Phosphorylation of TRPV1 by neurokinin-1 receptor agonist exaggerates the capsaicin-mediated substance P release from cultured rat dorsal root ganglion neurons

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Abstract.

The present study was conducted to determine whether the activation of neurokinin-1 receptor (NK-1R) by its agonist (GR73632) enhances the capsaicin-evoked substance P (SP) release using a radioimmunoassay. A pre-exposure to GR73632 enhanced the capsaicin-evoked SP release in a time- and dose-dependent manner. The augmentation of capsaicin-evoked SP release by GR73632 was completely inhibited by pharmacological blockade of NK-1R or transient receptor potential vanilloid receptor subtype 1 (TRPV1), and was partially attenuated by the inhibition of either protein kinase C (PKC), cyclooxygenase (COX) or phospholipase C (PLC), p38 or p42/44 mitogen-activated protein (MAP) kinase, but not protein kinase A. This augmentation of SP release was further increased by inhibition of c-Jun NH_2-terminal kinase. A short-term (10 min) exposure to GR73632 resulted in an increase in the TRPV1 phosphorylation. The increase in the TRPV1 phosphorylated forms induced by a 60-min exposure to GR73632 was completely abolished by the inhibition of either PKC, COX or PLC, p38 or p42/44 MAP kinases. Immunocytochemistry study demonstrated that the NK-1R and TRPV1 were mainly co-expressed in the small-sized neurons. These findings suggest that the activation of NK-1R by its agonist, by sensitizing the TRPV1 through the PKC phosphorylation of TRPV1, may play a role in the enhancement of the capsaicin-evoked SP release from cultured rat DRG neurons.

Keywords: Dorsal root ganglion neuron; Neurokinin-1 receptor; Phosphorylation of transient receptor potential vanilloid receptor subtype 1; Substance P release

1. Introduction

Recently, we have demonstrated that the activation of neurokinin-1 receptor (NK-1R) by its agonists, substance P (SP) and GR73632, modulates the SP release from cultured dorsal root ganglion (DRG) neurons accompanying NK-1R internalization (Tang et al., 2007). In fact, the released SP from DRG neurons is not taken into these cells again, it diffuses to the surrounding cells (including neuronal and non-neuronal cells) and tissues, thereby achieves its functional roles, such as regulating the biosynthesis and release of SP in DRG neurons and transmitting information about various noxious stimuli from the periphery to the central nervous system. We have therefore considered that the SP released from cultured DRG neurons contributes its own release by activating the NK-1R.

NK-1R is a member of the G_q protein-coupled receptor family, which was widely distributed in both central and peripheral nervous system (Li and Zhao, 1998; Harrison and Geppetti, 2001). The main endogenous ligand for the NK-1R is substance P (SP), an important neurotransmitter and primary afferent modulator belonging to the tachykinin family (Hastrup and Schwartz, 1996). After binding with SP, the NK-1R caused the activation of p38 and p42/44 mitogen-activated protein (MAP) kinases, nuclear factor-kappa B and protein kinase C (PKC), and thereafter to increase the production of prostaglandin E_2 and the expression of cyclooxygenase (COX)-2 (Fiebich et al., 2000; Ebner and Singewald, 2006; Koon et al., 2006). The NK-1R activation by SP also involved the modulation of phospholipase C (PLC) and adenylate cyclase, thereby increasing the consequent production of inositol trisphosphate (IP_3) and cyclic-AMP (Snijdelaar et al., 2000). Our latest findings demonstrated that the activation of NK-1R by exogenously added SP triggered the SP release associating with the activation of MAP kinases, PKC and COX-2 from cultured DRG neurons (Tang et al., 2007).

It is known that the activation of PLC, protein kinase A (PKA) and PKC, the

IP_3-dependent calcium release and COX by various inflammatory mediators in several signal transduction systems are involved in the sensitization and activation of transient receptor potential vanilloid receptor subtype 1 (TRPV1) being a non-selective cation channel with high calcium permeability (Premkumar and Ahern, 2000; Chuang et al., 2001; Tang et al., 2004; Oshita et al., 2005; Tang et al., 2006; Tang and Nakata, 2008). The sensitized TRPV1 can be further activated by capsaicin to evoke the SP release from cultured DRG neurons (Hong and Wiley 2005; Tang et al, 2006; Tang and Nakata, 2006). In addition, the colocalization of SP and TRPV1 has been detected in rat DRG neurons (Dinh et al., 2004; Price and Flores, 2007).

The present study has therefore examined the activation of NK-1R by its agonist enhances the capsaicin-evoked SP release through the TRPV1 activation detected by its phosphorylation.

2. Materials and Methods

2.1. Materials

The following drugs were used: PKCε translocation inhibitor peptide, Gö6976 and bisindolylmaleimide I (Calbiochem, Darmstadt, Germany); CP-96,345 (Pfizer Central Research, Groton, CT, USA); Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan); collagenase, phosphoramidon and bacitracin, captopril and indomethacin, SB222200, GR94800 and capsaicin, capsazepine, U73122, GR73632 and SP600125 (Sigma Chemical, St Louis, MO, USA); H89 (Seikagaku, Tokyo, Japan); [^{125}I]Tyr^{8}-substance P (81.4 TBq/mmol; New England Nuclear, Boston, MA, USA); trypsin (Invitrogen, Burlington, ON, Canada); U0126 and SB203580 (Promega, Madison, WI, USA).

2.2. Isolation and culture of rat DRG cells

DRGs of young adult Wistar rats (6–9 weeks of age) were dissociated into single cells (3 DRGs/dish) by the treatment of enzymes (collagenase and trypsin) according to a previously described method (Tang et al., 2004). All animal procedures were performed in accordance with the Guide for Animal Experimentation, Hiroshima University and the Committee of Research Facilities for Laboratory Animal Sciences, Graduate School of Biomedical Sciences, Hiroshima University, Japan.

2.3. Measurement of SP content

Except for some cells treated by peptidase inhibitors alone (as a control), other cells were exposed to GR73632 alone or together with various inhibitors in DMEM (serum free) containing peptidase inhibitors (1 μM phosphoramidon, 4 μg/ml bacitracin and 1 μM captopril) for a designated period of time at 37°C in a water-saturated atmosphere with 5% CO_2. After being washed two times with 1 ml of Krebs-HEPES buffer, those cells pretreated with GR73632 were continuously stimulated by capsaicin plus peptidase inhibitors or by peptidase inhibitors alone in Krebs-HEPES buffer for 10 min at 37°C. Thereafter, the SP content collected from Krebs-HEPES buffer was measured by a highly sensitive radioimmunoassay (Tang et al., 2005).

2.4. Western blot analysis

At the end of the SP release experiments, the cell samples were processed for Western blot analysis as previously described (Tang et al., 2006). Primary antibodies were raised against phosphorylated TRPV1 (1:500 dilution; anti rat phospho-TRPV1 polyclonal antibody; Cosmo Bio) or β-actin (1:10,000 dilution; the mouse monoclonal antibody for β-actin; Sigma). The horseradish peroxidase-conjugated anti-rabbit and

anti-mouse secondary antibodies (1:2,000 dilution; Cell Signaling, Beverly, MA) were used for chemiluminescence detection according to the manufacturer's instructions, respectively.

2.5. Immunofluorescence staining

Cultured DRG neurons on coverglasses were treated with various stimuli for a designated period of time, and rapidly fixed for 30 min at room temperature with 4% paraformaldehyde (Sigma). Fixed cells were washed three times in 10 mM phosphate buffered saline (PBS) containing 0.1% Triton X-100 (Sigma), and incubated overnight at 4°C with mouse anti-capsaicin receptor monoclonal antibody (1:1000 dilution; Chemicon, Temecula, CA) plus rabbit anti-SP receptor (1:2,000 dilution; Sigma) in PBS containing 1% BSA and 0.1% Triton X-100 (Tang et al., 2007). Coverslips were then incubated for 1 hour at room temperature with Alexa Fluor 488 goat anti-rabbit IgG (1:1,000; Molecular Probes, Eugene, OR, USA) plus Alexa Fluor 568 goat anti-mouse IgG (1:1,000; Molecular Probes), washed three times in PBS and visualized by a fluorescent microscope (Biozero BZ-8000, Keyence, Osaka, Japan).

2.6. Statistics

The data are presented as the mean ± SEM. Statistical analyses were performed by the multiple *t*-test with the Bonferroni correction following ANOVA. Significance was set at a value of $P < 0.05$ (two-tailed).

3. Results

3.1. Effect of pretreatment with GR73632 on the capsaicin-induced SP release

To investigate whether a pretreatment with GR73632 causes the enhancement of SP release evoked by capsaicin, the levels of capsaicin-induced SP release from cultured

DRG neurons pretreated with or without GR73632 were examined (Fig. 1). Based on the observations about the potency of a NK-1R antagonist, CP-96,345 (Snider et al., 1991; Dianzani et al., 2003), we therefore decided to use 1 μM as an appropriate concentration for examining the changes in the SP level in the present study. It was observed that capsaicin evoked a significant increase in the SP release in a CP-96,345 sensitive manner from the pre-exposure of cultured DRG neurons to GR73632 (1–100 nM) for 60 min in comparison to that induced by capsaicin alone (Fig. 1A). As observed in Fig. 1B, the pretreatment with GR73632 increased the SP release evoked by capsaicin in a time-dependent manner (10–60 min). The effect of pretreatment with GR73632 on the SP release evoked by capsaicin was completely attenuated by capsazepine (1 μM), a competitive antagonist of TRPV1 (Fig. 1C), whereas capsazepine did not have any effect on the SP release induced by the 60 min treatment with 10 nM GR73632 from cultured DRG neurons (Data not shown). Together with the results shown in Fig. 1, these findings indicate that NK-1R agonist GR73632 induces an enhancement of capsaicin-evoked SP release.

3.2. Characteristics of the enhancement of capsaicin-evoked SP release induced by GR73632

Our previous studies demonstrated the importance of several intracellular effectors, such as PKC, PKA and MAP kinases, COX and PLC in the SP release. It is therefore of interest to investigate whether these effectors are involved in the enhancement of capsaicin-evoked SP release from cultured DRG neurons exposed to long-term (60 min) treatment with GR73632. Fig. 2 shows that the GR73632-regulated enhancement of capsaicin-evoked SP release was significantly attenuated by either MAP kinase inhibitors 10 μM U0126 (a p42/44 MAP kinase inhibitor, Tang et al., 2006) and 15 μM SB203580 (a p38 MAP kinase inhibitor, Tang et al., 2006), by 10 μM U73122 (an

inhibitor of PLC, Tang et al., 2004), by COX inhibitors 1 μM NS-398 (an inhibitor of COX-2, Tang et al., 2005) and 1 μM indomethacin (a non-selective inhibitor of COX1/2, Tang et al., 2006), or by PKC inhibitors Gö6976 (an inhibitor for PKCα-, βI-isozymes, Suzuki et al., 2006), bisindolylmaleimide I (an inhibitor for PKCα-, βI-, βII- , γ-, δ-, ε-isozymes, Suzuki et al., 2006) and PKCε translocation inhibitor peptide (Huang and Walker, 2004), and was further enhanced by 30 μM SP600125, a c-Jun NH_2-terminal kinase (JNK) inhibitor (Tang et al., 2006). However, PKA inhibitor H89 (10 μM) did not inhibit the enhancement of that by GR73632. These above-mentioned inhibitors used alone did not have any effect on the SP release, except for SP600125 with a weak tendency to increase the SP release (Data not shown).

3.3. Involvement of NK-1R in the induction of TRPV1 phosphorylation

Next, the phosphorylation status of TRPV1 after the stimulation of GR73632 alone or together with CP-96,345 (1μM) using a specific anti rat phospho-TRPV1 polyclonal antibody (which reacts with phosphorylated TRPV1 at Ser800) were examined. Treatment with 10 nM GR73632 resulted in time-dependent (10–60 min) increases of the TRPV1 phosphorylated forms. The increases in phosphorylation levels of TRPV1 induced by GR73632 were significantly attenuated by pre-treatment with CP-96,345 (Fig. 3A). Especially, the increase in the TRPV1 phosphorylated forms induced by a 60-min exposure to 10 nM GR73632 was completely abolished by the pharmacological blockade of either 10 μM U0126, 15 μM SB203580, 10 μM U73122 or 1 μM NS-398, 1 μM indomethacin or 1 μM Gö6976, 1 μM bisindolylmaleimide I or 200 μM PKCε translocation inhibitor peptide, but not 30 μM SP600125 (Fig. 3B). However, no increases in the total TRPV1 protein levels were observed after a 60-min exposure of DRG neurons to GR73632 in comparison to control (Fig. 3C).

3.4. Co-expression of NK-1R and TRPV1 in cultured DRG neurons

Finally, the co-expression of NK-1R and TRPV1 in cultured DRG neurons after a 60-min exposure of DRG neurons to GR73632 was investigated. Despite the 60-min exposure of DRG neurons to GR73632, the NK-1R (green images) and TRPV1 (red images) were mainly co-expressed in the small-sized neurons. Consistent with our latest findings (Tang et al., 2007), the internalization of NK-1R was confirmed after the 60-min exposure of DRG neurons to GR73632 (see green photomicrograph in right panel of Fig. 4), in comparison to control. Alternatively, we tried to compare TRPV1 protein expression levels these cultured DRG neurons. Consistent with the western blot analysis (shown in Fig. 4), there was no significant difference in the TRPV1 protein expression levels in DRG neurons between the control and GR73632 groups (Data not shown).

4. Discussion

Our present results show that the long-term (60 min) exposure of cultured rat DRG neurons to the NK-1R agonist via the NK-1R resulted in the activation of several different intracellular effectors, and thereafter induced the sensitization of TRPV1 through the phosphorylation of TRPV1, leading to the enhancement of SP release triggered by capsaicin.

Previously, we established that bradykinin can activate its G_q protein-coupled bradykinin B_2 receptor to sensitize TRPV1 through the activation of second messenger signaling transduction cascades in cultured rat DRG neurons, thereby leading to the potentiation of capsaicin-evoked SP release (Tang et al., 2005, 2006). The sensitization of TRPV1 regulated by bradykinin requires the activation of PKA and COXs, and also depended on the IP_3-senstive calcium release, but is independent on the activation of MAP kinases in rat cultured DRG neurons (Oshita et al. 2005; Tang et al., 2006). The

present findings demonstrated that the activation of MAP kinases, PKCs and COX1/2 involved in the regulation of capsaicin-evoked SP release by a 60-min exposure of cultured DRG neurons to GR73632 alone (Fig 2). It is therefore possible to propose another similar sensitization mechanism of TRPV1 in the present study. Interestingly, some studies indicated that p42/44 and p38 MAP kinases can be rapidly activated by SP (Yang et al., 2002; Fiebich et al., 2000; Tang et al., 2007). These findings suggest the important roles for these MAP kinases, PKCs and COXs in the sensitization of TRPV1 and in the potentiation of capsaicin-evoked SP release by GR73632. Differed from several observations (Lopshire and Nicol 1998, Oshita et al., 2005), an inhibitor of PKA H89 did not attenuate the potentiation of capsaicin-evoked SP release by GR73632, suggesting the sensitization of TRPV1 by the activation of NK-1R is independent of PKA in our experimental conditions.

The phosphorylation of TRPV1 is now considered as an important index of its own sensitization (Zhang et al., 2005). TRPV1 has two phosphorylation sites for PKC-mediated phosphorylation: Ser502 and Ser800 (Tominaga and Caterina, 2004). In addition, the PKC-dependent phosphorylation of Ser800 in the TRPV1 C terminus potentiated capsaicin-evoked ionic selectivity changes (Chung et al., 2008). The potentiated response of SP release was significantly attenuated by three inhibitors for PKC isozymes (Fig. 2D), we therefore considered the probability of various PKC isozymes including ε-isozyme in the sensitization of TRPV1 by GR73632, through causing a change in ion permeability of TRPV1 to exaggerates the capsaicin-mediated substance P release from cultured DRG neurons. In the present study, a specific anti rat phospho-TRPV1 polyclonal antibody was selected to examine the phosphorylation level of TRPV1. This antibody reacts with phosphorylated TRPV1 at Ser800. As expected, an early (10 min) phosphorylation of TRPV1 was observed during a 60-min exposure of cultured DRG neurons to GR73632. Interestingly, the phosphorylation level of TRPV1

increased by the 60-min exposure to GR73632 was completely abolished by the inhibition of either PKC, COX or PLC, p38 or p42/44 MAP kinases (Fig. 3B). However, there was no alternation in total TRPV1 protein levels after a 60-min exposure of DRG neurons to GR73632, in comparison to control. These suggest the activation of NK-1R by GR73632 induces the TRPV1 sensitization through the PKC phosphorylation of TRPV1, which is associated with the activation of COX, PLC, p38 and p42/44 MAP kinases. Recently, the activation of PKC was also reported to induce the sensitization of TRPV1 (Amadesi et al., 2004; Ferreria et al., 2005), for example, the activation of PKC is directly involved in the phosphorylation of TRPV1 (Vellani et al., 2001); a PKC-dependent pathway is involved in the prostaglandin E_2 (1 μM, 1.5 min)-induced potentiation of capsaicin-activated currents in mouse DRG neurons (Moriyama et al., 2005). Furthermore, the activation of NK-1R by its agonist in primary sensory neurons induces heat hyperalgesia via a PKCε-mediated potentiation of TRPV1 (Zhang et al., 2007). Especially, the present study showed that the NK-1R and TRPV1 are co-expressed in the same small-sized neuron and also confirmed the internalization of NK-1R in these small-sized neurons exposed to GR73632 for 60 min (Fig. 4). The internalization of NK-1R is a pharmacologically specific index of NK-1R-SP interaction (namely the activation of NK-1R). This co-expression in the same neuron further supported that there is a cross-talk between these two receptors via these above-mentioned intracellular effectors. Therefore, we proposed a molecular mechanism from our results and the observations as described above that the activation of NK-1R by GR73632 induce the activation of p42/44 and p38 MAP kinases, and PKCs, thereafter increased the levels of COX-2, which contribute to the sensitization of TRPV1 through the PKC phosphorylation of TRPV1. As a consequence, the NK-1R activation in DRG neurons increases the sensitivity of TRPV1, leading to the potentiation of capsaicin-evoked SP release.

Alternatively, a PLC inhibitor U73122 significantly attenuated the potentiation of capsaicin-evoked SP release (Fig. 2B), and also completely abolished the increase in the TRPV1 phosphorylated forms induced by GR73632 (Fig. 3B). In addition, GR73632 can activate its G_q protein-coupled NK-1R to activate PLC which can hydrolyze plasma membrane phosphatidylinositol-4,5-bisphosphate (PIP_2) to yield inositol phosphates and diacylglycerol, and simultaneously sensitize the TRPV1 by removing constitutive inhibition of the vanilloid receptor 1 by PIP_2 (Mau et al., 1990; Chuang et al., 2001). These suggest that a PLC-dependent sensitization of TRPV1 should also be considered in the present study. These findings also provide further support for the important role of NK-1R which stimulates and sensitizes the peripheral nerve endings to pain (Zhang et al. 2007). However, we still do not know the exact reason for the enhancement of capsaicin-evoked SP release by a JNK inhibitor SP600125.

In conclusion, we have demonstrated that the activation of NK-1R by its agonist, can induce the activation of PKCs, p42/44 and p38 MAP kinases, PLC and COXs, thereafter sensitize the TRPV1 through the phosphorylation of TRPV1 at Ser800 site, and enhance the capsaicin-evoked SP release from cultured DRG neurons, and indicated that the JNK likely has an inhibitory effect on the SP release process. These observations offer a new evidence for the neuromodulatory actions of SP through the NK-1R in primary afferent neurons.

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Figure legends

Figure 1. Effect of pretreatment with GR73632 on the SP release evoked by capsaicin from cultured DRG neurons.

Some cells were left untreated as a control, all other cells were pretreated with GR73632 alone or together with CP-96,345 (1 μM) in serum-free DMEM for a designated period of time at 37°C. Next, those cells were stimulated by capsaicin plus peptidase inhibitors or by peptidase inhibitors only in Krebs-HEPES buffer for 10 min. The data are obtained from 4 (A), 3–4 (B) or 3–4 (C) separate experiments. *, ** and *** denote $P < 0.05$, 0.01 and 0.001, respectively.

Figure 2. Characteristics of the GR73632-enhanced release of SP induced by capsaicin from cultured DRG neurons.

Some cells were left untreated as a control, all other cells were pretreated with GR73632 alone or together with either 10 μM U0126, 15 μM SB203580 or 30 μM SP600125, 10 μM U73122 or 1 μM NS-398, 1 μM indomethacin or 1μM Gö6976, 1 μM bisindolymaleimide, 200 μM PKCε translocation inhibitor peptide or 10 μM H89 in serum-free DMEM for 1 hour at 37°C. Next, those cells were stimulated by capsaicin plus peptidase inhibitors or by peptidase inhibitors only in Krebs-HEPES buffer for 10 min. The data are obtained from 5 (A), 5–13 (B) or 8–9 (C), 4–9 (D) separate experiments. *, ** and *** denote $P < 0.05$, 0.01 and 0.001, respectively.

Figure 3. The phosphorylation of TRPV1 induced by GR73632 in cultured adult rat DRG neurons.

Representative blots of phosphorylated TRPV1 (P-TRPV1) expression in cultured DRG neurons exposed to GR73632 alone or together with CP-96,345 for 10 to 60 min (upside of A) or together with either 10 μM U0126, 15 μM SB203580 or 10 μM

U73122, 1 μM NS-398 or 1 μM indomethacin (IDM), Gö6976, bisindolylmaleimide I (Bis) or PKCε translocation inhibitor peptide (PKCεI), 30 μM SP600125 for 60 min (upside of B). Representative blots of TRPV1 expression in cultured DRG neurons exposed to GR73632 alone or together with CP-96,345 for 60 min (upside of C). All data have been quantified by normalizing the P-TRPV1 (downside of A; downside of B) or TRPV1 (downside of C) expression level of control. The data are obtained from 3-5 (A, B) or 3 (C) separate experiments. #, ##, ### denote $P < 0.05$, 0.01, 0.001 in comparison to the effect of respective GR73632 alone; *, **, *** denote $P < 0.05$, 0.01, 0.001 versus the effect of the untreated control.

Figure 4. Co-expression of NK-1R and TRPV1 in cultured adult rat DRG neurons.

Bright-field photomicrograph (top panels) of DRG neurons after the 60-min exposure to GR73632; representative immunofluorescence photomicrographs for NK-1R (green photomicrographs) and TRPV1 (red photomicrographs) in these DRG neurons of control (left panels) and GR73632 (right panels) groups; digitally merged photomicrographs (lowest panels). Bars: 15 μm. The red and white arrows indicate two different size neurons.

Figure 1A

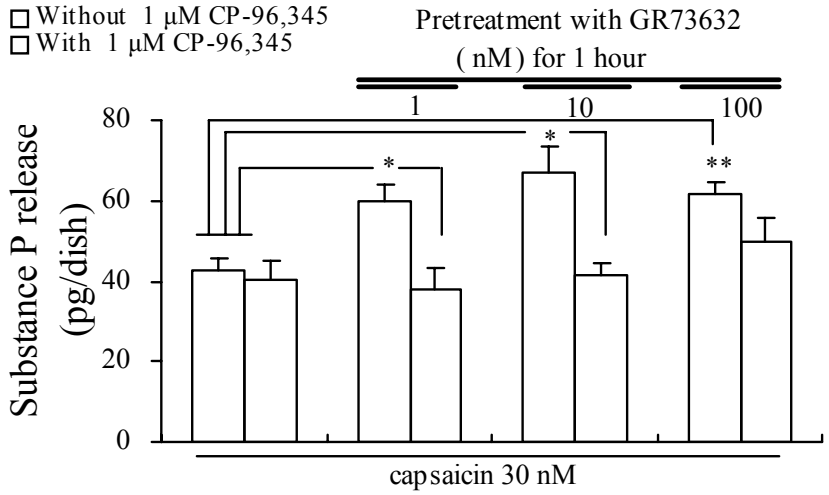

Figure 1B

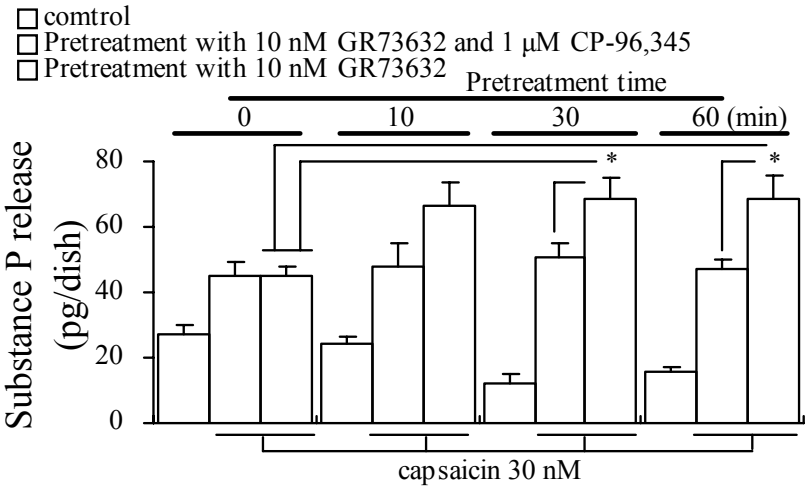

Figure 1C

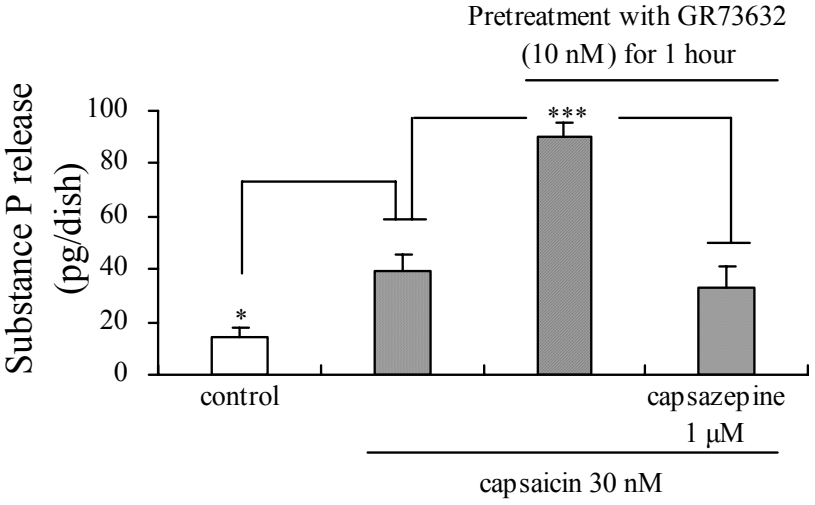

Figure 2A

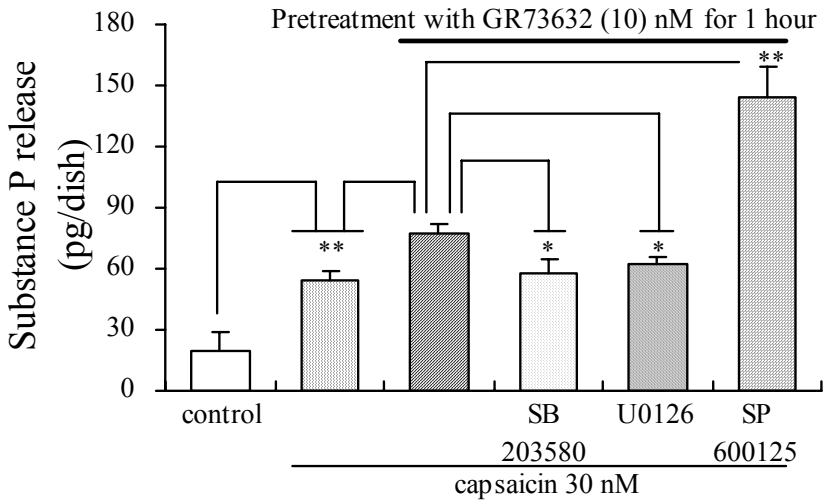

Figure 2B

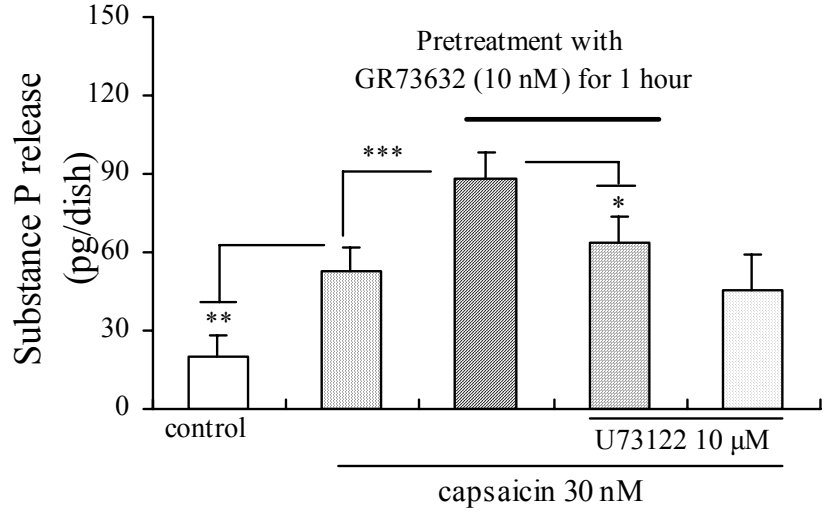

Figure 2C

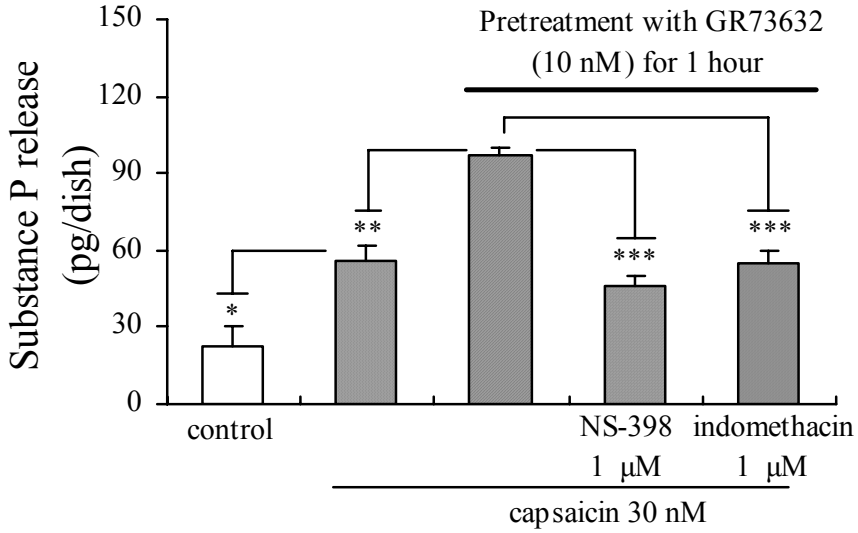

Figure 2D

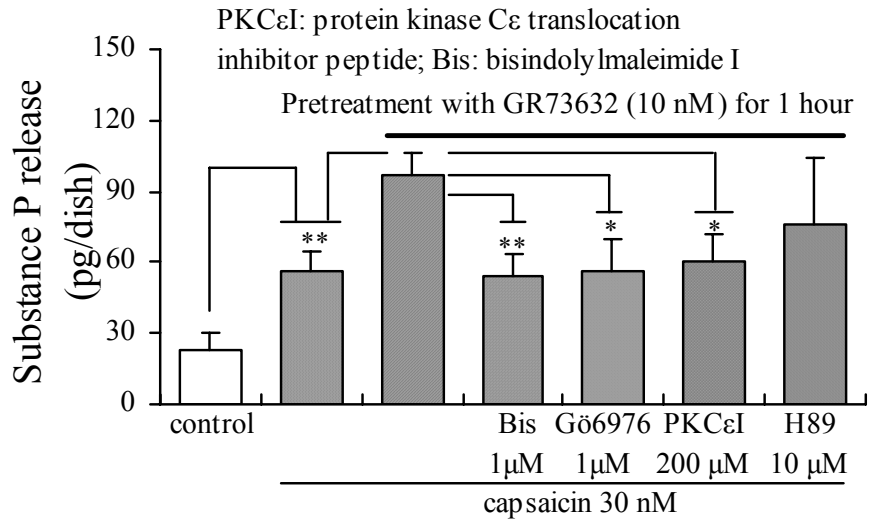

Figure 3A

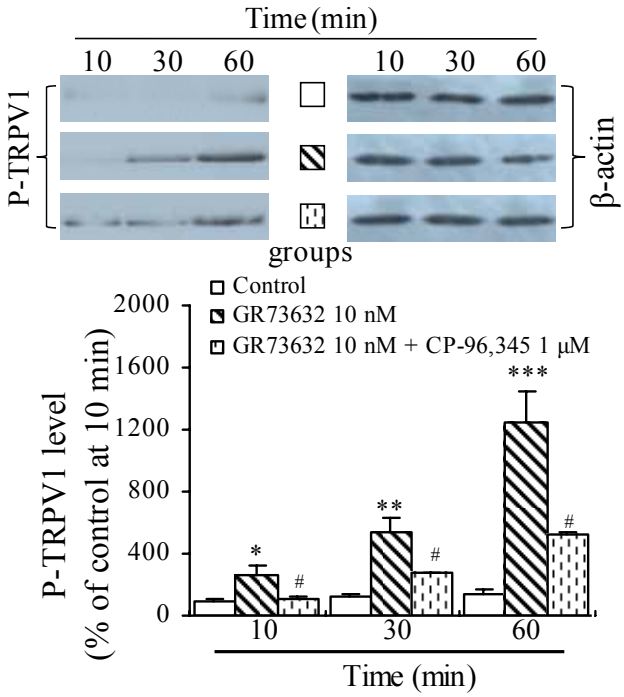

Figure 3B

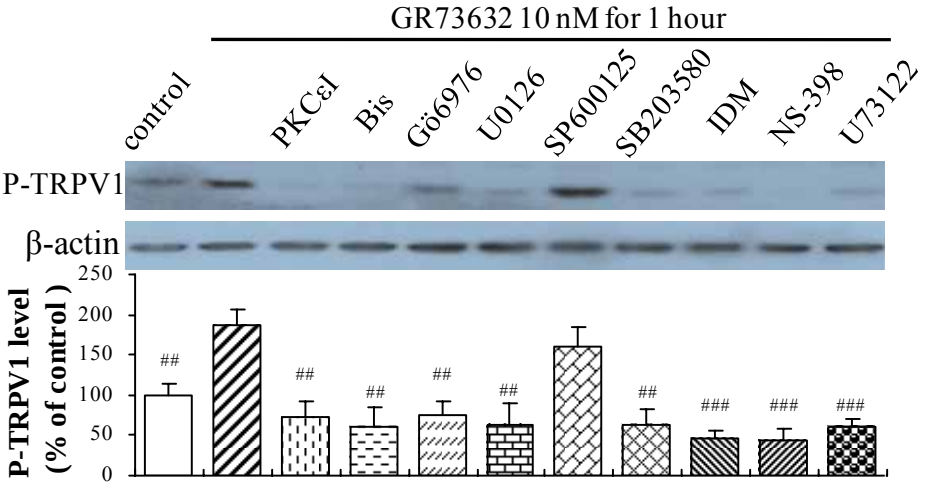

Figure 3C

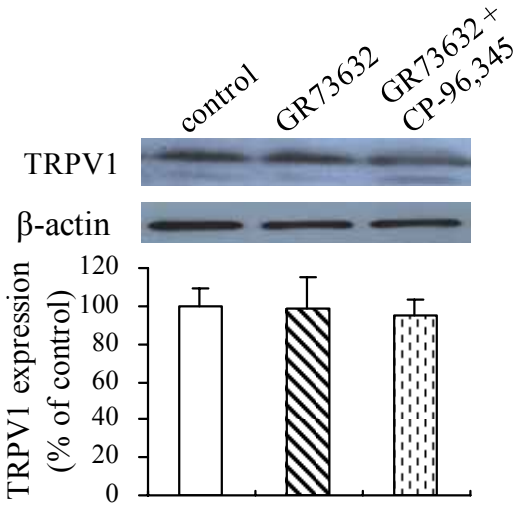
control
GR73632
GR73632 + CP-96,345
TRPV1
β-actin
TRPV1 expression (% of control)
120
100
80
60
40
20
0

Figure 4