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도파민 **D1** 수용체 작용제
SKF-38393에 의한
transient receptor potential
vanilloid-1 수용체 활성화

Activation of TRPV1 by D1R agonist SKF-38393
in mouse dorsal root ganglion neuron

2014년 8월

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ABSTRACT

Activation of TRPV1 by D1R agonist SKF-38393 in mouse dorsal root ganglion neuron

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Transient receptor potential vanilloid-1 (TRPV1) is a ligand-gated ion channel expressed on nociceptive sensory neuron, which transduces extracellular stimuli such as noxious heat, proton, and capsaicin. It is well known the activity of TRPV1 is modulated by a variety of intracellular signaling pathways. Dopamine is a neurotransmitter that plays important roles in motor control, cognition, and pain modulation in the central nervous system (CNS). Dopamine receptor is a class of G-protein coupled receptor (GPCR) that regulates cellular functions through its downstream intracellular signaling pathways. Previous studies have demonstrated the expression of D1-like receptors (D1R and D5R) in nociceptive sensory neurons. However, the effect of dopamine receptor on TRPV1 remains unknown.

This study was designated to examine the effects of D1R activation on TRPV1 in mouse dorsal root ganglion (DRG) neuron using Ca^{2+} imaging and immunofluorescence. It was found that D1R agonist SKF-38393 induces reproducible Ca^{2+} response in nociceptive sensory neurons. The SKF-38393-induced Ca^{2+} response results from influx through TRPV1 rather than Ca^{2+} mobilization from intracellular Ca^{2+} stores. Immunofluorescence analysis revealed co-expression of D1R and TRPV1 in DRG neurons. Whereas PKC inhibitor, diacylglycerol (DAG) lipase inhibitor, adenylyl cyclase (AC) inhibitor, and PKA inhibitor had no effects on SKF-38393-induced Ca^{2+} response, phospholipase C (PLC) specific inhibitor blocked SKF-38393-induced Ca^{2+} response. Taken together, these data suggest that Ca^{2+} response by SKF-38393 result from direct activation of TRPV1 by PLC/DAG-mediated membrane-delimited pathway.

In conclusion, these results provide strong evidence that trans-activation of TRPV1 following D1R activation may contribute to the modulation of pain signaling in nociceptive sensory neurons.

keywords : dopamine, dopamine receptor, D1R, SKF-38393, TRPV1, diacylglycerol (DAG), nociceptor

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INTRODUCTION

Transient receptor potential vanilloid subtype-1 (TRPV1) is a representative transducer of nociceptive sensory neuron that plays important roles in nociception. TRPV1 is activated by noxious heat, proton, and chemical such as capsaicin (Caterina et al., 1997; Tominaga et al., 1998; Szallasi and Blumberg, 1999; Clapham, 2003). Notably, the activation of TRPV1 is modulated by a variety of intracellular signaling pathways. cAMP-dependent protein kinase (PKA) regulates TRPV1 desensitization by direct phosphorylation (Bhave et al., 2002). Protein kinase C (PKC) is also involved on activation of TRPV1 (Premkumar and Ahern, 2000; Vetter et al., 2006). In our laboratory's previous study, we demonstrated direct activation of TRPV1 by DAG (Woo et al., 2008; Kim et al., 2009).

Dopamine (DA) is a neurotransmitter in a catecholamine family that plays numerous important roles in the nervous system (Cools and Vanrossum, 1980; Joyce, 1983). Dopamine exerts cellular effects by binding with a specific membrane receptor. Dopamine receptor is a class of G protein-coupled receptor (GPCR) that affects through various intracellular signaling pathways. Dopamine receptor has been classified into five subtypes, D1R to D5R, which are categorized into D1-like (D1R, D5R) and D2-like (D2R to D4R) subfamilies based on their molecular structures and functional properties (Jackson and Westlinddanielsson, 1994; Jaber et al., 1997; Neve and Neve, 1997; Missale et al., 1998). D1-like receptor activates adenylyl cyclase (AC)

and increases intracellular cAMP concentrations, while activation of D2-like receptor acts in opposite way (Huff, 1997; Andringa et al., 1999; Cools et al., 2002). Previous studies revealed that there is another D1-like receptor that elicits the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) into diacylglycerol (DAG) and inositol triphosphate (IP₃) through stimulation of phospholipase C (PLC) (Jin et al., 2003; Neve et al., 2004; Zhen et al., 2005).

It is well established that distribution and contribution of dopamine receptor in the central nervous system (CNS). Dopamine receptors were widely expressed in various brain regions, particularly in cerebral cortex, hippocampus, basal ganglia (Meador-Woodruff and Mansour, 1991; Weiner et al., 1991; Huang et al., 1992; Bergson et al., 1995; Yung et al., 1995; Gurevich and Joyce, 1999; Hurd et al., 2001; Kumar and Patel, 2007). The expression and functional contribution of dopamine receptors in the spinal cord have been demonstrated along with their underlying mechanism for pain transmission. Dopaminergic fibers that originate from area A11 in the diencephalon project to the dorsal horn of the spinal cord and represent the major source of dopamine in the spinal cord (Skagerberg et al., 1982; Skagerberg and Lindvall, 1985; Barraud et al., 2010). It was reported that dopamine in spinal cord exerts anti-nociceptive action by modulating activity of nociceptive transmission through D2-like receptors (Jensen and Yaksh, 1984; Barasi and Duggal, 1985; Barasi et al., 1987; Fleetwood-Walker et al., 1988; Tamae et al., 2005; Zhu et al., 2007; Taniguchi et al., 2011). Recent study shows that dopamine receptors were expressed not only in CNS, but also in dorsal root ganglion (DRG) neurons in peripheral nervous system (PNS). A subpopulation of DRG neurons expresses tyrosine

hydroxylase (TH), the rate-limiting enzyme for the synthesis of dopamine (Price and Mudge, 1983). mRNAs and alternatively spliced transcripts of dopamine receptors have been detected in peripheral sensory neurons (Xie et al., 1998; Brumovsky et al., 2006a), and the expression of dopamine receptor in DRG neurons was confirmed by immunofluorescence imaging (Brumovsky et al., 2012).

The expression of dopamine receptor was limited in small diameter DRG neurons, mainly nociceptive sensory neuron, as D1R and D5R were primarily expressed but D2R was not expressed (Galbavy et al., 2013). Dopamine influences on the properties of various ion channels in DRG neuron through intracellular signaling pathways (Li et al., 1998; 2000; Molokanova and Tamarova, 2003; Li et al., 2005; Galbavy et al., 2013). Moreover, it was reported that spontaneous glutamate release were enhanced by activation of D1-like receptor via PLC and TRPV1 in rat somatosensory cortical neurons (Chu et al., 2010). However, the effect of dopamine receptor on TRPV1 in nociceptive sensory neuron still remains poorly understood.

In this study, I hypothesized that D1-like receptor and TRPV1 are co-expressed in DRG neuron, and activation of D1-like receptor recruits intracellular signaling pathway, to modulate TRPV1 activity in nociceptive sensory neuron. To prove this hypothesis, the effects of D1R activation on TRPV1 in mouse DRG neurons were investigated by using Ca^{2+} imaging experiments with D1R agonist SKF-38393. Our results suggest that D1R activation causes trans-activation of TRPV1 through PLC/DAG pathway in nociceptive sensory neurons.

MATERIALS AND METHODS

All Surgical and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the School of Dentistry, Seoul National University (Seoul, Korea). Animal treatments were performed according to the ethical guidelines of the International Association for the Study of Pain (Zimmermann, 1983) and the Guide for the Care and Use of Laboratory Animals (National Research Council (US), 2011). Adult C57BL/6J wild-type male mouse (OrientBio, Sungnam, Korea) were used. Animals were housed in a conventional facility with a 12:12 hr light cycle (lights on 8.00am) and ad libitum access to water and chow. Mice were acclimatized for at least one week prior to experiments.

DRG Preparation and Primary Neuron Culture

DRG neurons were obtained from 6 weeks old adult mice. Animals were anesthetized with isoflurane and decapitated. Under aseptic condition, spinal cord including thoracic and lumbar segments were removed and placed in cold HBSS (Welgene). The bones surrounding spinal cord were removed and exposed DRGs were pulled out. After removing roots, DRGs were incubated with 1 mg/ml collagenase A (Roche) and 2.4 U/ml dispase-II (Roche) in HBSS for 40 min at 37°C. Subsequently, DRGs were digested with 0.25% trypsin (Sigma) for 5 min at 37°C, followed by 0.25% trypsin inhibitor (Sigma). DRGs were

then washed in DMEM (with 10% FBS, Welgene) and mechanically dissociated with a flame-polished Pasteur pipettes in the presence of 0.05% DNase I (Sigma). DRG neurons were plated on Poly-D-Lysine (PDL)-coated 12mm glass coverslip and grown in Neurobasal defined medium (with 5% B-27 supplement, Invitrogen) with 5% CO₂ at 37°C. DRG neurons were maintained for 24 hrs before use.

Ca²⁺ Imaging

Ca²⁺ response of DRG neuron was measured using the fluorescent Ca²⁺ probe (indicator) Fluo-3 AM (Enzo Life Science). DRG neurons were plated on PDL-coated 12mm glass coverslip 24 hrs before use. DRG neurons were loaded with Fluo-3 AM (5 μM) mixed with 1 μl Pluronic acid (20% solution in DMSO, Life technologies) in DMEM medium (with 10% FBS) for 40 min at 37°C. Fluo-3 loaded cells on coverslip were mounted onto the chamber (12mm ChamSlide AC, total volume of 500 μl, Live Cell Instrument, Korea), and placed onto an inverted microscope (Olympus IX70, Japan). Then, the cells were washed by bath medium for 5 min. We used an external bath medium (normal Tyrode solution) of the following composition (in mM): 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, and 10 N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), with pH adjusted to 7.3-7.4 using NaOH. All drugs and bath medium were applied via bath perfusion at a flow rate of 5 ml/min.

Cells were excited with a LED source pE-100 (CoolLED, UK) at 470 nm and emissions were recorded at 535 nm wavelengths. Fluorescent emission readings were recorded and stored on hard disk every 0.5 s.

Intracellular calcium concentrations ($[Ca^{2+}]_i$) were measured by digital video microfluorometry with an intensified charge-coupled-device (CCD) camera (QIClick, QImaging, Canada) coupled to the microscope and a computer with software (MetaMorph® NX, Molecular Devices, US).

Ca^{2+} responses were presented as a pseudoratio ($\Delta F/F$) to estimate comparison fluorescence intensity, because Fluo 3-AM is a non-ratiometric Ca^{2+} indicator. Single-wavelength values are different depending on the concentration of loaded dye in respective cells. Following formula indicates pseudoratio (Takahashi et al., 1999).

$$\Delta F/F = (F_1 - F_{base})/F_{base}$$

F_1 = measured intensity of the cell after stimulation

F_{base} = measured intensity of the cell before stimulation

Immunohistochemistry

Immunohistochemical processes were performed as previously described (Kim et al., 2008). Briefly, 6 weeks old male C57BL/ mouse were perfused with physiological saline and sequentially with 4% paraformaldehyde in 0.1 M phosphate buffer (PBS, pH 7.4). DRGs in lumbar segments were removed and immersed in the post-fixative at 4°C overnight and then transferred to 30% sucrose in PBS for 48 hrs. Serial frozen transverse sections (10 μ m thickness) were mounted on slides. All procedures were performed at room temperature (RT) unless otherwise stated. Slide were washed in PBS and then incubated in the blocking solution containing 5% normal goat serum, 2% BSA, 2% FBS,

and 0.1% Triton X-100 for 1 hr. The sections were incubated overnight at 4°C with primary guineapig anti-TRPV1 antibody (1:1000; Chemicon AB5566) and primary mouse anti-D1R antibody (1:200; Millipore MAB5290), washed 3 times with PBS. Then, incubated with CY3-conjugated donkey anti-guinea pig IgG antibody (1:1000; Jackson ImmunoResearch, West Grove, PA, USA) and FITC-conjugated donkey anti-mouse IgG antibody (1:500; Jackson ImmunoResearch, West Grove, PA, USA) for 1hr. After being washed with PBS, sections were mounted with VectaShield, mounting media (Vector Laboratories, Inc., Burlingame, CA, USA), which contained DAPI to detect nuclei and visualized using a confocal microscope using the appropriate filter sets (FV-300; Olympus, Tokyo, Japan).

Drugs

SKF-38393, SCH-23390, Capsaicin, Capsazepine, SQ 22536, H-89, RHC 80267, BIM, Thapsigargin were obtained from Sigma and U73122, U73343 were purchased from Tocris Bioscience.

Statistical Analyses

Statistical analyses of the significances in normalized Ca^{2+} responses (2nd SKF-38393 response relative to 1st SKF-38393 response) were carried out by SPSS software (SPSS Statistics 17.0, IBM). Data were compared by one-way ANOVA followed by Bonferroni's post-hoc test. Differences were considered to be significant when P value was less than .05. All data are presented as mean $\pm$ SEM; the numbers of cells tested are indicated in parentheses where applicable.

RESULTS

D1R agonist SKF-38393 induces Ca^{2+} response in DRG neuron in a dose-dependent manner

The effects of D1R activation in DRG neurons were determined with small and medium diameter cells. Firstly, we examined whether D1R agonist SKF-38393 induces Ca^{2+} response in DRG neurons using Fluo-3 AM-based Ca^{2+} imaging. A 10 s application of 1 μ M SKF-38393 induced reproducible Ca^{2+} response during sequential application of SKF-38393 ($83.74 \pm 4.53\%$; $n = 8$; $p > 0.05$) (Fig 1A). A 100 s pretreatment of 10 μ M SCH-23390, a D1R selective antagonist, inhibits SKF-38393-response, indicating that the response to SKF-38393 was mediated through activation of the D1R ($6.31 \pm 1.04\%$; $n = 8$; $p < 0.005$) (Fig 1B). SKF-38393-sensitive neurons exhibited concentration-dependent increase in $[Ca^{2+}]_i$, with the half-maximal concentration (EC_{50}) of SKF-38393, 517 nM (Fig 1D). Therefore, activation of D1R causes a transient increases in intracellular Ca^{2+} level with dose-dependent manner in a subpopulation of DRG neurons.

SKF-38393-induced Ca^{2+} response results from influx of extracellular Ca^{2+}

Next, we examined the source of increasing $[Ca^{2+}]_i$ by SKF-38393. To

exclude extracellular Ca^{2+} influx, 0 mM Ca^{2+} solution was applied for 100 s before 2nd SKF-38393 application. 2nd SKF-38393-induced Ca^{2+} response was blocked by pretreatment with 0 mM Ca^{2+} in the bath solution compared with the Ca^{2+} response to 1st SKF-38393 application ($4.70 \pm 1.12\%$; $n = 8$; $p < 0.005$) (Fig 2A). Next, we used thapsigargin, a selective inhibitor of the sarcoplasmic Ca^{2+} -ATPase, to exclude Ca^{2+} mobilization from intracellular Ca^{2+} stores (Kirby et al., 1992). SKF-38393-induced Ca^{2+} response did not change in the presence of 1 μM thapsigargin ($71.53 \pm 6.55\%$; $n = 8$; $p > 0.05$), and the depletion of intracellular Ca^{2+} stores was confirmed by a following little Ca^{2+} response to application of thapsigargin (Fig 2B). The results of 0 mM Ca^{2+} in bath solution abolished SKF-38393-induced Ca^{2+} response, not by the thapsigargin, suggest that most of SKF-38393-induced Ca^{2+} response is as a consequence of an influx of extracellular Ca^{2+} rather than Ca^{2+} release from intracellular Ca^{2+} stores.

SKF-38393 induces Ca^{2+} response through TRPV1 in nociceptive sensory neuron

We next examined whether SKF-38393-induced Ca^{2+} response was associated with TRPV1. The Ca^{2+} response was elicited by 1st SKF-38393 application, and 2nd SKF-38393-induced Ca^{2+} response was blocked by a pretreatment with a TRPV1 blocker, 10 μM capsazepine ($6.59 \pm 1.24\%$; $n = 8$; $p < 0.005$) (Fig 3A). To identify TRPV1-expressing nociceptive neurons, TRPV1 agonist, 300 nM capsaicin, was applied for a 5 s at the end of each experiment. In 86 out of 134 DRG neurons that had a capsaicin-sensitivity (64%; $n =$

86/134), we found 57 neurons responded to SKF-38393 (66%; n = 57/86). 29 out of 86 neurons (34%; n = 29/86) only showed capsaicin response. SKF-38393-only-responsive neuron was not observed.

TRPV1 is more permeable to Ca^{2+} than other cations (Caterina et al., 1997; Chung et al., 2008). To test whether voltage-activated Ca^{2+} channels (VACCs) contributed to SKF-38393-induced Ca^{2+} response, 100 μM CdCl_2 was pretreated in bath solution to non-selectively block all VACCs without influence on TRPV1 (Shoudai et al., 2010). SKF-38393 evoked Ca^{2+} response even in the presence of Cd^{2+} ($79.61 \pm 7.27\%$; n = 8; p > 0.05) (Fig 3B). Together, these results indicated that SKF-38393 activates TRPV1 channels in nociceptive sensory neurons.

Co-expression of D1R and TRPV1 in dorsal root ganglia

Our results strongly suggest that stimulation of D1R activates TRPV1, suggesting D1R and TRPV1 are expressed together in the same DRG neurons. Previous study reported that D1R and D5R were expressed in small diameter DRG neuron (Galbavy et al., 2013). To confirm whether D1R and TRPV1 were co-localized, we explored the expression of D1R and TRPV1 by immunofluorescent staining in intact dorsal root ganglia. D1R was homogeneously expressed in DRG neurons. The expression of D1R has no clear pattern and intensity of staining varied in DRG neurons. Consistent with previous reports, TRPV1 was primarily expressed in small diameter DRG neurons (Guo et al., 1999; Amaya et al., 2004). Overall, the expression of D1R and TRPV1 was consistent with previous studies, and most of TRPV1-positive DRG neurons were also expressed D1R.

SKF-38393-induced Ca^{2+} response in DRG neuron results from direct activation of TRPV1 by diacylglycerol (DAG)

Activation of D1R activates adenylyl cyclase (AC) and increases intracellular cAMP levels (Missale et al., 1998; Glovaci et al., 2014). Other study presents that there are D1R which stimulates PLC pathway and hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into Diacylglycerol (DAG) and inositol triphosphate (IP_3) (Jin et al., 2003; Chu et al., 2010). Both AC and PLC pathway can affect to function of TRPV1 by phosphorylation or directly (Bhave et al., 2002; Zhuang, 2004). To determine which signaling molecules mediate SKF-38393-induced TRPV1 activation, we examined with respective inhibitors of signal molecules. Pretreatment with 2 μ M U 73122, a specific PLC inhibitor abolished SKF-38393-induced Ca^{2+} response ($3.79 \pm 1.25\%$; $n = 8$; $p < 0.005$) (Fig 5A), whereas U 73343, an inactive-form of U 73122 at the same concentration did not influence on Ca^{2+} response by SKF-38393 ($74.74 \pm 5.36\%$; $n = 8$; $p > 0.05$) (Fig 5B). SKF-38393-induced Ca^{2+} response endured in the presence of 1 μ M Bisindolylmaleimide I (BIM), a specific PKC inhibitor ($77.20 \pm 3.73\%$; $n = 8$; $p > 0.05$) (Fig 5C) or 1 μ M RHC 80267, a DAG lipase inhibitor ($78.38 \pm 10.54\%$; $n = 8$; $p > 0.05$) (Fig 5D) or its cocktail ($77.92 \pm 4.58\%$; $n = 8$; $p > 0.05$) (Fig 5E), indicating that PLC pathway mediated SKF-38393-induced Ca^{2+} response, but not PKC- and DAG lipase-dependent activation of TRPV1. Previously we have shown that TRPV1 was directly activated by OAG, a membrane-permeable analog of DAG (Woo et al., 2008). Together these results, we conclude DAG

is the most appropriate candidate for downstream signaling molecule of PLC pathway which mediated SKF-38393-induced Ca^{2+} response. In addition, AC/PKA pathway also can be stimulated by D1R activation. Therefore, we tested whether AC/PKA signaling pathway was also involved in SKF-38393-induced Ca^{2+} response. Pretreatment with 10 μM SQ 22,536, an adenylyl cyclase inhibitor ($74.49 \pm 8.45\%$; $n = 8$; $p > 0.05$) (Fig 5F) and 10 μM H-89, a PKA inhibitor ($84.10 \pm 5.62\%$; $n = 8$; $p > 0.05$) (Fig 5G) had no effect to SKF-38393-induced Ca^{2+} response.

Taken together, these results indicate that D1R agonist SKF-38393 activates TRPV1 through PLC signaling pathway, and imply that DAG is a downstream signaling molecule of PLC pathway to activation of TRPV1 by SKF-38393.

Figure 1

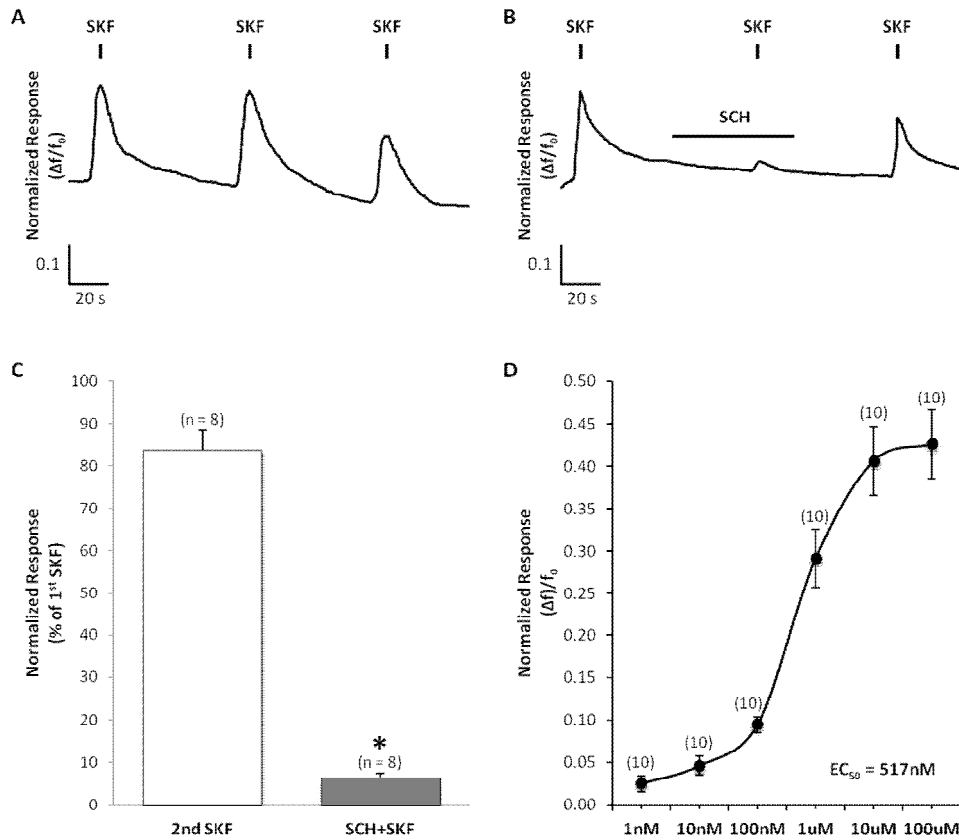


Figure 1. SKF-38393 induces Ca^{2+} response in DRG neuron. A, Sequential application of 1 μ M SKF-38393 (SKF) evokes Ca^{2+} responses with slight decreasing, but no differences ($n = 8$). B, SKF-38393-induced Ca^{2+} response was blocked by a 100 s pretreatment of 10 μ M SCH-23390 (SCH, $n = 8$). C, Summary of normalized Ca^{2+} response (2nd SKF-38393 response relative to 1st SKF-38393 response). (* $p < 0.005$; non-paired t test) D, Dose-response curve of SKF-38393-induced Ca^{2+} response in mouse DRG neuron showed that EC_{50} is 517 nM ($n = 10$ in respective concentration). Results are presented as the mean $\pm$ SEM.

Figure 2

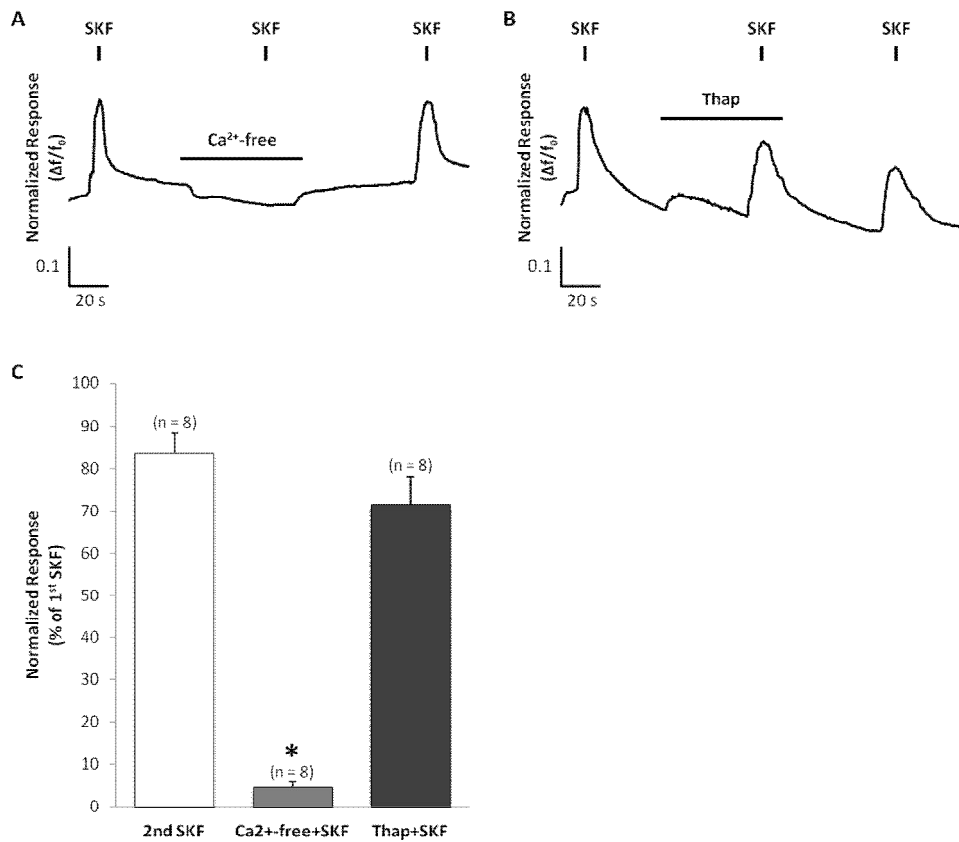


Figure 2. SKF-38393-induced Ca^{2+} response results from extracellular Ca^{2+} influx. A, SKF-38393-induced Ca^{2+} response was abolished by pretreatment of 0 mM Ca^{2+} in the bath solution (n = 8). B, SKF-38393 induces Ca^{2+} response without change in the presence of 1 μM Thapsigargin (Thap, n = 8). C, Summary of normalized Ca^{2+} response (2nd SKF-38393 response relative to 1st SKF-38393 response). (*p < 0.005; one-way ANOVA) Results are presented as the mean $\pm$ SEM.

Figure 3

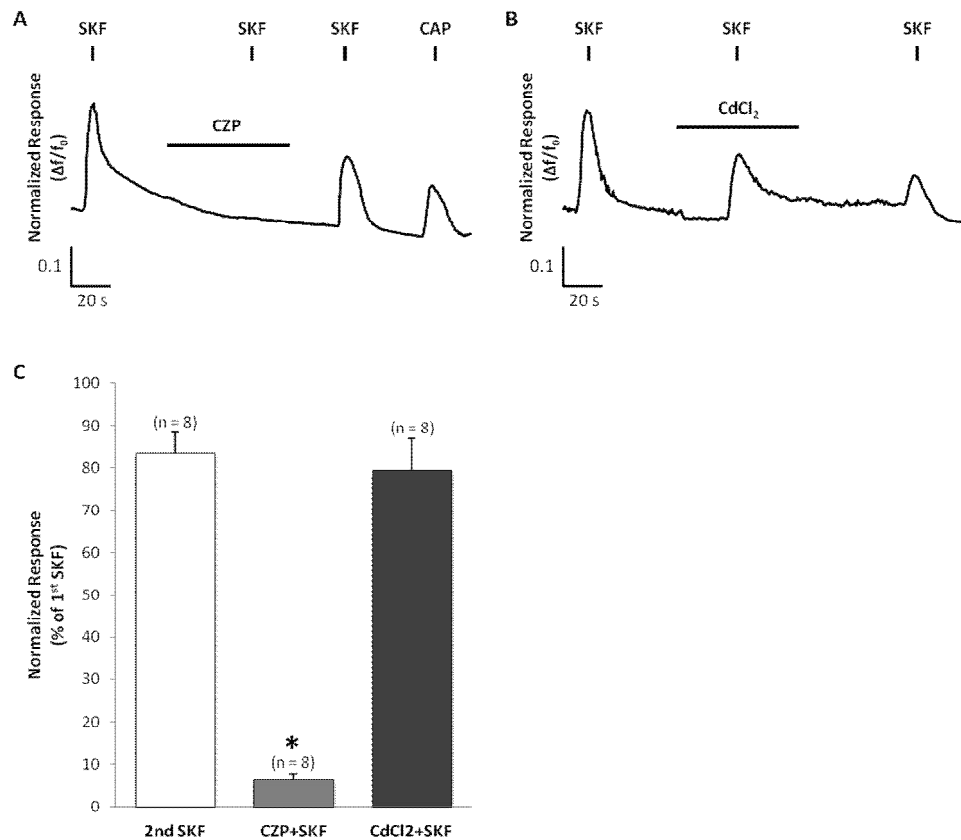


Figure 3. SKF-38393 induces Ca^{2+} response through activation of TRPV1. A, SKF-38393-induced Ca^{2+} response was blocked by the pretreatment of 10 μM capsazepine (CPZ, $n = 8$). B, SKF-38393-induced Ca^{2+} response maintained in the 1 μM Cd^{2+} condition ($n = 8$). C, Summary of normalized Ca^{2+} response (2nd SKF-38393 response relative to 1st SKF-38393 response). (* $p < 0.005$; one-way ANOVA) Results are presented as the mean $\pm$ SEM.

Figure 4

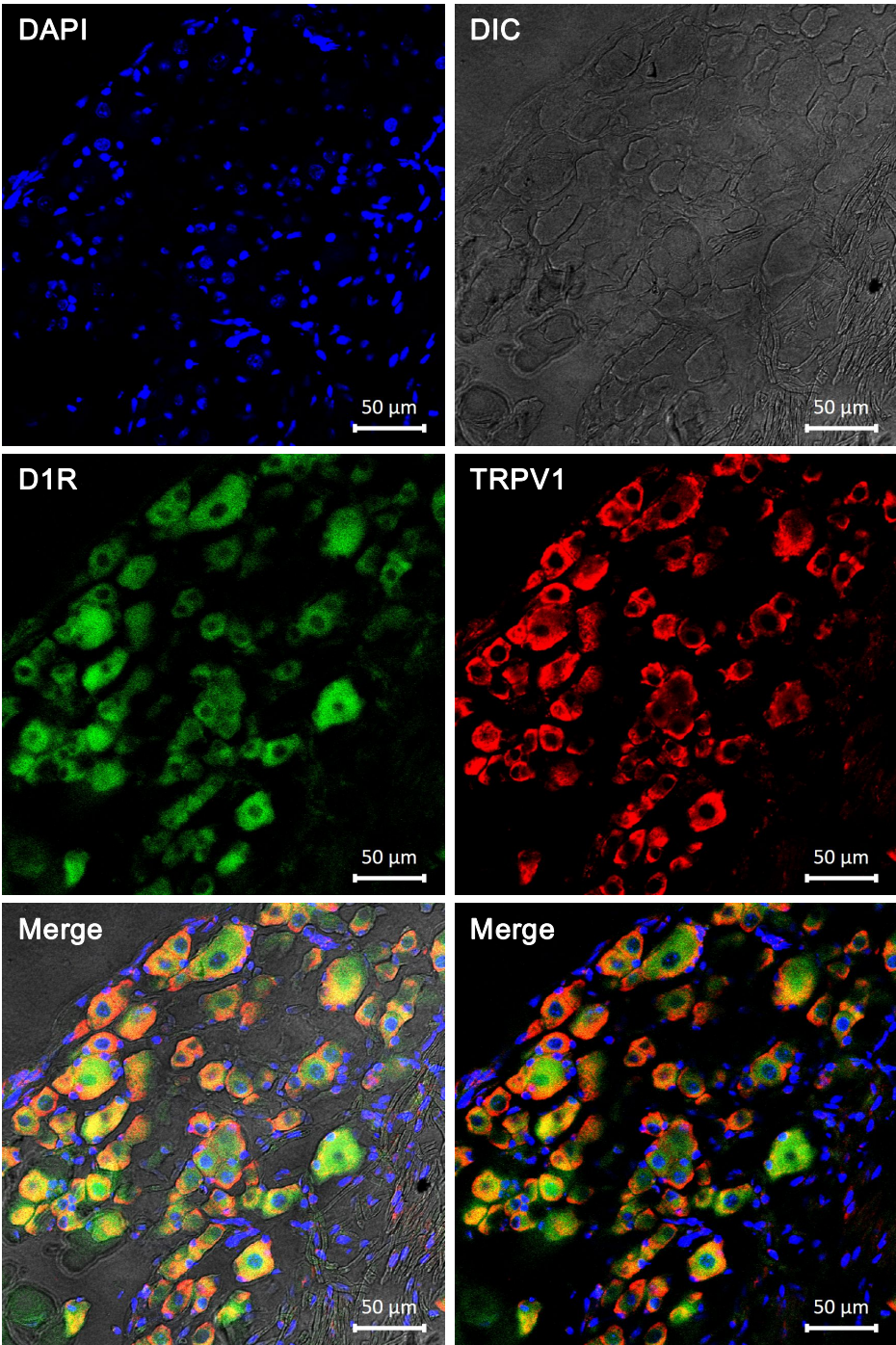
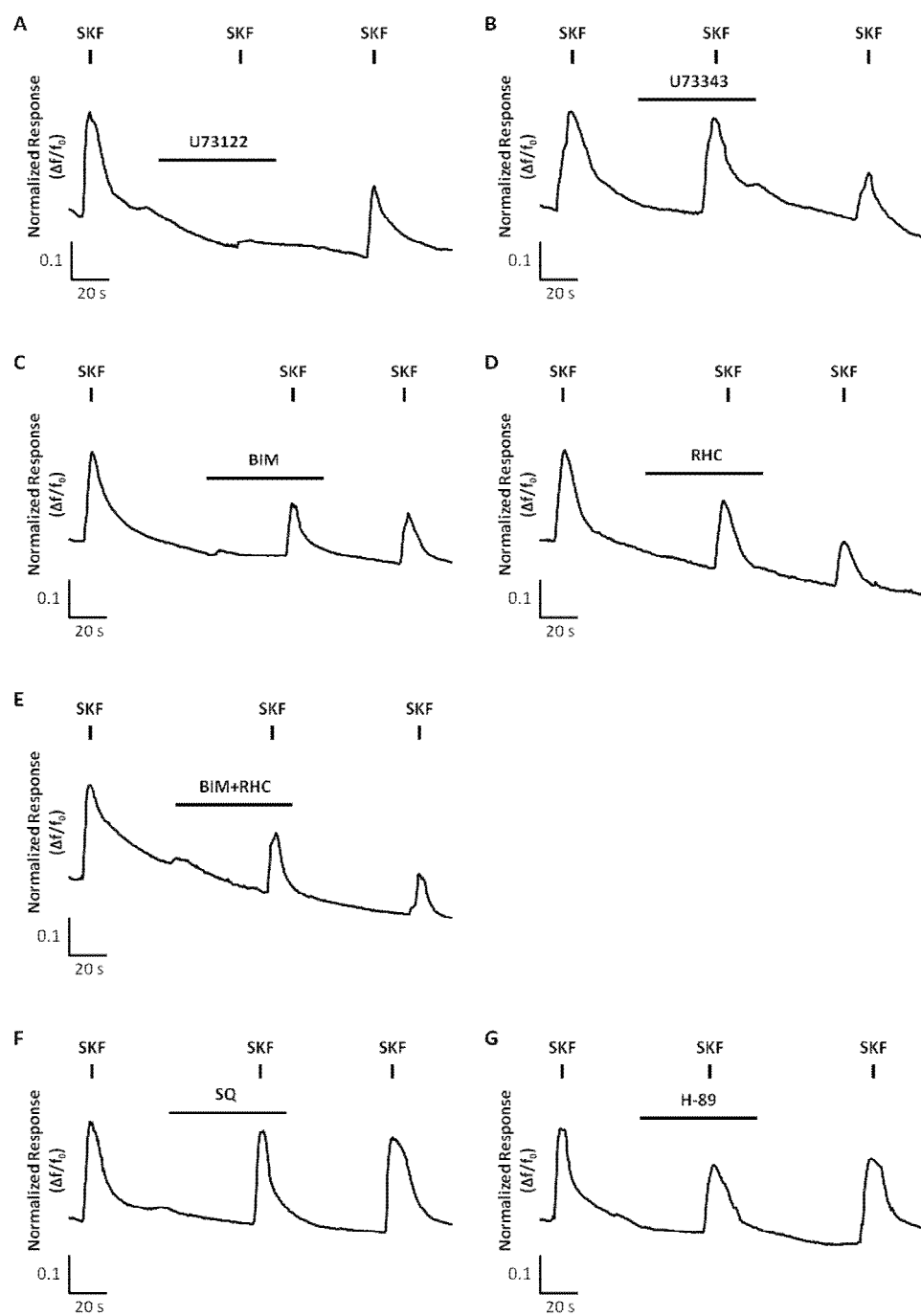


Figure 4. Co-expression of D1R and TRPV1 in dorsal root ganglia. 20 μm frozen sections were prepared from lumbar DRG taken from 6 weeks old C57BL/6 mouse. Nuclei of DRG neurons were stained by mounting media which contained DAPI (blue channel). Sections of dorsal root ganglia were respectively stained with monoclonal antibodies to either D1R (green channel) or TRPV1 (red channel), and FITC/CY3-labeled secondary antibody. Merged images indicate co-expression of D1R and TRPV1 (DIC^{+/−}). Scale bars shown are 50 μm .

Figure 5



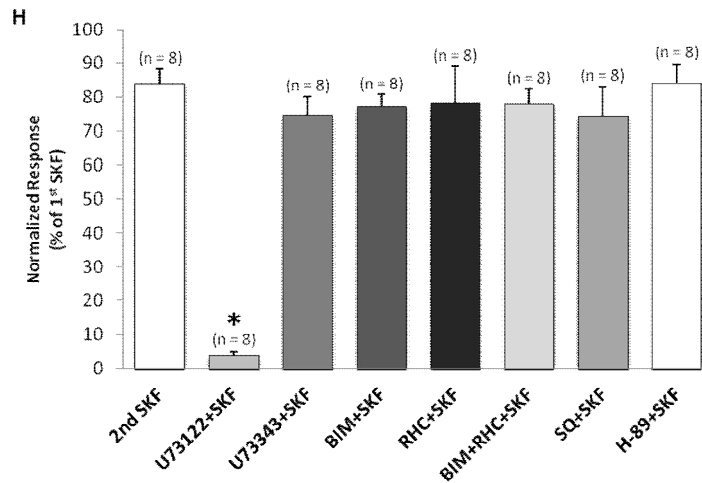


Figure 5. SKF-38393 induces Ca^{2+} response in consequence of direct activation of TRPV1 by PLC/DAG pathway. A, SKF-38393-induced Ca^{2+} response was eliminated in the presence of 2 μM U 73122 ($n = 8$). B, 2 μM U 73343 had no effect on SKF-38393-induced Ca^{2+} response ($n = 8$). C,D,E, 1 μM Bisindolylmaleimide I ($n = 8$), 1 μM RHC 80267 ($n = 8$), and mixture of both ($n = 8$) also had no effect on SKF-38393-induced Ca^{2+} response. F,G, SKF-38393-induced Ca^{2+} response was not blocked by the pretreatment with 10 μM SQ 22,536 ($n = 8$) or 10 μM H-89 ($n = 8$). H, Summary of normalized Ca^{2+} response (2nd SKF-38393 response relative to 1st SKF-38393 response). (* $p < 0.005$; one-way ANOVA) Results are presented as the mean $\pm$ SEM.

DISCUSSION

In the present study, it was found that stimulation of D1R trans-activates TRPV1 in mouse DRG neurons. Calcium imaging revealed that SKF-38393, a specific D1R agonist, increased $[Ca^{2+}]_i$ in subpopulation of capsaicin-sensitive DRG neurons, which was blocked by TRPV1 antagonist, capsazepine, indicating the involvement of TRPV1. Immunofluorescent staining further revealed co-expression of D1R and TRPV1 in DRG neurons. By using pharmacological analysis with the calcium imaging, endogenous DAG produced by D1R activation was found to lead to TRPV1 activation.

In the present study, we focused on the excitatory effect of dopamine in DRG neuron by using a specific D1R agonist SKF-38393. The biphasic effect of dopamine depends on the subclass of dopamine receptor, D1-like or D2-like. Excitatory effect of dopamine was mediated by D1-like receptor through intracellular signaling pathways. In contrast, Inhibitory effect was mediated by D2-like receptor (Mahan et al., 1990; Marshall et al., 1997; Sidhu, 1998; Neve et al., 2004). Recent studies characterized the expression of dopamine receptors in DRG neurons by Western blot and immunofluorescence imaging. It was reported that D1R and D5R were primarily expressed in small diameter DRG neuron, but expression of D2R was not observed (Galbavy et al., 2013). Based on these reports, we inferred that excitatory effect of dopamine would be major in nociceptive sensory neuron. It was shown that stimulation of D1R trans-activated TRPV1

and increased intracellular Ca^{2+} concentration. However, further experiments will require to test whether dopamine indeed induces identical excitatory effect via D1-like receptor.

Our results in DRG neuron show that the effect of D1R activation was mediated by TRPV1. SKF-38393-induced Ca^{2+} response was blocked by capsazepine. This result was consistent with previous study about activation of D1R in rat somatosensory cortical neuron (Chu et al., 2010). The possible contribution of intracellular Ca^{2+} release was excluded by the pretreatment of thapsigargin. Thapsigargin empties intracellular Ca^{2+} stores, endoplasmic reticulum (ER), as a result of inhibiting Ca^{2+} uptake pathway (Kirby et al., 1992). Intracellular Ca^{2+} store were depleted by thapsigargin, however, SKF-38393 response was not changed.

Since voltage-activated Ca^{2+} channels (VACCs) were also concerned as another possible portion of Ca^{2+} response, we verified the irrelevance of VACC to SKF-38393-induced Ca^{2+} response by using 100 μM CdCl_2 to inhibit VACC without influences on TRPV1 (Shoudai et al., 2010). In the presence of Cd^{2+} , SKF-38393-induced Ca^{2+} response was no different from SKF-38393 only response. In addition, taken from this result, inhibitory effect of capsazepine (at micro-molar concentrations) on VACCs could be negligible under our experimental conditions (Docherty et al., 1997).

SKF-38393 response was prevented by PLC inhibitor, but not by a specific PKC inhibitor, DAG lipase inhibitor, and the cocktail of both. The existence of phosphatidylinositol-linked D1-like receptor was

described in previous studies (Missale et al., 1998; Neve, 2005). However, it is unclear which downstream signaling molecule activates TRPV1. Previously we reported that endogenous DAG directly activates TRPV1 which produced by GPCR-activation (Woo et al., 2008). Moreover, we have recently shown that group I mGluR5 activates TRPV1 channel in a membrane-delimited manner and it was mediated by DAG (Kim et al., 2009). Together these data, it seems clear that DAG, produced by D1R activation, is the most plausible endogenous ligand for direct activation of TRPV1.

In addition, we found that the effect of SKF-38393 persisted in the presence of the adenylyl cyclase (AC) inhibitor or PKA inhibitor. This result is in contrast with previous study. It was reported that capsaicin-induced currents were abolished by AC and PKA inhibitor in mouse DRG neurons (Park et al., 2011). Even the experimental conditions were different, as a indirect activation through intracellular signaling pathway and direct activation by specific agonist, it seems that more studies are needed to better characterize the intracellular signaling pathways to TRPV1 modulation.

It has been demonstrated that dopaminergic nerve in dorsal horn (DH) of the spinal cord originated from the A11 area in the diencephalon (Barraud et al., 2010). It may seem that dopamine was released from dopaminergic nerve in dorsal horn to DRG neurons. Previous studies were well established that descending pain modulation of dopamine is related with the dopaminergic nerve and D2-like receptors (Jensen and Smith, 1982; Barasi et al., 1987; Shimizu et al., 2004). However, descending modulation of dopamine is still

debating whether direct effect on DRG neurons or indirect effect through interneurons (Fleetwood-Walker et al., 1988; Millan, 2002; Ossipov et al., 2010). Previous observations support that dopamine may be released by itself in DRG neuron (Price and Mudge, 1983; Philippe et al., 1993; Brumovsky et al., 2006b; 2012). Recently, it has been reported that epidermis cells such as keratinocytes or melanocytes expressed tyrosine hydroxylase (TH), the rate-limiting enzyme for the dopamine metabolic pathway (Ramchand et al., 1995; Chang et al., 1996; Gillbro et al., 2004). If dopamine receptors are expressed in the epidermal nerve fibers, especially unmyelinated afferent fibers, epidermis cell also can be another candidate of dopamine source to DRG neuron. Moreover, it might be possible explanation to itching sensations in Parkinson's patients (Giuffrida et al., 2005). In-vitro experiments are needed to understand the meaning of trans-activation of TRPV1 by D1R activation in DRG neuron. And In-vivo studies are also needed to explore the role of dopamine in peripheral pain modulation.

In conclusion, our present study demonstrates that activation of D1R trans-activates TRPV1 in mouse DRG neuron. Most of D1R-expressing DRG neurons were nociceptive sensory neurons, expressing TRPV1. D1R agonist SKF-38393 induces Ca^{2+} response through direct activation of TRPV1 by PLC/DAG intracellular signaling pathway without involvement of AC/PKA cascade. Our finding on the effect of D1R agonist in nociceptive sensory neurons provides insight into role of dopamine in pain modulation.

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국 문 초 록

도파민 D1 수용체 작용제 SKF-38393에 의한 **transient receptor potential vanilloid-1** 수용체 활성화

Transient receptor potential vanilloid-1 (TRPV1) 수용체는 리간드-결합 이온통로로 통증감각신경세포에서 주로 발현하는 통증 수용체이다. TRPV1은 유해한 열 또는 산성자극, 그리고 캡사이신과 같은 화학자극에 의해 활성화되고 신경신호를 전달한다. 이러한 TRPV1이 다양한 세포 내 신호경로에 의해서 그 기능이 조절된다는 사실은 이미 많은 연구를 통해 밝혀졌다. 신경전달물질의 한 종류인 도파민은 중추신경계에서 운동조절과 인지, 그리고 통증조절까지 다양한 부분에 걸쳐 중요한 역할을 담당하고 있다. 도파민 수용체는 G단백질 연결 수용체로 세포 내 신호경로를 조절함으로써 세포에 그 영향을 미친다. 최근, 이러한 도파민 수용체의 한 종류인 도파민 D1-like 수용체 (도파민 D1/D5 수용체)가 중추신경계뿐만 아니라 말초신경계인 척수후근신경절세포, 특히 통증감각신경세포에서도 발현한다는 보고가 있지만 TRPV1에 대한 도파민 수용체의 영향에 대한 연구는 전무한 수준이다.

이번 연구에서는 세포 내 신호경로를 활성화시키는 도파민 D1 수용체가 TRPV1에 미치는 영향을 마우스의 척수후근신경절세포에서 알아보았다. 이를 위해 도파민 D1 수용체 작용제인 SKF-38393과 다양한 억제제를 사용하여 세포 내 칼슘 농도의 변화를 칼슘 이미징 방법으로 비교하였다.

실험 결과, SKF-38393에 의한 도파민 D1 수용체의 활성화는 세포 내 칼슘 농도를 증가시켰다. 이때의 칼슘 반응은 세포 내 칼슘 분비보다는 대부분 TRPV1을 통한 외부 칼슘의 유입에 기인하였다. 또한 도파민 D1 수용체가 척수후근신경절세포에서 TRPV1과 함께 발현하고 있음을 면역형광염색 분석을 통해 확인하였다. 단백질인산화효소C 억제제와 디아실글리세롤지방가수분해효소 억제제, 아데니닐고리화효소 억제제, 그리고 단백질인산화효소A 억제제는 SKF-38393에 의한 칼슘 반응에 아무런 영향을 미치지 않았지만, 인지질가수분해효소C 억제제에 의해서는 칼슘 반응이 차단되었다. 이러한 결과를 바탕으로 SKF-38393에 의한 TRPV1 활성화는 인지질가수분해효소C에 의해 생성된 디아실글리세롤이 관여한다는 것을 확인할 수 있었다.

이상의 실험 결과들은 척수후근신경절의 통증감각신경세포에서 도파민 D1 수용체 작용제 SKF-38393이 인지질가수분해효소C 세포 내 신호경로를 통해 TRPV1을 활성화시킴을 보여주며, 도파민 수용체의 활성화가 TRPV1을 통해 통증신호 조절에 기여할 수 있다는 가능성을 제시한다.

주요어 : 도파민, 도파민 수용체, **SKF-38393**, **TRPV1**, 디아실글리세롤, 통증감각신경세포

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