Phospholipase C and protein kinase A mediate bradykinin sensitization of TRPA1: a molecular mechanism of inflammatory pain

Shenglan Wang, Yi Dai, Tetsuo Fukuoka, Hiroki Yamanaka, Kimiko Kobayashi, Koichi Obata, Xiuyu Cui, Makoto Tominaga and Koichi Noguchi

1Department of Anatomy and Neuroscience, Hyogo College of Medicine, Nishinomiya, Hyogo 663-8501, 2Department of Pharmacy, School of Pharmacy, Hyogo University of Health Sciences, Kobe, Hyogo 650-8530, Japan, 3Institute for Biomedical Sciences of Pain, Capital Medical University, Beijing 100069, P.R. China and 4Section of Cell Signaling, Okazaki institute for Integrative Bioscience, National Institutes of Natural Sciences, Okazaki, Aichi 444-8787, Japan

Correspondence to: Koichi Noguchi, MD, PhD, Department of Anatomy and Neuroscience, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan
E-mail: noguchi@hyo-med.ac.jp

Bradykinin is an inflammatory mediator that plays a pivotal role in pain and hyperalgesia in inflamed tissues by exciting and/or sensitizing nociceptors. TRPA1 is an important component of the transduction machinery through which environmental irritants and endogenous proalgesic agents depolarize nociceptors to elicit inflammatory pain. Here, using electrophysiological, immunocytochemical and behavioural analyses, we showed a functional interaction of these two inflammation-related molecules in both heterologous expressing systems and primary sensory neurons. We found that bradykinin increased the TRPA1 currents evoked by allyl isothiocyanate (AITC) or cinnamaldehyde in HEK293 cells expressing TRPA1 and bradykinin receptor 2 (B2R). This potentiation was inhibited by phospholipase C (PLC) inhibitor or protein kinase A (PKA) inhibitor, and mimicked by PLC or PKA activator. The functional interaction between B2R and TRPA1, as well as the modulation mechanism, was also observed in rat dorsal root ganglia neurons. In an occlusion experiment, the PLC activator could enhance AITC-induced TRPA1 current further even in saturated PKA-mediated potentiation, indicating the additive potentiating effects of the PLC and PKA pathways. These data for the first time indicate that a cAMP-PKA signalling is involved in the downstream from B2R in dorsal root ganglia neurons in addition to PLC. Finally, subcutaneous pre-injection of a sub-inflammatory dose of bradykinin into rat hind paw enhanced AITC-induced pain behaviours, which was consistent with the observations in vitro. Collectively, these results represent a novel mechanism through which bradykinin released in response to tissue inflammation might trigger the sensation of pain by TRPA1 activation.

Keywords: TRPA1; bradykinin; PLC; PKA; inflammation

Abbreviations: AC = adenylate cyclase; AITC = allyl isothiocyanate; BK = bradykinin; Cap = capsaicin; DAG = diacylglycerol; DMEM = Dulbecco’s Modified Eagle’s Medium; DRG = dorsal root ganglia; EBSS = Earle’s balanced salt solution; FBS = foetal bovine serum; FSK = Forskolin; GF = GF109203X; HEK = human embryonic kidney; IP3 = inositol triphosphate; PIP2 = phosphatidylinositol-4,5-bisphosphate; PKA = protein kinase A; PKC = protein kinase C; PLC = phospholipase C; PMA = phorbol 12-myristate 13-acetate; TRP = transient receptor potential


Introduction

The nonapeptide bradykinin (BK) appears to act as a mediator of a wide variety of physiological and pathophysiological responses including pain and inflammation (Farmer and Burch, 1992; Hall, 1992). BK directly excites nociceptive dorsal root ganglia (DRG) neurons and causes pain in animals and human (Steranka et al., 1988; Mizumura et al., 1990; Manning et al., 1991; Levine et al., 1993; Couture et al., 2001). Two BK receptors, B1R and B2R, have been identified. It is commonly accepted that most of the physiological and
pathophysiological actions of BK involve the B2 receptor (Farmer and Burch, 1992; Hall, 1992). The B2 receptor has been cloned from different species and found to belong to the G protein-coupled receptor family (McCachern et al., 1991; Hess et al., 1992, 1994), and also implicated in BK-induced nociceptor activities and nociceptive behaviors (Dray and Perkins, 1993). Stimulation of the B2 receptor is known to result in activation of the phospholipase C (PLC) or adenylate cyclase (AC)/cyclic AMP (cAMP) signalling system, which leads to consequential activation of protein kinase C (PKC) or protein kinase A (PKA), respectively (Liebmann and Bohmer, 2000).

The transient receptor potential (TRP) channel A1 (Jaquemar et al., 1999) has been reported to be activated by icilin, a chemical that induces a cooling sensation, and by temperatures \( \leq 17^\circ C \) (Story et al., 2003). This channel was also reported to be activated by some pungent chemicals, such as horseradish, mustard oil, cinnamon oil, allin and methyl paraben (Story et al., 2003; Bandell et al., 2004; Jordt et al., 2004; Macpherson et al., 2005; Fujita et al., 2007) and an endogenous aldehyde, 4-hydroxynonenal (Trevisani et al., 2007). These noxious compounds activate TRPA1 through covalent modification of cysteines (Hinman et al., 2006; Macpherson et al., 2007). TRPA1 is expressed by a subset of small-sized DRG or trigeminal ganglia neurons in neonatal rats, adult rats and mice (Jordt et al., 2004; Kobayashi et al., 2005; Nagata et al., 2005). A recent report indicated that TRPA1 mediates formalin-induced pain (McNamara et al., 2007). Studies using knockout mice demonstrated that TRPA1 is an important component of the transduction machinery through which environmental irritants and endogenous proalgesic agents depolarize nociceptors to elicit inflammatory pain (Bautista et al., 2006; Kwan et al., 2006). Taking the above into account, it is clear that this channel is one of the important transducers of noxious stimuli in the primary afferents.

Inflammatory pain is initiated by tissue damage/inflammation and is characterized by hypersensitivity, both at the site of damage and in adjacent tissue. In the context of inflammation, stimuli that normally would not produce pain do so (allodynia), whereas previously noxious stimuli evoke even greater pain responses (hyperalgesia). One mechanism underlying these phenomena is the modulation (sensitization) of ion channels, such as the TRPV1, that detect noxious stimuli at the nociceptor terminal. How does BK act to enhance pain sensation? BK sensitizes TRPV1 through the G protein-coupled receptor, B2R (Chuang et al., 2001; Sugiuara et al., 2002), but evidence implies this pathway is insufficient to account for BK action (Kollarik and Undem, 2004; Rong et al., 2004). In the present study, we asked whether BK could sensitize another TRP family member, TRPA1. We observed a functional interaction between B2R and TRPA1, both in heterologous expressing systems and in rat DRG neurons, which was also confirmed at the behavioural level.

Materials and Methods
Mammalian cell culture
Human embryonic kidney-derived (HEK) cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 10% foetal bovine serum (FBS), 2 mM glutamax, penicillin and streptomycin. HEK cells were transfected with 1 \( \mu g \) of a human TRPA1 (hTRPA1) cDNA and 0.5 \( \mu g \) of a mouse B2R (mB2R) cDNA by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The hTRPA1 cDNA was a generous gift from Dr Ardem Patapoutian, and the mB2R cDNA was a generous gift from Dr Haruhiro Higashida. To identify transfected cells, an enhanced green fluorescence protein reporter plasmid was also transfected at 0.1 \( \mu g \). For primary culture of DRG neurons, DRGs were collected from the adult Sprague-Dawley rats (100–200 g) using sterile techniques, and placed in ice-cold Earle’s balanced salt solution (EBSS, Sigma). Adhering fat and connective tissue were removed and each DRG was placed immediately in a medium consisting of 2 ml of EBSS and 1.25 mg/ml of collagenase P (Sigma) and kept at 37°C for 60 min with occasional agitation. After dissociation of the DRG cells, this cell suspension was centrifuged for 5 min at 1000 r.p.m. and the cell pellet was re-suspended in EBSS supplemented with 10% FBS, 2 mM glutamax, penicillin and streptomycin. Recombinant rat NGF (100 ng/ml, Sigma) was added to the medium.

Electrophysiology
Whole-cell patch-clamp recordings were carried out at 2 days after transfection of cDNAs to HEK293 cells or at 1 day after dissociation of the DRG neurons. Voltage-clamp experiments were performed at \(-60 \) mV holding potential, and recordings were sampled at 5 kHz and filtered at 2 kHz. Normalized currents (currents were normalized to the currents evoked initially by an agonist) or current densities (\( \text{pA/pF} \)) were measured. A normalized current was obtained just in case the initial current produced by agonist was smaller than 200 \( \text{pA} \) (for HEK cell recording) or 400 \( \text{pA} \) (for DRG neuron recording) to prevent a large current-induced desensitization. The current magnitude was quantified by peak current amplitude in all experiments. In experiments with DRG neurons, after allyl isothiocyanate (AITC, Nacalai tesque, Kyoto, Japan) application, capsaicin (1 \( \mu M \)) was applied at the end of recording to identify whether the AITC-induced current was mediated by TRPA1 channels. Data were obtained just in case the DRG neuron was sensitive to both AITC and capsaicin application, since an AITC-activated current in capsaicin-sensitive DRG neurons is certainly a TRPA1-mediated event (Kwan et al., 2006). Standard bath solution contained 140 mM NaCl, 5 mM KCl, 2 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM HEPES and 10 mM glucose, pH 7.4 (adjusted with NaOH). In some experiments, BK (20 nM–1 \( \mu M \)) and/or intracellular signal inhibitors or activators were contained in the bath solutions. The pipette solution contained 135 mM K-gluconate, 5 mM KCl, 2 mM MgCl\(_2\), 0.5 mM CaCl\(_2\), 5 mM MgATP, 5 mM EGTA and 5 mM HEPES, pH 7.2 (adjusted with Tris-base). In some experiments, a water-soluble phosphatidylinositol-4,5-bisphosphate (PI(4,5)P\(_2\), 10 \( \mu M \)) was included in the pipette solution. AITC (30–300 \( \mu M \)) or cinnamaldehyde (500 \( \mu M \)) were used to stimulate TRPA1. All patch-clamp experiments were performed at room temperature (~25°C, RT). The solutions containing drugs were applied to the chamber (180 \( \mu l \)) by a gravity system at a flow rate of 3–4 ml/min.
Calcium imaging study
Ratiometric calcium imaging was performed using an Olympus fluorescence microscope equipped with a variable filter wheel (Sutter Instruments, Novato, CA, USA) and a cooled digital CCD camera (Hamamatsu, Shizuoka, Japan). Dual images (340 and 380 nm excitation, 510 nm emission) were collected and pseudocolour ratiometric images were monitored every 5 s during the experiment using the Axon Imaging Workbench 5.0 (INDEC BioSystems, Inc. Mountain View, CA, USA). Rat DRG neurons were cultured on poly-L-lysine-coated glass coverslips for 18–24 h, and then they were loaded with 4 μM Fura-2 acetoxyethyl ester (Nacalai tesque, Kyoto, Japan) for 40 min at 37°C. BK (20 μM) was first applied for 60 s, then after washing out for 90 s, AITC (100 μM) was applied for 40 s. Finally, cells were perfused by capsaicin (1 μM) followed by KCl (50 mM). Threshold of activation was defined as 20% above baseline. DRG neurons in one randomly selected microscopic field (20–50 cells) in one glass coverslip were measured. The number of agonists-responding neurons relative to KCl-responding neurons in each selected microscopic field was calculated and summed.

cAMP measurement
Rat DRG neurons were cultured on poly-L-lysine-coated glass coverslips for 18–24 h. cAMP release was evoked by BK. The BK (200 nM or 1 μM) was applied to the neurons for 1 min at RT. All samples were treated with IBMX (1 μM, 1 min), an inhibitor of cyclic nucleotide phosphodiesterase with subsequent inhibition of cyclic nucleotide hydrolysis, to increase the intra-cellular cAMP level. The intracellular cAMP level was examined using the cAMP Biotrak Enzymeimmunoassay System following the non-acetylation protocol according to the manufacture’s directions (Amersham Biosciences, Bucks, England, UK). Total protein of each sample was examined using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All samples were normalized by their optical density (OD) values of total protein, and then the values of the samples treated with BK divided by the average value of control.

Translocation of PKA in DRG neurons
BK (1 μM, 1 min) or vehicle solution was applied to rat DRG neurons at RT after 18–24 h culture. Forskolin (FSK, 10 μM) was applied for 0.5 min as a positive control. All samples were fixed for 20 min with 3.7% paraformaldehyde. A polyclonal primary antibody (Cell Signaling Technology, Inc., Danvers, MA, USA) for PKA catalytic subunits (PKAc) at 1:50 was applied in the presence of 0.1% Triton X-100 and 0.1% Tween-20 for 20 min with 3.7% paraformaldehyde. A polyclonal primary antibody against PKAc at 1:50 was applied in the presence of 5% normal goat serum, 0.1% Triton X-100 and 0.1% Tween-20 in PBS for 20 h at 4°C. Alexa Fluoro 488-conjugated secondary antibody (1:1000, Molecular Probes, Eugene, OR, USA) was applied in the presence of 0.1% Triton X-100 and 0.1% Tween-20 in PBS for 2 h at 4°C. After washing, the nuclei were stained by Hoechst 33342 (1:10,000, Invitrogen) for 1 min at RT. Images were acquired using a confocal laser scanning microscope (LSM 510 version 2.8, Carl Zeiss Microimaging, Inc., Germany) with the water Plan-Neofular 40× objective lens. Small-sized DRG neurons (diameter was less than ~25 μm) were randomly selected for calculation. The fluorescence intensity of neurons was measured by selecting a straight line across the neuronal soma and using the plot profile function of Imaging J 1.37v software (Wayne Rasband, National Institutes of Health, USA, http://rsb.info.nih.gov/ij/).

The total average fluorescence intensity over the cell diameter was calculated. To quantify the redistribution of PKAc in neurons, the average fluorescence intensity of 10% segments of the total intensity profile length in the peripheral or central regions was normalized to the total average intensity, and then compared between peripheral and central regions of neurons.

Behavioural study
Twenty-two male adult Sprague-Dawley rats (200–250 g) were used for the behavioural analyses. After adaptation, 50 μl BK (20 μM, in saline) or saline was injected intra-plantarly into the left hind paw of each rat. Five minutes after injection, rats received intra-dernal injection of 50 μl of allyl isothiocyanate (AITC, 3% in liquid paraffin, Wako Pure Chemical Industries Ltd., Osaka, Japan), to the same area of BK-injected plantar surface of the left hind paw. The rats were placed in a wire mesh cage immediately after the injection and the numbers and durations of hind paw lifts per 15 min interval during the initial 1 h post-injection (AITC) period were measured. The total number of lifts and durations of flinches during the entire initial 1 h post-injection were also calculated.

Compounds
8-Br-cAMP, ET-18-OCH₃, FSK, m-3MFSBS, PMA, poly-L-lysine, IBMX, [Hyp³]-bradykinin, capsaicin and cinnamonaldehyde were purchased from Sigma-Aldrich (St Louis, MO, USA); GF109203X and PIP₂ were from Calbiochem (La Jolla, CA, USA); H89 was from Biomol (Plymouth Meeting, PA, USA). KT5720 was from Tocris (Ellisville, MO, USA); BK was from Bachem (King of Prussia, PA, USA). AITC, camphor and Fura 2-AM were from Nacalai tesque (Kyoto, Japan); Glutamax, FBS, Penicillin–streptomycin, MEM Vitamin solution and OPTI-MEM were from Invitrogen (Carlsbad, CA, USA).

Statistical analysis
All results are expressed as mean ± SEM. An unpaired t-test was used to compare the electrophysiological data between the two groups. Two-way repeated ANOVA followed by Fisher’s PLSD was applied to the behavioural data. A difference was accepted as significant if the probability was <5% (P<0.05).

Results
BK potentiates TRPA1 in heterologous expression systems
BK may modulate TRPA1 by binding to its receptor on sensory neurons and activating second messenger signalling cascades. To address this possibility, we expressed TRPA1 and B2R together in human embryonic kidney (HEK293) cells and examined their sensitivity to TRPA1 agonists before and after exposure to BK using a whole-cell patch clamp technique. In the voltage-clamp experiments, the BK at 20 nM did not induce any significant current in either HEK cells transfected with B2R, or HEK cells transfected with B2R and TRPA1. The AITC-activated inward current in the TRPA1-transfected HEK293 cells displayed outward rectification and was sensitive to ruthenium red (data not shown). The current underwent tachyphylaxis, giving
First, we tested the participation of PLC signalling on potentiation of TRPA1 by BK. ET-18-OCH₃ (2 μM), a PLC inhibitor, significantly inhibited the potentiation of AITC-activated currents (0.70 ± 0.12-fold, n = 8 for ET-18-OCH₃, P < 0.05 versus BK). This finding conformed to the observation that m-3M3FBS (10 μM), a PLC activator potentiated the AITC-activated current (1.97 ± 0.35-fold, n = 17 for m-3M3FBS, P < 0.05 versus control) (Fig. 2A). Unexpectedly, GF109203X (GF), a highly potent and specific PKC inhibitor, did not significantly inhibit the effect of BK at a high concentration (1.34 ± 0.15-fold n = 6 for GF at 10 μM, P > 0.05 versus BK). This result was supported by the lack of effect of PMA (1 μM, 1 min), a potent PKC activator, on TRPA1 potentiation (0.63 ± 0.13-fold, n = 8 P > 0.05 versus control) (Fig. 2A). These data suggest that BK-induced potentiation of TRPA1 responsiveness is dependent on PLC activation, but independent of PKC activation.

The B2R also couples to Gs protein, and activation of B2R activates Gs followed by an increase in cAMP production and PKA activation (Liebmann and Bohmer, 2000). Therefore, we next examined whether the BK-induced potentiation of TRPA1 was regulated by the PKA pathway. KT5720 (1 μM) and H89 (10 μM), both are potent PKA inhibitors, significantly prevented the potentiation of AITC-evoked TRPA1 currents in HEK cells (0.89 ± 0.01-versus control, 0.47 ± 0.10-fold, n = 6 for H89, P < 0.05 versus BK) (Fig. 2A). In addition to the inhibitory effect of PKA inhibitors, FSK (10 μM), a potent PKA activator, and 8-Br-cAMP (100 μM), potentiated the AITC-activated currents (1.46 ± 0.25-fold, n = 7 for FSK, 1.40 ± 0.18-fold, n = 5 for 8-Br-cAMP, P < 0.05 versus control) (Fig. 2A and B).

**BK potentiates TRPA1 channels in DRG neurons**

We have shown that BK can sensitize TRPA1 in heterologous expression systems. To determine if this interaction is physiologically relevant, we performed calcium imaging to determine if BK- and AITC-responsive profiles overlap in cultured DRG neurons. We found that AITC-activated 31.67% of the total neurons, and 65.22% of BK-responsive neurons, whereas BK activated 16.37% of the total neurons, and 37.04% of the AITC/capsaicin-corresponsive neurons (Fig. 3A and B). These results suggest that a majority of TRPA1-expressing neurons also express BK receptors, providing the physiological possibility of functional interaction between TRPA1 and BK receptors in the rat DRG neurons.

We then tested whether BK would potentiate TRPA1 channels in sensory neurons. We performed voltage-clamp experiments in cultured rat DRG neurons, and examined the effects of BK on the AITC-activated currents. A recent study indicates that there are still AITC-sensitive cells in TRPA1 knockout mice, but these are not the TRPV1-expressing cells where most TRPA1 is expressed (Kwan et al., 2006).
To confirm the AITC-evoked current in DRG is certainly a TRPA1-mediated event, capsaicin (1 mM), the selective agonist of TRPV1 was applied at the end of recording using the patch-clamp preparation (see Materials and methods section). To further know the selectivity of AITC in DRG neurons when used in 300 μM, we tested if camphor, a specific antagonist for TRPA1 (Xu et al., 2005; Macpherson et al., 2006; Bang et al., 2007; Sawada et al., 2007), could block the AITC-activated action. We found that camphor in 2 mM completely and reversibly inhibited the AITC- or cinnamaldehyde-induced currents in DRG neurons (Supplementary Fig. 1). In the voltage-clamp experiments, similar to that observed from HEK cells, the AITC (300 μM)-activated inward currents generally underwent tachyphylaxis, giving smaller responses on repeated applications. However, after a 1-min pre-treatment with BK (1 μM) or [Hyp 3]-bradykinin (1 μM), a more selective agonist of B2R in rodent (Leeb-Lundberg et al., 2005), the same dose of AITC produced larger current responses than the first application of AITC, where there was no pre-treatment with BK or [Hyp3]-bradykinin (0.83 ± 0.09-fold, n = 12 for control; 1.77 ± 0.24-fold, n = 13 for BK; 1.84 ± 0.39-fold, n = 5 for [Hyp3]-bradykinin; P < 0.01, control) (Fig. 4A–C). Similar to that in the HEK cells, the PLC inhibitor, ET-18-OCH 3 (2 μM) and the PKA inhibitor, H89 (1 or 10 μM) significantly prevented the potentiation by BK application in DRG neurons (0.95 ± 0.18-fold, n = 6 for ET-18-OCH 3; 0.96 ± 0.26-fold, n = 8 for H89 1 μM; 0.95 ± 0.07-fold, n = 8 for H89 10 μM, P < 0.05 versus BK) (Fig. 4C). Because PLC-dependent hydrolysis of PIP 2 has been shown to regulate the activity of TRPA1 (Dai et al., 2007), we asked whether

Fig. 2 BK potentiates AITC-activated currents in HEK cells transfected with TRPA1 and B2R in a PLC and a PKA-dependent manner. AITC (30 μM) was perfused for 20 s in all experiments. (A) AITC was re-applied 20 s after exposure to the bath solution with or without BK (20 nM). Currents were normalized to values first induced by AITC application in the absence of BK. A PLC inhibitor, ET-18-OCH 3 (ET, 2 μM); two PKA inhibitors, KT5720 (1 μM) and H89 (10 μM); or a PKC inhibitor, GF09023X (GF 10 μM) was perfused for 2 min before BK application. In some experiments, instead of BK, a PLC activator, m-3M3FBS (10 μM) for 90 s; PKA activators 8-Br-cAMP (100 μM) for 100 s, or Forskolin (FSK 10 μM) for 90 s; a PKC activator, PMA (1 μM) for 60 s was perfused before re-application of AITC. (B) Representative trace showing that AITC-activated currents were potentiated after perfusion of FSK, Vh = −60 mV in all experiments. Numbers in parentheses indicate cells tested. *P < 0.01 versus control (con); #P < 0.05 versus BK; unpaired t-test.

Fig. 3 BK and allyl isothiocyanate co-activated cultured DRG neurons. (A) Radiometric calcium imaging of representative DRG neuron responses to BK (BK, 20 μM), allyl isothiocyanate (AITC, 100 μM) and capsaicin (cap, 1 μM). (B) BK and allyl isothiocyanate response profiles.
BK-mediated potentiation of TRPA1 channel activity was regulated by PIP2. When water-soluble PIP2 was diluted in the pipette solution to a final concentration of 10 μM, the effect of BK was almost completely inhibited (0.88 ± 0.13-fold, n = 7, P < 0.05 versus BK) (Fig. 4C). The AITC-induced current density was significantly inhibited by intra-cellular PIP2 application (data not shown), which is consistent with our previous study (Dai et al., 2007).

To make sure that BK would activate the PKA signalling pathway in DRG neurons, we first determined the effects of BK on intra-cellular cAMP levels in DRG neurons using enzyme immunoassay. BK at 1 μM, the same concentration of BK that potentiated AITC-activated currents in DRG neurons, significantly increased intra-cellular cAMP level (1.81 ± 0.18-fold, n = 6 for BK; P < 0.01 versus control) (Fig. 5). Activation of PKA results in the translocation of its catalytic subunits (PKAc) to the cell periphery in neurons (Rathee et al., 2002). Therefore, we next determined the translocation of PKAc in cultured DRG neurons after BK stimulation (Fig. 6). In control neurons, staining of PKAc shared the same immunofluorescence intensity between peripheral regions and central regions of neurons (0.95 ± 0.03 U, for peripheral, versus 0.94 ± 0.03 U, for central regions, n = 21, P > 0.05). BK stimulation induced the translocation of PKAc to the periphery of the cells in the majority of small-sized neurons (1.35 ± 0.08 or 1.36 ± 0.07 U, for peripheral, versus 0.59 ± 0.06 U, for central regions, n = 25, P < 0.01). As a positive control, FSK treatment also induced the translocation of PKAc (1.46 ± 0.11 or 1.44 ± 0.11 U, for peripheral, versus 0.63 ± 0.06 U, for central regions, n = 26, P < 0.01) (Fig. 6), which are consistent with the previous report (Rathee et al., 2002). These observations confirm that BK activates PKA pathway in DRG neurons.

We have demonstrated that BK may sensitize TRPA1 through the PLC and PKA signal pathways. However, after B2R activation, whether these signalling pathways are activated in parallel or one is upstream of another remains to be clarified. We then performed an occlusion experiment with DRG neurons to answer this question. The PLC activator, m-3M3FBS at 100 μM and the PKA activator, FSK at both 10 μM and 100 μM potentiated the AITC-induced currents in DRG neurons (1.87 ± 0.32-fold, n = 8 for m-3M3FBS, 1.99 ± 0.46-fold, n = 6 for FSK 10 μM, 1.67 ± 0.27-fold, n = 5 for FSK 100 μM, P < 0.005 versus control, respectively). No significant difference of the normalized currents was observed between FSK at 10 μM and 100 μM (Fig. 7A), suggesting that FSK at 100 μM could
Fig. 6 Translocation of PKAc after BK stimulation in cultured DRG neurons. Microphotographs (upper panels) show examples of neurons double stained with PKAc antibody (green) and Hoechst 33342 (blue) without (control) or after BK (1 μM, 1 min) or FSK (10 μM, 0.5 min) stimulation. Confocal images indicate the position of the line scan profile used for calculations in line graphs (middle panels). Confocal line scan profile was taken with the Image J software. Columns (lower panels) show the average fluorescence intensity in the peripheral (P) or central (C) regions divided by the total average fluorescence intensity of the cell. *P < 0.01 indicates significant differences of fluorescence intensity between peripheral and central regions. Scale bars, 10 μm.

Fig. 7 Occlusion experiments indicated that PLC and PKA pathways potentiated TRPA1 activity in an additive manner in DRG neurons. AITC (300 μM) was perfused for 20 s in all experiment. (A) A PKA activator FSK (10 μM or 100 μM), a PLC activator m-3M3FBS (100 μM) or a mixture of the FSK (100 μM) and the m-3M3FBS (100 μM) was applied for 1 min before AITC re-application. Currents were normalized to the values first evoked by AITC in the absence of activators. Vh = −60 mV in all experiments. *P < 0.005 versus control (con), #P < 0.05 versus FSK 100 μM and P < 0.05 versus m-3M3FBS; unpaired t-test. Numbers in parentheses indicate cells tested. (B) Representative trace of TRPA1-activated currents which was potentiated after the mixture of FSK (100 μM) and m-3M3FBS (100 μM) application for 1 min.
saturate the PKA-dependent potentiation. However, application of a mixture of FSK (100 μM) and m-3M3FBS (100 μM) significantly increased the normalized current compared to either application alone of FSK (100 μM) or of m-3M3FBS (3.70 ± 0.75-fold, n = 6 for FSK + m-3M3FBS, P < 0.05 versus FSK or m-3M3FBS) (Fig. 7A and B). These results indicate an additive potentiation effect of the PLC signal to the PKA signal pathway.

**AITC-evoked pain behaviour is enhanced by BK**

Activation of TRPA1 by pungent natural products suggests a nociceptive role for TRPA1. To test whether BK could enhance TRPA1-mediated pain behaviours, we made intraplantar injections of AITC after a sub-painful dose injection of BK (Ferreira et al., 2004) and recorded nocifensive behaviours of rats. AITC-injection induced significant flinching and lifting behaviours of the injected hind paw during the 1-h post-injection period, whereas such behaviours were not observed in vehicle-injected rats (data not shown). The sub-painful dose injection of BK into the hind paw as well as saline did not cause any inflammatory reactions such as redness, swelling, heat and acute nocifensive behaviours (e.g. paw lifting, flinching or licking, Fig. 8A and B). The AITC was injected into the same area of hind paw 5 min after saline or BK pre-treatment. Five minutes after pre-treatment with the BK, intraplantar injection of AITC induced a significant increase of the number of paw lifts and the duration of paw flinches in the initial 1-hour post-injection period, compared to those of rats pre-treated with saline (Fig. 8A and B).

**Discussion**

BK directly excites nociceptive DRG neurons and causes hyperalgesia (Levine et al., 1993; Couture et al., 2001). Previous studies have showed that activation of B2R leads...
to sensitization of TRPV1 activity, lowers the threshold temperature for heat activation of TRPV1 (Chuang et al., 2001; Sugiu et al., 2002). However, evidence implies this pathway is insufficient to account for BK action (Kollarik and Undem, 2004; Rong et al., 2004). Recent reports have showed that the TRPA1 null mice exhibit pronounced deficits in BK-evoked nociceptor excitation and pain hypersensitivity (Bautista et al., 2006; Kwan et al., 2006); indicating an indispensable role of TRPA1 in the BK-mediated pain. BK at a dose of no <1 μM can activate TRPA1 in heterologous HEK cells, or at a dose of 5 μM can activate TRPA1 in DRG neurons (Bandell et al., 2004). In the present results, we showed that BK at low doses, which have been reported to be more closely to its concentration in vivo (Barlas et al., 1985; Kumakura et al., 1988; Langber et al., 2002; Shah et al., 2005), did not activate TRPA1, but sensitized TRPA1 to agonist stimulation in the transfected HEK cells and in primary sensory neurons.

In the present study, TRPA1 currents evoked by either of two different stimuli (AITC or cinnamaldehyde) were potentiated by BK in HEK cells transfected with B2R and TRPA1; suggesting B2R is the possible target through which these effects could be mediated. In the case of DRG neurons, although BK and a more selective activator of B2R, [Hyp3]-bradykinin, potentiated the AITC-activated currents; a role of B1R in the potentiation could not be completely ignored. It has been shown that B1R is constitutively expressed in DRG neurons in the rat and human (Ma et al., 2000; Wotherspoon and Winter, 2000). Although admittedly there is a debate about the possible function of the B1R in the normal animal (Brand et al., 2001), B1R is rapidly upregulated and plays a functional role in the case of cell damage and inflammation (Ferreira et al., 2001; Ma and Heavens, 2001; Fox et al., 2003).

In the present experiments, the lack of potentiating effect of BK in cells treated with the PLC inhibitor, and the similar potentiating effect of the PLC activator, indicate that a PLC-dependent pathway is involved in BK receptor-mediated TRPA1 sensitization. PLC activity plays a major role in the regulation and activation of many TRP channels (Minke, 2001). One of the consequences of PLC activation is the breakdown of PIP2 into diacylglycerol (DAG) and inositol triphosphate (IP3). IP3 then releases calcium from internal stores, while DAG activates PKC and can also be converted to arachidonic acid by DAG lipase. We found that the PKC inhibitor did not prevent the potentiation of AITC-evoked current by BK. The PKC activator did not potentiate the AITC-evoked current. These observations suggest that PKC activation does not contribute to the sensitization of TRPA1, which is different from the TRPV1 sensitization mechanism. We have recently reported that proteinase activated receptor 2-activation mediated sensitization of TRPA1 activity in a PLC, but not PKC-dependent mechanism (Dai et al., 2007), which is consistent with the BK-mediated potentiating mechanism of TRPA1 in the present study.

The B2R may couple to different classes of G proteins (including Gs), resulting in simultaneous or consecutive initiation of different signalling chains (Liebmann and Bohmer, 2000). In addition to PLC, a Gs-cAMP-PKA signalling involved the downstream from B2R in several cell lines has been indicated by numerous studies (Stevens et al., 1994; Liebmann et al., 1996; Graness et al., 1997; Bae et al., 2003). The PKA pathway has been proposed to be involved in sensitization of TRPV1 (Lopshire and Nicol, 1998; De Petrocellis et al., 2001; Hu et al., 2002; Rathee et al., 2002; Vetter et al., 2006), and in the development of inflammatory hyperalgesia (Naik, 1984; Malmberg et al., 1997). cAMP levels are elevated in inflamed tissues (Elattar and Lin, 1981; Malmberg et al., 1997). PKA or adenyl cyclase (AC) activators lower nociceptive thresholds, while PKA inhibitors can be anti-hyperalgesic (Taiwo and Levine, 1991). The cAMP/PKA pathway might be essential in sensitizing inflammatory nociception and contribute to the development of inflammatory hyperalgesia induced by pro-inflammatory mediators (Naik, 1984; Malmberg et al., 1997).

In the present study, we demonstrate for the first time that BK stimulation can activate the PKA signal pathway in DRG neurons. Application of BK to cultured DRG neurons increased the intra-cellular level of cAMP and induced translocation of the PKA catalytic subunit. These findings corroborate the correlative electrophysiological recordings from sensory neurons, i.e. application of PKA inhibitors completely abolished the potentiation of AITC-evoked current by BK, while the AITC-evoked currents were potentiated similarly by PKA activators to that by BK. These results indicate the PKA signalling pathway is also important in the sensitization mechanism of TRPA1. In the occlusion experiment, the PLC activator potentiated AITC-induced TRPA1 current additively under saturation of the PKA pathway. Therefore, after activation of BK receptors, PLC and PKA may regulate TRPA1 in an additive or synergistic manner.

Calcium sensitive ACs, such as AC1, may be activated by calcium leading to the production of cAMP (Ferguson and Storm, 2004). Thus, it is possible that PLC activation leads to an increase in intra-cellular calcium, which in turn stimulates calcium-sensitive ACs, leading to cAMP production and PKA activation. However, we do not interpret our results by this mechanism, because intra-cellular calcium was tightly chelated with the 5 mM EGTA included in the pipette solution in the present study. After inflammation, BK is released from the local tissue and then binds to its receptor, resulting in the initiation of both Gq and Gs signal pathways. The Gq-mediated PLC activation and the Gs/cAMP-mediated PKA activation contribute to the sensitization of TRPA1 in an additive manner.

If BK can sensitize TRPA1, pain sensation that is caused through the TRPA1 channel may also be potentiated by the BK receptor activation. Topical application of AITC has been reported to excite sensory nerve fibres, thereby
producing acute pain (Bandell et al., 2004; Jorđt et al., 2004; Bautista et al., 2006). In our behavioural studies, we found that pre-treatment of BK in a sub-painful dose led to an increase in AITC-evoked nocifensive behaviours in rats. These results indicate that BK, at a dose, which does not cause nociception by itself, consistently potentiates TRPA1 activity, not only at the cellular level, but also at the behavioural level. TRPA1 is not expressed by non-neural components in the skin (Nagata et al., 2005), while B2 receptor is ubiquitously and constitutively expressed throughout most mammalian tissues and cells (Hall, 1992; Campos et al., 2006). Therefore, we cannot completely exclude contribution of non-neural cells to the increased nocifensive behaviours. However, because BK used in the behavioural experiments was sub-inflammatory, which did not cause redness, heat, swelling and pain (see Results section), BK-mediated releasing of inflammatory mediators, such as cytokines or nitric oxide from endothelial or immune cells or fibroblasts may not be considered.

BK may be released from peripheral tissue and plasma in inflammatory diseases. Our results represent a novel mechanism through which BK released in response to tissue inflammation might trigger the sensation of pain through TRPA1 activation. Because our present data demonstrate that BK functionally sensitzizes TRPA1, which has been reported to be involved in pathological pain sensation and proalgesic actions of environmental irritants, compounds interfering with the interaction between TRPA1 and BK receptor may be useful in the treatment of inflammatory pain.

**Supplementary material**

Supplementary material is available at *Brain* online.

**Acknowledgements**

We gratefully acknowledge technical assistance from Nobumasa Ushio and Noriko Kusumoto. We thank Dr D. A. Thomas for correcting the English usage on this article. This work was supported in part by Grants-in-Aid for Scientific Research, and the Open Research Center grant, Hyogo College of Medicine, both from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

**References**


Potentiation of TRPA1 by bradykinin


Kollarik M, Undem BJ. Activation of bronchopulmonary vagal afferent nerves with bradykinin, acid and vanilloid receptor agonists in wild-type and TRPV1 knockout mice. J Physiol 2004; 555: 115–23.


