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Molecules and Cells

NMDA Receptor, PKC and ERK Mediate Fos Expression Induced by the Activation of Group I Metabotropic Glutamate Receptors in the Spinal Trigeminal Subnucleus Oralis

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Fos, a protein product of immediate early gene c-*fos*, has been used as a marker for activation of nociceptive neurons in central nervous system including spinal trigeminal nucleus (Vsp). By noxious stimulation applied to orofacial area, the expression of Fos occurred in the Vsp pars oralis (Vo), the subnucleus receiving inputs from trigeminal primary afferents that predominantly innervate intraoral receptive fields. The present study demonstrates that the *in vitro* activation of group I metabotropic glutamate receptors (mGluRs; mGluR1 and 5) by bath-application of their well-known agonist (*S*)-3,5-dihydroxyphenylglycine (DHPG) increased the number of Fos-expressing neurons in the Vo area. In addition, bath application of DHPG caused inward currents, a parameter of neuronal excitation, in the Vo neurons held at -70 mV in voltage-clamp mode of whole-cell recordings. In further experiments characterizing two phenomena, the increased Fos expression in the Vo was mediated by an additive activation of both mGluR1 and mGluR5, which required the activation of *N*-methyl-*D*-aspartate (NMDA) receptors, protein kinase C (PKC) and extracellular signal-regulated kinase (ERK). In contrast, the inward currents were mediated only by mGluR1, but not by others. The data resulting from this *in vitro* study indicate that the DHPG-induced membrane depolarisation or neuronal excitation may be upstream to, or skip, the NMDA receptor, PKC and ERK pathways for the DHPG-induced Fos expression.

INTRODUCTION

Fos is a protein product of the immediate early gene c-*fos*, and functions as a transcriptional factor. Since it was first recognized that the spinal dorsal horn (DH) neurons express Fos protein by sensory stimulation (Hunt et al., 1987), it has also been demonstrated in the spinal trigeminal subnucleus caudalis (Vc), the area known as anatomically or functionally homologous to the spinal DH (Bereiter et al., 2000), that noxious mechanical (pinch), thermal (55°C) and chemical (capsaicin) stimuli to the facial skin induces Fos expression (Strassman and Vos, 1993). Later, the Fos-expressing noxious stimuli in the Vc further includes electrical stimulation of tooth pulp (Iwata et al., 1998) or trigeminal ganglion (Takemura et al., 2000), transection of the inferior alveolar nerve (Nomura et al., 2002), mustard oil (Iwata et al., 1998), or formalin injected into the lip or the tongue (Sugiyo et al., 2009). Although some of the strong stimuli caused Fos expression located in the deeper layer of the Vc, Fos-expressing neurons induced by noxious stimuli were exclusively concentrated in the superficial layer, i.e., laminae I and II, of the Vc (Strassman and Vos, 1993). Because the pattern of Fos expression in the Vc by noxious stimulation overlapped with the termination sites of trigeminal primary nociceptors, it was interpreted that the Fos expression might be due to the monosynaptic inputs from the activated primary nociceptors (He et al., 2000; Sugimoto et al., 1998a). On the other hand, it has also been shown that the spinal trigeminal subnucleus oralis (Vo) responds to noxious stimulation in orofacial areas, and expresses Fos protein (He et al., 2000; Sugimoto et al., 1998a; 1998b). Interestingly, the Fos-expres-sing neurons were prominently located in the dorsomedial part of the Vo when noxious stimulation was applied to intraoral structures (Sugimoto et al., 1998a; 1998b), and could be prevented by the pre-destruction of unmyelinated primary afferent neurons by capsaicin (He et al., 2000), indicating that the monosynaptic input through unmyelinated fibers is an important contributor to the Fos expression.

As yet, the expression of Fos has been used for deciphering neuronal activation following nociceptive stimuli or locating the brain area integrating nociceptive somatic sensations (Coggeshall, 2005; Harris, 1998). Beside of the use of Fos as the neuronal activation marker, to date, numerous molecules and various types of electrical stimuli have been known to induce Fos expression (Coggeshall, 2005). The Fos-inducing molecules include neurotransmitters, neuropeptides, inflammatory mediators, and exogenous pain-inducing chemicals. Among them, glutamate, the major excitatory neurotransmitter in the central

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nervous system, has been known to be a critical inducer of the Fos expression in second-order DH neurons (Coggeshall, 2005; Soyguder, 2005). Given that noxious electrical stimulation of primary afferent fibers may release a sufficient amount of glutamate into the spinal DH and cause a spill-over of glutamate outside of the synaptic structure, it has been suggested that group I metabotropic types of glutamate receptor (mGluRs) existing at extrasynaptic sites within the spinal DH are critical contributors of Fos expression in the spinal DH (Giles et al., 2007). The group I mGluRs have been also known to increase the excitability of DH neurons by membrane depolarisation (Zhong et al., 2000), and mediate slow excitatory transmission (Galik et al., 2008). Therefore, in the present study, using pharmacological tools, if the Fos expression and neuronal excitability induced by the activation of group I mGluRs have any correlation from the viewpoint of mediation of *N*-methyl-*D*-aspartate (NMDA) receptors, subtypes of group I mGluRs and signalling protein kinases was investigated.

MATERIALS AND METHODS

Slice preparation

The Animal Care and Use Committee of Kyungpook National University approved these experiments. Horizontal brainstem slices (400-450 μm) were prepared from 6-14 day-old Sprague-Dawley rats of either sex, as described previously (Han et al., 2008). Briefly, the brain and part of the cervical spinal cord were removed under deep urethane anaesthesia (1.5 g/kg, i.p.), then immediately transferred into an ice-cold Krebs' solution (composition in mM: 117 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, and 11 glucose; pre-oxygenated with a mixture of 95% O_2 and 5% CO_2; pH 7.4). The prepared slices, using a Vibratome 1000$^+$ (Vibratome, USA), were incubated at room temperature (23-25°C) for at least an hour with continuous oxygenation to wash away hazardous or bioactive molecules released from the preparation processes.

Immunohistochemistry and quantification of Fos-like immunoreactive cells

A horizontal brainstem slice was transferred into a chamber used for electrophysiological recordings, fixed with small pieces of silver metal bars and perfused with the oxygenated Krebs' solution (at room temperature, 23-25°C) containing bicuculline methiodide (BMI, 5 μM) and strychnine (1-2 μM) to block inhibitory influence on the Vo neurons, mediated by γ-aminobutyric acid type A ($GABA_A$) and glycine receptors, respectively. (*S*)-3,5-dihydroxyphenylglycine (DHPG, 10 μM), known as a selective group I mGluR agonist, was applied for 5 min. After 15 min from the start of DHPG application (i.e., 10 min washout of DHPG), the slices were immediately transferred into a 4% paraformaldehyde solution in 0.1 M phosphate buffer (PB) (pH 7.4), and fixed at 4°C overnight. Then the slices were cryo-protected overnight in a 30% sucrose solution [in 0.1 M phosphate buffered saline (PBS)] at 4°C. The brainstem slices were then sectioned with a cryostat (30 μm; 1-3 sections/slice), and floated in the PBS. Non-specific binding was blocked using 10% normal goat serum for 1 h, and the sections were incubated overnight with rabbit polyclonal antibody to c-Fos (1:500; ab7963, abcam, Cambridge, UK) and subsequently incubated for 2.5 hours with the secondary biotinylated goat anti-rabbit IgG antibody (1:200; BA-1000, Vector, USA). For visualization, VECTASTAIN® ABC Reagent (Vector, USA) for 1.5 h and 3,3′-diaminobenzidine (DAB) were used. Fos-like immunoreactive (Fos-LI) cells were determined only when a distinctive immunolabeling was shown compared to the background without considering the intensity of the staining. After the Vc area was identified by a translucent band that designates the lamina II area of the Vc, the spinal trigeminal nucleus subuncleus interpolaris (Vi) was located rostrally to the Vc. Since the area of the Vi has relatively distinct border in the immunolabelled sections, the Vo was rostrally identified to the Vi. Then, the number of Fos-LI cells was counted by locating rectangular box (270 μm × 270 μm) that could be positioned within the Vo (200×) (Fig. 1A). The counts were represented as mean ± standard error of mean (SEM) for each group (*n*-value, number of sections), and statistically compared using Student's *t*-test ($P < 0.05$ or $P < 0.01$).

Electrophysiological recordings

Blind whole-cell recordings with patch pipettes (borosilicate glass, TW150F; WPI, Sarasota, FL, USA) were made from the Vo area identified in the horizontal brainstem slices as previously demonstrated (Han et al., 2008). The resistance of the patch pipettes was typically 8-12 MΩ when filled with internal solutions (composition in mM: Cs_2SO_4 110, $CaCl_2$ 0.5, $MgCl_2$ 2, EGTA 5, HEPES 5, TEA chloride 5, ATP-Mg salt 5; pH 7.2). All recordings were made under a continuous perfusion of the preoxygenated Krebs' solution (2-3 ml/min), the same as the slice preparation solution, at room temperature. BMI (5 μM) and strychnine (1-2 μM) were always used in all the recordings to block inhibitory synaptic responses. For Ca^{2+}-free external solution, $CaCl_2$ was omitted from the Krebs' solution, and as much $MgCl_2$ was added. Whole-cell formation was identified by the appearance of capacitance transients upon voltage pulses (-5 mV), and neurons by spontaneous events at a holding potential of -70 mV that are mostly excitatory postsynaptic currents (EPSCs). Recordings were amplified with a Multiclamp 700A amplifier (MDS Inc., Canada), sampled at 5-10 KHz, and filtered at 1-2 KHz. pClamp software (version 9 or 10; MDS Inc., Canada) was used for data acquisition and analysis. After a stable baseline holding current, DHPG (10 μM) was bath-applied for 5 min. In the antagonist experiments, each antagonist at the concentration indicated was always maintained inside the Krebs' solution throughout experiment, i.e., before, during and after the application of DHPG. The peak amplitude of whole-cell inward current was determined as the difference between baseline and the peak of inward current for individual neurons. The measurements were represented as mean ± SEM. Statistical comparisons were made using Student's *t*-test (*$P < 0.05$; **$P < 0.01$).

Drugs

Drugs were as follows: D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5), BMI, DHPG, 2-[1-(3-dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl)maleimide (GF109203X), (+)-2-methyl-4-carboxyphenylglycine (LY367385), 2-methyl-6-phenylethynyl-pyridine (MPEP), 2-(2-amino-3-methoxyphenly)-4H-1-benzopyran-4-one (PD98059) and strychnine from Tocris Cookson (USA), and tetrodotoxin (TTX) from Sigma (USA).

RESULTS

To investigate if the activation of group I mGluRs could cause the expression of Fos protein, DHPG (10 μM) was bath-applied for 5 min to the horizontal brainstem slices, and then the slices were subjected to immunohistochemical staining at 15 min from the start of the application. When Fos-LI cells were counted in a rectangular box (270 μm × 270 μm) located in the Vo area of the spinal trigeminal nucleus photographed under a light microscope (200×) at each section, the number of Fos-LI cells were significantly increased by the bath application of DHPG (83.5 ±

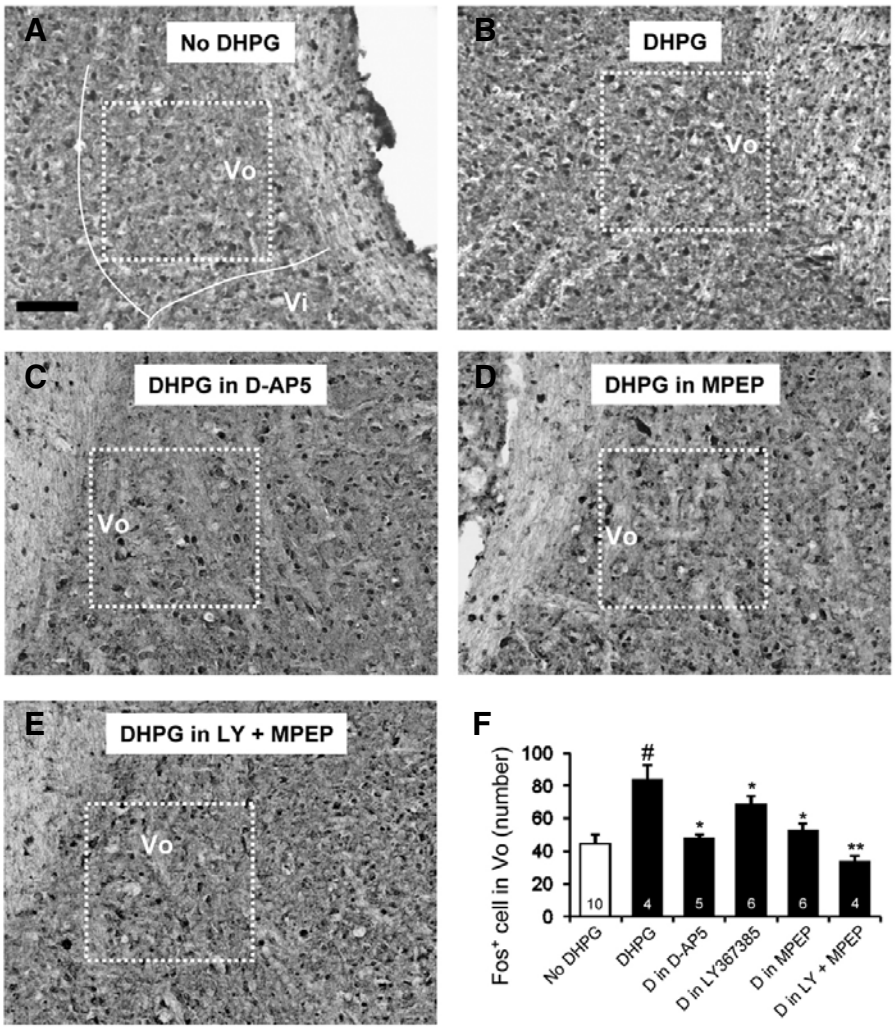

Fig. 1. Involvement of NMDA receptor, mGluR1 and mGluR5 in the Fos expression induced by bath application of DHPG *in vitro*. Bath application of DHPG significantly increased the number of Fos-LI cells in the Vo area (B), compared to that with no treatment of DHPG (A). The increased number of Fos-LI cells was significantly reduced in the presence of the NMDA receptor antagonist D-AP5 (C), the mGluR5 antagonist MPEP (D), and the mGluR1 antagonist LY367385 plus MPEP (E). (F) A histogram demonstrates significant reductions of the DHPG-induced Fos expression by the antagonists used (*P < 0.05 and **P < 0.01 vs. DHPG; #P < 0.05 vs. no DHPG). The white lines (A) indicate the borders of Vi and Vo. Dotted boxes on the photographs indicate the counted areas in the Vo. The numbers on the bars indicate the numbers of sections counted. Scale bar, 100 μm.

9.3, P < 0.05; Figs. 1B and 1F), when compared to that expressed in the basal condition (44.2 ± 6.1; Figs. 1A and 1F). Interestingly, the increased expression of Fos protein by DHPG was almost completely blocked in the presence of the competitive NMDA receptor antagonist D-AP5 (50 μM; 47.6 ± 2.8, P < 0.05 vs. DHPG; Figs. 1C and 1F), indicating an important contribution of the NMDA receptors to the DHPG-induced Fos expression. On the other hand, the increased number of Fos-LI cells by DHPG was significantly, but only partially, reduced by the mGluR1 antagonist LY367385 (100 μM; 68.3 ± 5.6, P < 0.05 vs. DHPG; Fig. 1F), and, more effectively than LY367385, reduced by the mGluR5 antagonist MPEP (10 μM; 52.7 ± 4.1, P < 0.05 vs. DHPG; Figs. 1D and 1F). Further, the co-presence of LY367385 and MPEP completely blocked the increased number of Fos-LI cells by DHPG (33.8 ± 3.4, P < 0.01 vs. DHPG; Figs. 1E and 1F), or even lowered the basal level of Fos expression although the difference was minimal. Further, this study also tested the involvements of protein kinase C (PKC) and extracellular signal-regulated kinase (ERK) in the DHPG-induced increase of Fos expression using their inhibitors of activation, respectively, GF109203X (1 μM) and PD98059 (50 μM). The increased number of Fos-LI cells by DHPG was significantly reduced by the presence of GF109203X (54.1 ± 3.7, P < 0.05 vs. DHPG) or PD98059 (46.0 ± 6.4, P < 0.05 vs. DHPG) (Fig. 2).

After establishing stable baseline holding currents under whole-cell configurations of Vo neurons whose neuronal properties were identified by capacitive transients upon rectangular voltage pulses (-5 mV) and an occurrence of spontaneous EPSCs at the holding potential of -70 mV, the group I mGluR agonist DHPG was bath-applied at a concentration of 10 μM for 5 min. As a result, DHPG produced inward currents in the amplitude range of 5 to 73 pA (33.1 ± 6.7 pA, n = 10; Figs. 3A and 3B). The inward currents induced by DHPG was reduced only partially by D-AP5 (21.1 ± 5.5 pA, n = 7, P > 0.05), but significantly by LY367385 (6.0 ± 1.5 pA, n = 7, P < 0.01; Fig. 3B). This significant inhibiting effect was further repeated in the co-presence of LY367385 and MPEP (5.8 ± 2.5 pA, P < 0.01) but not with MPEP alone (16.3 ± 7.6 pA, P < 0.05; Fig. 3B), indicating the predominant mediation of mGluR1 subtype in the DHPG-induced inward currents of the Vo neurons. To exclude an indirect influence on the recorded Vo neurons, which originated from global depolarization of other neurons existed in the horizontal brainstem slice, the DHPG effect was tested in TTX (1 μM)-containing or Ca^{2+}-free external solution, both block synaptic transmission by blocking action potential conduction or synaptic vesicular release, respectively. In these conditions, DHPG still induced whole-cell inward currents (TTX, 28.0 ± 4.6

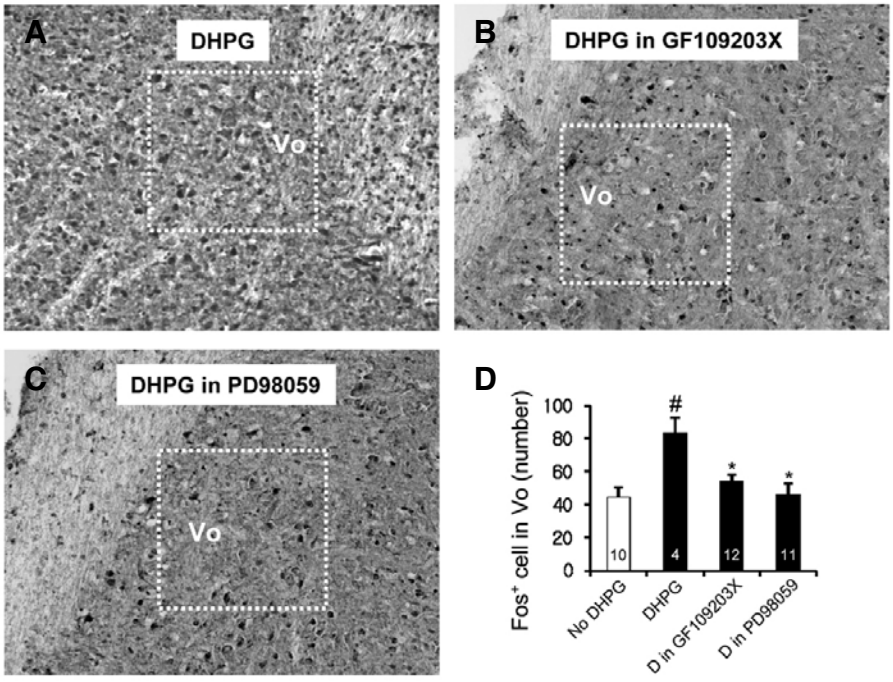

Fig. 2. Involvement of PKC and ERK in the Fos expression induced by bath application of DHPG *in vitro*. The increased number of Fos-LI cells by bath application of DHPG was significantly reduced in the presence of the PKC antagonist GF109203X (B) or in the prevention of ERK activation by PD98059 (C), compared to that with no treatment of DHPG (A; same as Fig. 2A). (D) A histogram demonstrates significant reductions of the DHPG-induced Fos expression by the antagonists used (*P < 0.05 vs. DHPG; #P < 0.05 vs. no DHPG). In the histogram, no DHPG and DHPG groups were borrowed from Fig. 1. Dotted boxes on the photographs indicate the counted areas in the Vo. The numbers on the bars indicate the numbers of sections counted. Scale bar (A), 100 μm.

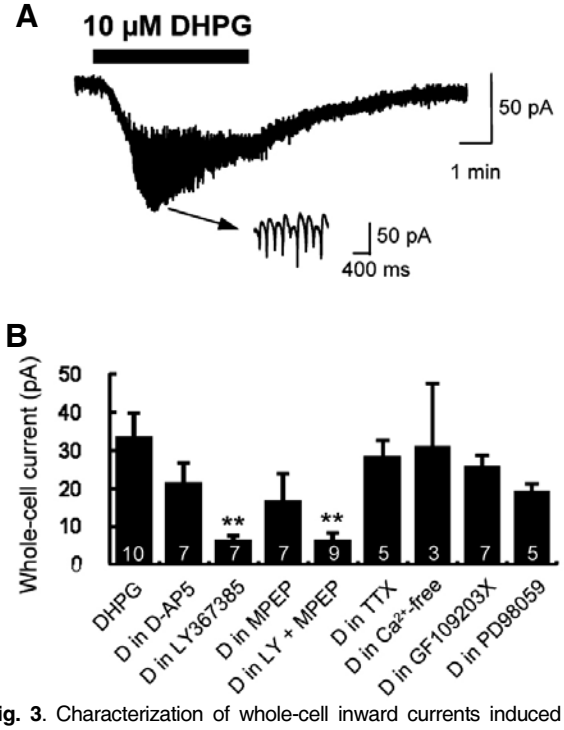

Fig. 3. Characterization of whole-cell inward currents induced by the activation of group I mGluRs. (A) Bath application of DHPG (10 μM), the group I mGluRs, induced whole-cell inward current in a Vo neuron at a holding potential of -70 mV. In this neuron, small spikes (*inset*) occurred during the DHPG application. (B) A histogram demonstrating pharmacological characterization of whole-cell inward currents induced by DHPG in Vo neurons (**P < 0.01 vs. DHPG). Only LY367385, the mGluR1 antagonist, alone or with MPEP, the mGluR5 antagonist, significantly inhibited the DHPG-induced inward current. The numbers on the bars indicate the numbers of Vo neurons recorded.

pA, n = 5, P > 0.05; Ca^{2+}-free, 30.8 ± 16.8 pA, P > 0.05; Fig. 3B). Further, GF109203X (1 μM) or PD98059 (50 μM) could not significantly reduce the inward currents induced by bath-applied DHPG (GF109203X, 25.4 ± 3.3 pA, n = 7, P > 0.05; PD98059, 19.0 ± 2.2 pA, n = 5, P > 0.05; Fig. 3B), indicating no involvement of PKC or ERK1/2 in the DHPG-induced, mGluR1-mediated inward currents.

DISCUSSION

The present study demonstrates that the increased Fos expression and the whole-cell inward currents, both induced by the activation of group I mGluRs in the Vo, may use different receptor subtypes and signalling mechanisms. The enhancement in the number of Fos-LI neurons by the selective group I mGluR agonist DHPG was mediated by both mGluR1 and mGluR5, and significantly inhibited by the blockades of NMDA receptors, PKC or ERK. However, the whole-cell inward currents induced by DHPG were only mediated by mGluR1, but not by mGluR5, NMDA receptors, PKC or ERK. These results suggest that, upon the mGluR1 activation by DHPG, membrane depolarisation (slow inward current in voltage-clamp mode) is upstream to, and does not require, the activations of NMDA receptor, PKC and ERK, whereas cooperative activations of them are necessary for the enhanced Fos expression in the Vo neurons.

In the present study, the expression of Fos was observed at 15 min following DHPG application because the peak inward currents typically occur within 10 min from DHPG application. Although the acute activation of c-fos occurs within 5 min and its mRNA peaks at 30-45 min following stimulation (Greenberg and Ziff, 1984; Greenberg et al., 1985; Mitsikostas and Sanchez del Rio, 2001), the time window observed is in some way faster than other *in vivo* studies that have been mostly performed at 2-4 h from the application of stimuli in the Vo (He et al., 2000; Sugimoto et al., 1998a; Takemura et al., 2000; Yoo et al., 2009). In addition, most of the staining profiles in the *in vitro*

study showed Fos staining in both nucleus and cytoplasm, rather than wholly in nucleus of Fos-LI cells as shown in other *in vivo* studies. This difference in the cellular location of Fos may be due to the difference of the time window. As demonstrated in a previous cell biological study (Bussolino et al., 2001), the first wave of Fos expression, following the stimulation of NIH 3T3 fibroblasts by fetal calf serum, peaked at 7.5 min, and the Fos proteins at this time stayed in the cytoplasm without entering the nucleus by the association with AP-1 transcription factor. The cytoplasmic Fos was associated with endoplasmic reticulum, and led to phospholipid activation. Therefore, it would be possible that Fos proteins in early period of expression were more concentrated in the cytoplasm, but those in later period in nucleus, supporting this study showing the cytoplasmic staining of Fos proteins in the Fos-LI cells when observed at 15 min from the start of the group I mGluR activation by DHPG.

An early report demonstrated that depolarisation by an increased extracellular concentration of potassium chloride triggered c-fos transcription (Greenberg et al., 1986). The depolarisation spreads through membrane of neuronal cell types, and activates NMDA receptor and L-type voltage-gated calcium channels, causing a localized calcium influx (Cohen and Greenberg, 2008). The localized calcium influx is capable of inducing c-fos expression via the calcium/calmodulin-dependent protein kinases (CaMKs) and cAMP response element binding protein (CREB) pathway existed in neuronal cells (Flavell and Greenberg, 2008). Although this study does not address the role of CaMKs and CREB, it demonstrates the precedence of the depolarisation process to the NMDA receptor activation in the signalling pathway for the DHPG-induced Fos expression since the inhibition of NMDA receptors did not block the DHPG-induced inward currents but prevented the enhancement of c-fos expression.

Although Fos can be activated by low threshold stimuli that do not induce central sensitization, a neurophysiological phenomenon playing a role in persistent pain conditions (Hunt et al., 1987; Latremoliere and Woolf, 2009), it has been vastly used as a marker to trace nociceptive neurons excited by various electrical, mechanical, chemical, and thermal stimuli (Coggeshall, 2005). Since the neuronal excitation is typically manifested by membrane depolarization or the increased number of spike generation, it would be interesting to investigate if membrane depolarization itself can correlate with the expression of Fos, further supporting the notion of neuronal activity-dependent gene expression and plasticity in the nervous system (Flavell and Greenberg, 2008). In the spinal cord, it has been known that *in vitro* application of DHPG induces membrane depolarization (Zhong et al., 2000) and also Fos expression (Giles et al., 2007). However, it has not yet been studied if the DHPG-induced depolarization uses a similar mechanism with, or contribute to, the DHPG-induced Fos expression. Thus, in this study to find out a correlation between whole-cell inward current, which is the reflection of membrane depolarisation, and Fos expression, it was investigated if PKC and ERK, which are known for the downstream targets of group I mGluRs (Kim et al., 2008), are involved in both phenomena. Consequently, the data, showing no accordance of group I mGluR subtypes, NMDA receptor and protein kinases in the DHPG-induced Fos expression and whole-cell inward currents, indicate that both phenomena existed in series, or uses different receptor subtypes and signalling pathways for their induction. In another way, these results rather suggest that synaptic activity or intracellular calcium rise are more readily connected with Fos expression (Flavell and Greenberg, 2008). Therefore, augmentation of spontaneous synaptic responses in amplitude which is the PKC- and ERK-dependent phenomenon (Song et al., 2009), may be a critical factor for the enhancement of Fos expression in the Vo.

The Vo neurons directly receives inputs from the central terminals of the trigeminal primary neuron that innervate the intraoral structures (Takemura et al., 1991). Therefore, it has been postulated that the Fos expression in the Vo area is the result of monosynaptic activation of nociceptive neurons predominantly by intraoral nociceptors. This notion has been reinforced by a result showing that the same area receives dense projection of trigeminal primary afferent neurons containing calcitonin gene-related peptide, a marker for primary nociceptors (Sugimoto et al., 1997). In the spinal DH, strong repetitive stimulation of primary afferents caused slow inward currents that are mediated by group I mGluRs (Galik et al., 2008). Thus, the activation of nociceptive primary afferents, innervating the intraoral area, may also produce inward currents via glutamate spillover and the activation of group I mGluRs at extrasynaptic sites in the Vo. Therefore, *in vitro* DHPG application in the present study resembles the strong *in vivo* activation of the trigeminal nociceptors. In this regard, the Fos expression by *in vitro* DHPG in the Vo could be a reflection of that induced by *in vivo* noxious activation of trigeminal nociceptors using electrical or chemical stimuli (He et al., 2000; Sugimoto et al., 1998a; Takemura et al., 2000). Hence, both PKC and ERK mediation in the Fos expression in the Vo area of *in vitro* horizontal brainstem slices would be also plausible for the Fos expressed by the trigeminal nociceptors *in vivo*. In addition, the *in vitro* slice models would be useful to further elucidate complex mechanisms of Fos induction that underlie an establishment of central sensitization in the pain induction or maintenance pathways (Latremoliere and Woolf, 2009).

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