the expression of ERK in the sensory nucleus of the brain stem was noted. However, the studies on the seconday sensory neurons associated with the bladder sensation have not been reported yet.

We performed this study through the ERK immunohistochemistry in the spinal cord after the physical and chemical stimulation of the bladder; (1) to identify the spinal sensory neurons responsible for the bladder sensation, (2) to elucidate the neurochemical characteristics of the spinal neurons, and (3) to elucidate the anatomical characteristic of the primary afferents terminate on the spinal neurons.

MATERIALS AND METHODS

Experimental animals and groups

Experimental animals used in the experiments were 300 -400 g healthy Sprague-Dawley male rats, and during the experiment period, drinking water and feed were supplied ad libitum. Experimental animals were divided into; 1) naïve control group, 2) the sham control group 3) the saline injection group 4) the mustard oil injection group, and 5 animals per group were assigned in each group.

Physical and chemical stimulation of the bladder

To assess neurons expressing pERK during the distention of the bladder, normal saline was injected into the bladder. Anesthesia was induced by the intraperitoneal injection of the mixture of Ketamine (90 mg/Kg) and Xylazine (10 mg/Kg). The longitudinal skin incision of 2 cm in length was made along the lower abdomen, the muscle layers and the peritoneum were dissected to expose the bladder. A PE10 tubing attached to a 3 ml syringe was inserted to the fundus of the bladder and ligated. One mililiter of saline was injected into the bladder twice with 10 minutes interval. The animals were sacrifice by perfusion fixation at 10 minutes after second injection.

To assess neurons expressing pERK in the spinal cord in response to the chemical stimulation of the bladder, mustard oil was injected into the bladder. Animals were anesthetized by the identical method used for the injection of saline, the bladder was exposed, and after the insertion of a 26-gauge needle to the bladder fundus, 0.3 ml of 20% mustard oil (Sigma, USA, in propylene glycol) was injected. The bladder was emptied prior to the injection of mustard oil, and the inferior end of the bladder was ligated to avoid the urethral irritation. For the animals in the sham control group, 0.3 ml propylen oxide was injected to the bladder. Animals were sacrificed at 20 minutes after the injections of mustard oil or propylne oxide.

Tissue preparation and immunohistochemistry

For immunohistochemical staining of spinal cord, all experimental animals were intracardially perfused with paraformaldehyde. After deep anesthesia with pentobarbital (60 mg/Kg, i.p.) a catheter was inserted to the left ventricle, and rinsed with 100 ml saline, followed by 500 ml 4% paraformaldehyde [in 0.1 N PB (phosphate buffer)]. After perfusion fixation, the spinal cords at the L6-S1 level were removed, post-fixed for 2 days in the same fixatives and rinsed with PBS. Transverse or parasagittal sections of the spinal cord at 40-50 μm in thickness were made using a Vibratome and stored in PB.

Multiple fluorescent immunohistochemical staining

To assess spinal sensory neurons that express pERK in response to the physical distention and chemical stimulation of the bladder, pERK immunohistochemical staining was performed. To assess the characteristic of cells expressing pERK, double staining of neruokinin I (NK1) that is expressed on projective sensory neurons primarily, and neuronal nitric oxide synthase (nNOS) that is expressed in a type of interneuron with the function controlling sensory transmission was performed. In addition, to characterize the primary afferents that provide the afferent input to pERK positive cells, together with pERK and NK1, triple immunofluorescent staining for pERK, NK1 with synaptophysin or vanilloid receptor 1 (VR1) were performed.

All incubation and reaction procedures for multiple immunohistochemical staining were performed at room temperature and on a shaker. To enhance the penetration of antibody to tissues, spinal cord sections were reacted with 50% ethanol for 30 minutes and rinsed with PBS for 5 minutes 3 times, and to block the non-specific reaction of secondary antibody, the samples were treated with 10% normal donkey serum (NDS, Jackson Immunoresearch, USA), 1-3 types of primary antibody was combined and reacted overnight.

The type of primary antibody, the manufactures, and

the dilution used were as shown in Table 1. After the completion of the reaction with primary antibody, tissues were rinsed with PBS for 5 minutes 3 times, treated with 2% NDS for 10 minutes, and depending on primary antibody, FITC, Cy3 or Cy5 fluorescent material-conjugated donkey anti-rabbit, donkey anti-guinea pig, donkey anti mouse, donkey anti-rat, or donkey anti-goat (Jackson Immuoresearch, USA) second antibody was diluted to 1:100, reacted for 3 hours, sections were rinsed with PBS, and mounted with Vectashield (Vector Lab, USA). The images were obtained by a confocal microscope (Leica TCX, Leica, Germany), the brightness and contrast was adjusted with Adobe photoshop (version 7.0, Adobe, USA), and the final illustarations were prepared by Corel Draw (Version 10, Corel, Canada).

Table 1. The host, source and working dilutions of the primary antibodies used in the single, double or triple immunohistochemistry

Primary antibody	Host	Company or Source	Dilution
pERK (p44/42 MAPK)	Rabbit	Cell Signaling	1:200
pERK	Mouse	Cell Signaling	1:200
NK1	Guinea pig	Chemicon	1:1000
nNOS	Rabbit	Sigma	1:1000
Synaptophsin	Mouse	Sigma	1:1000
VR1	Goat	Santa Cruz	1:1000

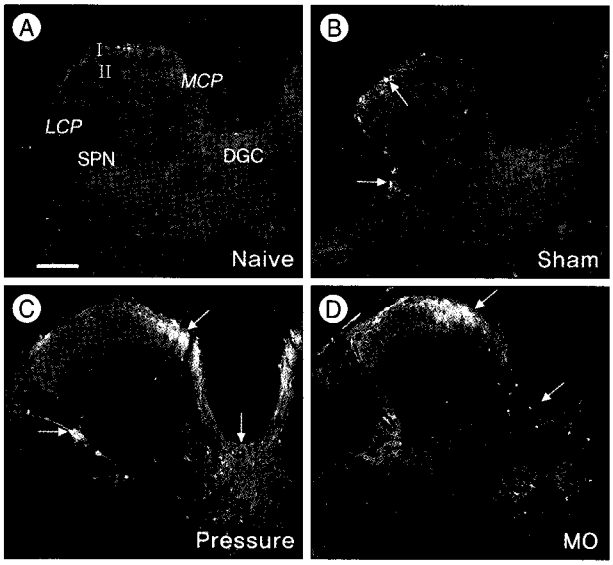

Fig. 1. Photomicrograph of immunohistochemistry for pERK in the spinal cord of rats of naive control (A), Sham control (B), intravesical saline injection (C) intravesical mustard oil (MO) injection (D). A : In naive animal, virtually no immunoreaction for pERK but a few neurons in sacral parasympathetic nucleus (SPN) is observed. B : A few neurons in superficial laminae and SPN are positive for pERK in sham control group. C, D : After injection of saline and mustard oil into the bladder, neurons in lamina I (I), medial dorsal horn, dorsal gray commissure (DGC) and SPN were positive for pERK. LCP : lateral collateral path, MCP : medical collateral path. Scale bar=100 μm.

RESULTS

pERK immunohistochemistry

In the spinal cord of L6 level of the naïve rats, the immunoreaction for pERK was detected in the neurons of the sacral parasympathetic nucleus (SPN). pERK-positive neurons were not detected in the all lamina of dorsal horn or dorsal gray commissure (DGC) (Fig. 1A). In the spinal cord of the sham control rats injected propylene glycol to the bladder, the immunoreaction for pERK was detected in a few neurons located in the lamina I and lamina II of dorsal horn, and also SPN, as in the untreated control group. The pERK immunoreaction in the Lamina I-II was not constant, and the number was 1-2 cells per sections. It was thought to be induced by the local injury of the bladder wall by injection needle (Fig. 1B). In the spinal cord of the rats injected with saline into the bladder, the immunoreaction of pERK was detected in the lamina I, lamina V, the medial dorsal horn (MDH), the DGC, and the SPN of the spinal cord. In the SPN, the number of the pERK-positive cells and the intensity of the immunoreaction were increased in comparison with the sham control group. The processes of pERK positive cells in the SPN were extended laterally to the lateral collateral path (LCP) and medially to the DGC (Fig. 1C). In the spinal cord of the rats injected mustard oil to the bladder, the distribution pattern of the neurons with pERK immunoreaction were similar with that of saline injection (pressure) group. The pERK positive neurons were present in the lamina I, the lamina V, the MDH, the DGC, the SPN, and the LCP (Fig. 1D).

Double immunofluorescent staining of pERK and NK1

To assess the characteristics of cells expressing pERK after physical and chemical stimulation of the bladder, the double immunofluoresecne for pERK and NK1 was performed. In the animial injected with saline and mustard oil into the bladder, pERK-positive cells in the lamina I, the lamina V and the SPN of the spinal cord also expressed NK1. Immunoreaction for pERK was detected in the nucleus of cells, cytoplasm, and dendritic processes, while the immunoreaction for NK1 was detected in the cytoplasm and typically along the membrane of cytoplasmic processes (Fig. 2A,B,C). In the lamina I, double labeled cells were in the cell body of the lamina I and their cell processes arranged in mediolateral or dorsoventral direction. In the lamina II, a number of cell processes were stained with either pERK or NK1 but most of these processes were not double stained (Fig. 2A). The process of pERK positive cells present in the SPN was extended to the LCP, these stains were mostly confined to

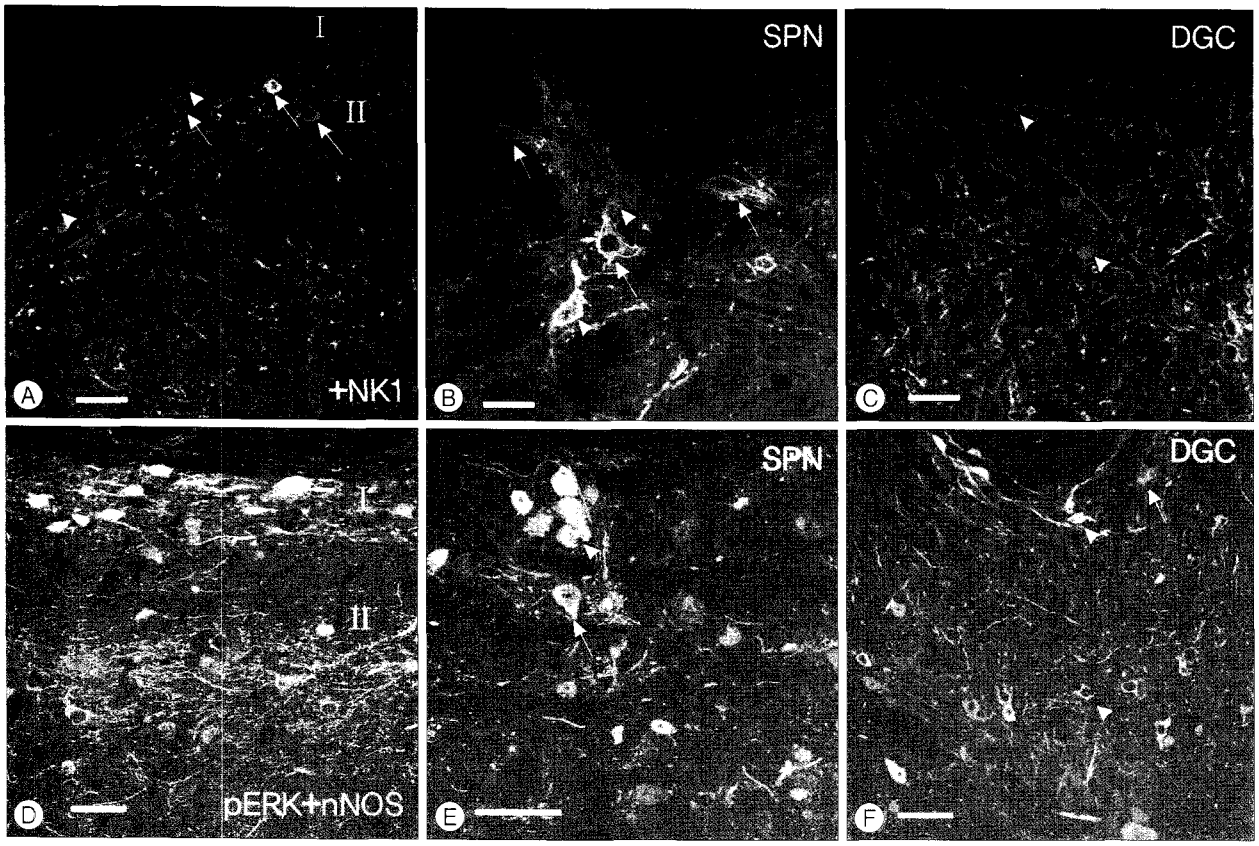

Fig. 2. Confocal microscopy of the spinal cord double stanied for pERK and NK1 (A-C), and pERK and nNOS (D-F) in superficial dorsal horn (A and D), SPN (B and E) and DGC (C and F) after saline or mustard oil injection. A-B : Majority of pERK-positive cells and dendrites in lamina I, SPN, and LCP are also stained for NK1 (arrows) while single stanied cells for NK1 or pERK are also observed (arrowheads). C : In DGC, cells positive for pERK (arrowheads) are not stained for NK1. D : pERK-positive cells are distributed in lamina I and lamina II while nNOS positive cells are scattered in lamina I and condensed in deeper laminae. pERK and nNOS positive cells are not colocalized in the superficial laminae after intravesical mustard oil injection. E : Majority of pERK-positive cells is located in dorsal part of SPN while nNOS-positive cells are in ventral part. In intermediate part of SPN a few cells and processes are double stained for both antigens (arrows). F : In the DGC, pERK cells were located in upper part and near central canal and nNOS positive cells were located intermediate region. Virtually no colocalization between two antigens were observed. Scale bars=30 ㎛ in A- C, 50 ㎛ in D-F.

the cytoplasm and nuclear stain was not observed. Most of pERK positive cells in SPN were also labeled with NK1 on their cytoplasmic membrane (Fig. 2B). In the MDH and the DGC, both pERK and NK1 positive cells were present. However, in contrast with the findings in lamina I and SPN, virtually no pERK positive cells were also stained for NK1 (Fig. 2C).

Double immunofluorescent staining of pERK and nNOS

Double immunostain for pERK and nNOS were performed to study the expression of pERK in nNOS-expressing interneurons. The neurons positive for nNOS were distributed in the entire dorsal horn including lamina I, lamina II, DGC, and SPN, but co-expression of pERK in these cells were very rare in these cells. In lamina I and lamina II, most nNOS positive cells were distinct from pERK positive cells after physical or chemical stimulation of the bladder. (Fig. 2D). In the SPN, pERK positive cells were located primarily in the dorsal part of the SPN and nNOS positive cells were located in the ventral part, and in only a few cells in the intermediate location, the pERK and nNOS immunoreaction were colocalized (Fig. 2E). In the dorsal portion of the DGC, cells positive for two antigens were distinct in location, i.e. nNOS positive cells were located in the ventral side of pERK positive cells, and in the vicinity of the central canal area (Fig. 2F).

Multiple immunostain for pERK, NK1, VR1 and synaptophysin

To assess the characteristics of the primary afferents conveying visceral sensory input to the spinal neurons expressing pERK after physical or chemical stimulation of the bladder, multiple immunostaining for pERK, NK1 and VR 1 was performed. At the L6 level of the spinal cord, the strong immunoereaction for VR1 was detected in the primary afferents extended from Lissauer's tract to deep laminae of the dorsal horn, lamina I, the MCP, the LCP. The fibers with a relatively weak

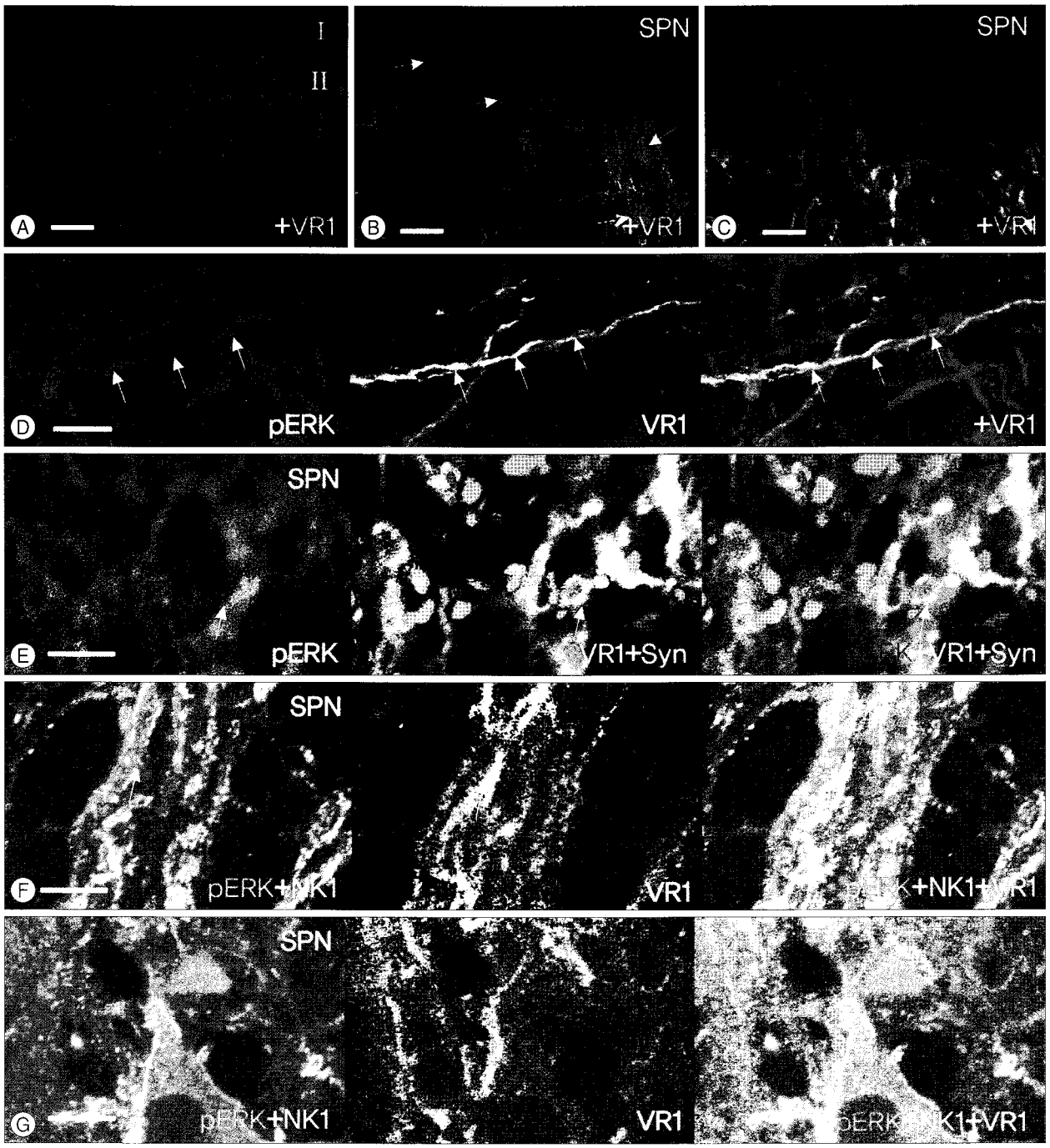

Fig. 3. Confocal microscopy of the spinal cord double or triple stained for pERK, NK1, VR1 and synaptophysin after intravesical mustard oil injection. A-D : Double labeling for pERK and VR1 in superficial lamina (A), SPN (B, C) and deep laminae (D). Fibers with strong VR1-immunoreactivtiy are dense in lamina I and sparse in deeper laminae and fibers are intermixed with pERK-positive cells and processes (arrows in B). SPN, pERK-positive cells in SPN (arrows in B) are aligned along the primary afferents fibers positive for VR1. In deeper laminae, VR1-positive fibers are in contact with pERK-positive neuronal process along their course (arrows in D). E : Triple labeling with pERK, VR1 and synaptophysin (Syn) in SPN. Terminal boutons stained with VR1 on its membrane and contain immunoreaction for synaptophysin inside (arrows) are contacted with pERK-positive neuronal processes. F-G : Triple labeling with pERK, NK1 and VR1 in SPN. pERK-positive perikaryon and processes are also stained for NK1 along their membrane (arrows) and contacted with VR1-postitive primary afferents. Scale bars = 100 μm in A, 30 μm in B, 10 μm in C-D and 5 μm in E-G.

immunoreaction were distributed in lamina II. Double labeling with VR1 and pERK showed that the pERK positive cells in the lamina I, lamina II, and the SPN of the spinal cord of the rats after either distention or chemical irritation were closely contacted with VR1 positive primary afferents (Fig. 3A, B, C). VR1 positive nerve fibers with the strong immunoreaction in the deep lamina formed bouton en pasant contact with long dendritic processes positive for pERK immuno-

reaction (Fig. 3D).

Triple labeling with VR1, pERK and plus synaptophysin, a marker for synaptic vesicle, was done for the verification of the synaptic contact between VR1 positive terminals and pERK positive cells. In the lamina I and SPN, VR1 positive terminals which have synaptophysin immunoreaction inside were contacted with pERK positive cell body or dendritic processes (Fig. 3E). High magnification of the triple labeling with VR1, NK1 and pERK showed that pERK expressing dendritic processes with NK1 immunoreaction on the membrane were in close contact with VR1 positive primary afferents (Fig. 3F, G), implying that VR1 positive fibers are responsible for the activation of NK1 positive secondary neurons after stimulation of urinary bladder.

DISCUSSION

In this study, the bladder was physically or chemically stimulated, and characterization of the neruoanatomical pathways responsible for the bladder sensation was tried through the assessment of; (1) the anatomical distribution of pERK positive cells in the spinal cord, (2) neurochemical characterization of pERK positive cells by multiple staining of NK1 receptors and nNOS, and (3) the primary afferents providing the afferent input to these cells by multiple staining for VR1. Control of the visceral pain has a significant meaning in the clinical field. However, in contrast with relatively well defined somatosensory pathways originated from the skin, the anatomical and functional characteristics of the pathway of the visceral pain is not clearly understood[5]. One of the reasons is that since the signals from visceral pain induce the excitation of various neurons through the polysynaptic pathway in a wide level of the spinal cord, and also converge to the neurons belong to the sensory pathway of the skin, a specific pathway of visceral sense has not been proved[4]. Second, because of the complexity and difficulty of the experimental animal models of visceral pain of specific visceral organ, recent studies on pain has mostly focused on the somatosensory of the skin. Visceral sensory fibers are mixed in the autonomic motor nerves and unmyelinated fibers are predominant[23]. The major sensory nerves distributed to the bladder are the group C and group Aδ fibers. The group Aδ fibers respond to the passive dilatation and active contraction of the bladder, and are also sensitive to the distention of the bladder mucosa[20] while group C fibers mainly respond to the chemical stimulation of the mucosa and also to strong mechanical stimulation[14].The nociceptive group C fibers in the viscera are also referred as the "silent nociceptors" because they are inactive under normal conditions, and show voluntary firing in inflammation the bladder, and increase their firing rate during the distention of the bladder.

In this study, both physical and chemical stimulation induced the activation of the ERK in the identical sensory neurons in the spinal cord and this finding is in agreement with former studies using cFos expression after stimulation of the bladder. The morphological identifications of spinal sensory neuron in response to the stimulation of visceral organs have been preformed previously with the immunostain or in situ hybridization of cFos[8,38]. Birder et al.[2] have reported that upon the physical stimulations of the bladder, the Fos immunoreaction was exhibited in the medial and lateral superficial dorsal horn, the SPN, and the lamina X in the vicinity of the central canal. pERK used in this study has several advantages in comparison with cFos. First, the specificity of pERK is higher than cFos. cFos is expressed in a population of cells in spinal cord without any noxious stimulus and thus the response have to be assessed by quantitative comparison while baseline expression of pERK in spinal cord is negligible except in a few cells in SPN which is possible related with anesthesia[32], Second, since pERK stains the entire perikaryon, it can provide further detailed anatomical information of the activated neurons including synapse with other neurons while cFos stains only nuclei. In our study, the physical and chemical stimulations of the bladder induced the expression of pERK in the lamina I, lamina V, SPN, MDH and DCG in spine, which is in agreement with the result of previous studies.

Neurons of the Lamina I receive the primary sensory information for the pain, and project to the higher center for nociception[7]. In rats, 80% of projection neurons in the lamina I express NK1[24,34], and these neurons project to the lateral parabrachial nucleus (LPB), caudal ventrolateral medulla (CVLM) and medullary dorsal reticular nucleus[1,31]. In our study, most pERK positive cells in the Lamina I, the lamina V and the SPN expressed NK1, implying that the noxious stimulation of the bladder is mediated by substance P positive primary afferents and also that most pERK positive cells are the neurons project to the brain. In the SPN and intermediolateral nucleus (IML) in the thoracolumbar cord (data not shown), a few cells with pERK immunoreaction was shown even in the control animal. Both SPN and IML have a common feature that they are preganglionic autonomic neurons. Exact mechanism of this phenomenon is not clear but two possibilities are expected. First, the effect of autonomic influence during perfusion may induced pERK expression. Second, there may be a constant tone of the autonomic reflex arc under normal condition.

In this study, to elucidate the characteristic of pERK positive cells, together with NK1, the immunostaining of nNOS was

performed. Nitric oxide (NO), a gaseous neuromodulatory substance, is widely distributed in the CNS, and in the spinal cord it has the function related with cardiovascular control and also transmission or modulation of the nociception[6,10,42]. nNOS is the enzyme that produces NO in the pre- and post-synaptic site of neurons. In according to NDPH diaphorase histochemistry and nNOS immunohistochemistry, nNOS is present in the lamina I-III, IML, SPN and DGC of dorsal horn, and dorsal root ganglion cells[28,36,37,43]. In this study, pERK positive cells in the dorsal horn, SPN and MGC after stimulation of the bladder rarely expressed nNOS. Therefore, it was confirmed that cells expressing pERK after physical or chemical stimulations of the bladder are mostly projection neurons, not the interneurons expressing nNOS.

The SPN was recently found to be composed of cells of a few functional classes, i.e. preganglionic autonomic neurons, sensory neurons projecting to the higher centers and interneurons are included[2]. In this study, nNOS positive cells were located in the ventral area of the SPN and most of them did not expressed pERK, except a few cells in the intermediate areas of the SPN. This is believed due to that pERK is expressed in the NK1 positive projection neurons as in lamina I. In support of our interpretation, Hamilton et al.[15] have reported that the projection neurons located in the dorsal to preganglionic neurons, two types of cells did not overlap, and NOS was only in a portion of preganglionic neurons.

Stimulation of the bladder also induced the expression of pERK in the neurons present in the MDH and the DGC, but most of these cells did not express NK1 and nNOS. It has been reported that the DGC is well developed in the area below L6 and it has a role in nociception and visceral sensation[26] but its precise mechanism has not been known yet. The DGC has bilateral somatic and visceral receptive field and it receives numerous propriospinal fibers, and it is believed to play a role in the convergence of somatic and visceral sensations[17,39]. Miura et al.[27] reported that in the L6 and S1 spinal cord, the DGC neurons contribute to the somato-visceral reflex such as the micturition reflex. Therefore, it is suggested that pERK positive cells in the MDC and the DGC are functionally different from those in lamina I and SPN. Combined study with stimulation of visceral organs and electrophysiology are needed for this nucleus.

In this study, to assess the primary afferents that provide the sensory input to pERK positive cells, the immunostaining for VR1 was performed. VR1 is the capsaicin receptor, it is a non-specific cation channel belongs to the transient receptor potential (TRP) family, and it reacts also to the heat within a certain range and hydrogen ion[3]. In dorsal root ganglia, VR1 is expressed in small cells associated with the group C unmyelinated nerve fibers and medium sized cells associated with the group Aδ myelinated nerve fibers[35]. VR1 is synthesized in sensory ganglion cells and subsequently transported peripherally to the skin and viscera and also centrally to the spinal cord[13,16]. VR1 in spinal cord is recognized as a marker of the primary afferents involved in nociception. In regard to relation of VR1 and visceral sensation, Hwang and Valtschanoff[19] reported that VR1 immunoreaction was stronger in visecral affernets than somatic. In this study, VR1 positive terminals were present primarily in the Lissauer's tract, the lamina I, the medial collateral path (MCP) and the lateral collateral path (LCP). Concerning the neurons in the Lamina I, Hwang et al.[18] proved that terminal of VR1 positive primary afferent forms synapse with NK1 neuron of the lamina I, and these cells project to the LPB. In present study, synaptic contact of VR1 positive terminals in LCP and SPN with pERK and NK1 positive cell body or dendritic process was confirmed morphologically by triple labeling including synaptophysin, a marker for synaptic vesicle.

Taken together, it was considered that the physical distention and chemical stimulation of the bladder induce the activation of ERK in NK1 positive sensory projection neurons in the lamina I-V, SPN, MDH and DGC of L6 spinal cord, and the activation of these spinal neurons is mediated by the VR1-positive visceral primary afferents.

CONCLUSION

In this study, the lumbosacral neurons activated by mechanical and chemical stimulation of the urinary bladder was identified with expssion of the pERK, and also provide the evidence that VR1-positive primary afferents may mediate the activation of these neurons.

• Acknowledgement
This work was supported by Korea Science & Engineering Foundation (Grant-2004-000-10801-0).

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