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Contribution of TRPV1 to the bradykinin-evoked nociceptive behavior and excitation of cutaneous sensory neurons

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ABSTRACT

Bradykinin (BK), a major inflammatory mediator, excites and sensitizes nociceptor neurons/fibers, thus evoking pain and hyperalgesia. The cellular signaling mechanisms underlying these actions have remained unsolved, especially in regard to the identity of channels that mediate acute excitation. Here, to clarify the contribution of transient receptor potential vanilloid 1 (TRPV1), a heat-sensitive ion channel, to the BK-evoked nociceptor excitation and pain, we examined the behavioral and physiological BK-responses in TRPV1-deficient (KO) mice. A nocifencive behavior after BK injection (100 pmol/site) into mouse sole was reduced in TRPV1-KO mice compared with wild-type (WT). A higher dose of BK (1 nmol/site), however, induced the response in TRPV1-KO mice indistinguishable from that in the WT. BK-evoked excitation of cutaneous C-fibers in TRPV1-KO mice was comparable to that in WT. BK clearly increased intracellular calcium in cultured dorsal root ganglion (DRG) neurons of TRPV1-KO mice, although the incidence of BK-sensitive neurons was reduced. BK has been reported to activate TRPA1 indirectly, yet a considerable part of BK-sensitive DRG neurons did not respond to a TRPA1 agonist, mustard oil. These results suggest that BK-evoked nociception/nociceptor response would not be simply explained by activation of TRPV1 and A1, and that BK-evoked nociceptor excitation would be mediated by several ionic mechanisms.

1. Introduction

Tissue damage due to injury, infection, and other pathological conditions releases chemical mediators responsible for inflammatory responses including pain and hyperalgesia. A nona-peptide, bradykinin (BK), is one of the major mediators for these responses. BK not only triggers various inflammation processes, such as vasodilatation and plasma extravasation, but also causes nociceptor depolarization and sensitization to thermal and mechanical stimuli (Kumazawa and Mizumura, 1980; Mense and Meyer, 1988; Neugebauer et al., 1989; Kumazawa et al., 1991; Koda and Mizumura, 2002). The action of BK on nociceptors is mediated by the activation of the metabotropic BK receptor subtype B2 (B2R) in both normal and some chronic inflammatory conditions (Banik et al., 2001). The intracellular signaling and ionic mechanisms coupling BK receptors to the excitation and sensitization of nociceptors have been intensively studied and divergent mechanisms have been proposed (Miller, 1987; Burgess et al., 1989; Cesare et al., 1999; Premkumar and Ahern, 2000; Chuang et al., 2001; Sugiura et al., 2002; Shin et al., 2002). In these studies, transient receptor potential vanilloid 1 (TRPV1) is considered to be a highly plausible candidate for the target ion channel of BK signaling pathways to depolarize the neurons.

TRPV1 is a non-selective cation channel preferentially expressed in a subset of sensory neurons with small diameter, presumably with C-fiber axons (Caterina et al., 1997). TRPV1 is activated by noxious heat (>43 °C), acid (pH <6) and capsaicin (CAP) (Caterina et al., 1997; Tominaga et al., 1998), and studies using TRPV1-deficient (knockout, KO) mice have revealed its involvement in the normal sensations of acid and noxious heat (Caterina et al., 2000) and inflammatory thermal hyperalgesia (Caterina et al., 2000; Davis et al., 2000).

As for the molecular basis of BK-induced nociceptor excitation, protein kinase C (PKC)-dependent sensitization of TRPV1 has been proposed from several lines of experiments (Reeh and Pethö, 2000). For examples, the activation of B2R by BK lowered the heat

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threshold of TRPV1 to a level below the skin temperature (to ~31 °C) through the PKC-dependent pathway (Sugiura et al., 2002). Similarly, in the excised patch of the dorsal root ganglion (DRG) neuron, BK opens the capsaicin-sensitive channel, presumed to be TRPV1, at room temperature only in the presence of PKC (Premkumar and Ahern, 2000). Besides, two other pathways independent of PKC have been also suggested. First, BK leads production of an endogenous TRPV1-agonist, 12-hydroperoxyeicosatetraenoic acid, through the phospholipase A_2-lipoxygenase pathway (Shin et al., 2002). Another is the phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2), which serves as an inhibitory module of TRPV1 (Chuang et al., 2001; Prescott and Julius, 2003). Although these results proposed the possible mechanisms of BK-evoked excitation of nociceptors, it is still unclear whether TRPV1 activation is the major mechanism for BK-induced excitation of sensory neurons and nociception.

Importance of TRPV1 in BK-evoked nociception has been reported using a TRPV1-antagonist in normal animals (Shin et al., 2002; Ferreira et al., 2004). On the other hand, recently, another member of the TRP channel family expressed in DRG, TRPA1, was shown to be a receptor-operated ion channel, which is activated and sensitized by BK (Jordt et al., 2004; Bandell et al., 2004; Wang et al., 2008). The BK-evoked nociception was examined on TRPA1-deficient mice, and revealed to be attenuated at the behavioral level (Kwan et al., 2006) as well as at the neuronal level (Bautista et al., 2006), but not examined on TRPV1-KO mice. Thus, it is still not clear to what extent these two TRP channels contribute to BK-evoked nociception.

In this report, to evaluate the contribution of TRPV1 and TRPA1 to BK-evoked nociception, first, we examined BK-responses of TRPV1-deficient mice by three different methods: (1) nocifensive behavioral assay; (2) single C-fiber recording; and (3) Ca^{2+}-imaging of cultured DRG neurons. In addition, we examined the possible involvement of TRPA1 in BK-evoked excitation of the sensory neurons.

2. Materials and methods

2.1. Animals

TRPV1-deficient (designated KO) C57BL/6 mice were generated by Caterina et al. (2000). Wild-type (WT) C57BL/6 mice were purchased from Charles River Laboratories, Japan. Adult (8–27 w) male mice were used for all experiments except the single-fiber recording experiment, in which we used females. In preliminary experiments, we confirmed that there was no difference in single C-fiber responses to BK or heat between WT male and female mice. Mice were kept under a 12 h-light/dark cycle in an air-conditioned room (24 °C). All experiments were approved by the Animal Care Committee of Nagoya University, and carried out in accordance with the ethical guidelines of The International Association for the Study of Pain (Zimmermann, 1983). All efforts have been made to minimize the number of animals used and their suffering.

2.2. Chemicals

BK (Peptide Institute, Osaka, Japan) was dissolved at 10 mM in the saline or bath solutions used in each experiment (see below), kept frozen (≤−20 °C), and diluted with those same solutions just before use. Capsaicin (CAP, Sigma, St. Louis, MO, USA) and mustard oil (MO, TOKYO KASEI, Tokyo, Japan) were dissolved in ethanol at 10 mM and 100 mM, respectively and diluted 1000-fold with the bath solutions.

2.3. Paw-licking behavioral assay

Mice were individually acclimated in cylindrical test chambers made of clear plexiglas (20 cm in diameter, 20 cm in height) for ~5 min, beneath the floor of which a mirror was set at a 45° angle to allow a clear view of their paws. BK solutions (5 μl saline containing 10, 100, or 1000 pmol of BK) or saline (vehicle) pre-warmed at 34 °C was injected into one side of the sole using a 29 G needle. After the injection, the mice were placed in the test chamber, recorded on videotape and analyzed their behavior offline with the observer blinded to the genotypes. Licking of the treated paw was observed for 1 min immediately after BK injection, because it completely disappeared thereafter. Individual mice received only one injection of a single dose of BK or vehicle, with 12–13 mice used for each dose. The experiment was conducted in a quiet and air-conditioned room at 24 °C.

2.4. Single-fiber electrophysiology

Ten WT and six KO female mice were used. The techniques for rat skin-nerve *in vitro* preparation and single-fiber recording (Reeh, 1986; Banik et al., 2001) were utilized. Mice were anesthetized with sodium pentobarbital (55 mg/kg, i.p.), and the hairy skin of hind paw was dissected with the saphenous nerve attached. The skin was placed epidermal-side down in the organ bath, and continuously superfused with modified Krebs–Henseleit solution (in mM: 110.9 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2SO_4, 24.4 $NaHCO_3$ and 20 glucose, temperature 34 ± 0.5 °C) equilibrated with a 95% O_2 and 5% CO_2 gas mixture. The nerve bundle was repeatedly teased until single-fiber activity could be obtained. The receptive field of a single identified afferent fiber was searched by mechanically probing the corium side of the skin with a blunt glass rod. The fibers studied here were thus categorized into mechano-sensitive afferents. The identified receptive field was isolated with a small metal ring (inner diameter 4 mm; volume ~0.09 ml) to apply BK solutions. Ten-fold serial dilution of BK (10^{-8} to 10^{-4} M) dissolved in the modified Krebs–Henseleit solution were sequentially applied, from the low to high concentrations, to the isolated receptive field by superfusion (2.6 ml/min) for 1 min at >7-min intervals. The receptive field was electrically stimulated to measure conduction velocity of the fibers; those conducting less than 1.2 m/s were considered to be unmyelinated C-fibers. Action potentials were amplified, filtered, and continuously recorded on videotape. The data were later analyzed with SPIKE/SPIDI software (C. Foster, University of Erlangen-Nurnberg, Germany). If a C-fiber produced at least six spikes during 5 min after the onset of BK application, it was considered responsive to BK. To calculate net discharges of spontaneously discharging C-fibers, the background activities measured during the pre-exposure period (1 min) were multiplied by 5, assuming that they continued for the whole recording episode, and then subtracted from the total activities during the post-exposure period (5 min).

2.5. Cell culture and intracellular calcium imaging

Five WT and nine KO mice were used. Methods of cell culture and calcium measurement were based on a previous report (Senba et al., 2004) with partial modifications. Briefly, the mice were acutely decapitated, and the lumbar and thoracic DRGs were dissected out. After the enzymatic digestion of DRGs with 0.2% collagenase (Wako, Osaka, Japan) at 34 °C for 60 min, the DRG cells were mechanically dissociated by gentle tapping with a needle and plated onto small glass coverslips pre-coated with poly-L-lysine. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (Wako), 100 ng/ml mouse NGF-7S (Sigma), and 2 mM

GlutaMAX™-I Supplement (Invitrogen, Carlsbad, CA, USA) for 2 days at 34 °C in a humid 5% CO_2 atmosphere.

The intracellular Ca^{2+} level was monitored using a Ca^{2+}-sensitive dye, Oregon Green™ 488 BAPTA-1 AM (Molecular Probes, Leiden, The Netherlands), and a confocal laser scanning microscope system (Oz/Intervision, Noran Instruments, USA) equipped with an inverted microscope (Axiovert S100, Zeiss, Germany). Cultured DRG neurons on the cover slips were incubated for 2 h at 37 °C in a bath solution (HEPES-Ringer solution: in mM; 140 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose, 10 HEPES, ~4 NaOH, pH 7.4) containing 0.8 μM Oregon Green, 0.001% Pluronic F-127 (Molecular Probes), and 0.1% dimethyl sulfoxide (DMSO, Wako). The dye was excited at 488 nm, and emitted fluorescence exceeding 500 nm was detected through an LP500 long-path filter. Sixteen consecutive scanned images (33 ms/scan) were averaged to reduce random noise and stored every 3 s. The cells were continuously superfused with the bath solution (~5 ml/min), and the chemical solutions were applied by switching the normal perfusate to each chemical solution in the following order: BK (10 μM) for 30 s; high K^+ (45 mM) for 15 s; MO (100 μM) for 15 s; CAP (10 μM) for 15 s. The time interval between each stimulus was ≥5 min to reduce the possible effects of cross-desensitization or sensitization and to ensure that the fluorescence intensity returned close to its pre-stimulus level. The duration of the stimulus with BK was set to last twice as long as that for the others, since slow calcium responses were often observed (see Fig. 4). To make the high K^+ solution, 40 mM NaCl of the bath solution was substituted with equimolar KCl. The basal fluorescence level (F_0) is defined as an averaged intensity for 15 s just before the application of each chemical. The chemical responsiveness of the neuron was judged as positive if the fluorescence intensity (F) increased by ≥10% and ≥4S.D. (standard deviation) of the basal level (F_0). Experiments were performed at room temperature. Images were analyzed by 2D-analysis software (Noran Instruments). Only the data from high K^+-sensitive cells were included in the analysis, because they were considered to be living neurons.

2.6. Statistics

Data were expressed by a mean ± standard error of the mean (S.E.M.). For statistical analysis of the results in behavioral tests and calcium imaging, the χ^2-test (for Figs. 1A and 3B) or Student's t-test (Figs. 1B and 4B) were used. For single-fiber recording, we utilized Fisher's exact probability test (Fig. 2B) or Mann–Whitney's U test (Fig. 2C). A difference was judged to be significant when $P < 0.05$.

3. Results

3.1. TRPV1-deficient mice had behaviorally diminished nociceptive sensitivity to bradykinin

To assess the contribution of TRPV1 in BK-evoked nociception at the behavioral level, a typical nocifensive paw-licking behavior was observed after an intraplantar injection of BK in wild-type (WT) and TRPV1-deficient (KO) mice. Both percentage of animals that licked the treated hindpaw for ≥1 s (Fig. 1A) and the time spent licking (Fig. 1B) were compared. The BK-evoked behavior showed some dose-dependent increase in both genotypes (Fig. 1). In WT mice, injection of a low dose of BK (10 pmol/site) or a control vehicle induced either no or only a brief licking behavior, respectively, whereas 100 pmol of BK induced clear responses (Fig. 1B) in about two-thirds of the animals (Fig. 1A). A 10-fold higher dose of BK (1000 pmol/site), however, did not cause further increase in the responses of WT animals. On the other hand, at a dose of 100 pmol BK, only one fourth of TRPV1-KO animals showed a licking response to BK, a significantly decreased incidence of BK-responders (Fig. 1A; KO, 25% vs. WT, 67%; n = 9–12; $P < 0.05$ by χ^2-test), and a shorter licking duration compared to that of WT (Fig. 1B; KO, 1.7 ± 0.9 s vs. WT, 4.6 ± 1.5 s; n = 12–13; P = 0.10 by t-test). At the highest dose of BK (1000 pmol/site), however, KO and WT mice displayed similar responses. Thus TRPV1-KO mice exhibited a diminished sensitivity to BK, indicating that TRPV1 is an important component in BK-evoked nociception. However, the fact that KO mice showed clear nocifensive responses to BK, especially at high doses, also suggests the involvement of TRPV1-independent mechanisms.

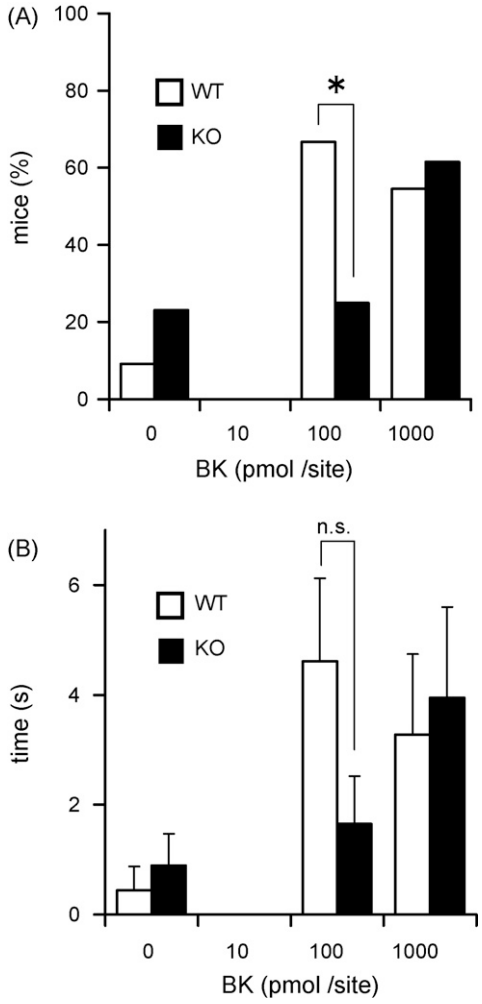

Fig. 1. Dose-dependency of bradykinin (BK)-evoked acute nocifensive behavior in wild-type (WT) and TRPV1-deficient (KO) mice. Each dose of BK (10, 100, and 1000 pmol/5 μl) was intraplantarly injected into a hind paw. Nocifensive behavior was observed for 1 min after BK injection at room temperature (24 °C). (A) Incidence of mice that showed paw-licking behavior (>1 s) evoked by BK injection. An asterisk denotes significant difference ($P < 0.05$, χ^2-test). (B) Total duration of paw-licking behavior evoked by BK injection. Results are shown as means + S.E.M., and n.s. means not significant (P = 0.10, unpaired t-test). WT, n = 9–12; KO, n = 12–13.

3.2. Single C-fibers of TRPV1-deficient mice responded normally to bradykinin

We then examined whether cutaneous afferent fibers of TRPV1-KO mice would respond to BK applied to their receptive fields on the skin. BK excited 13 single C-fibers from WT and 17 from KO mice (Fig. 2). As depicted in the sample recordings in Fig. 2A, C-fibers in both KO and WT animals exhibited clearly concentration-dependent

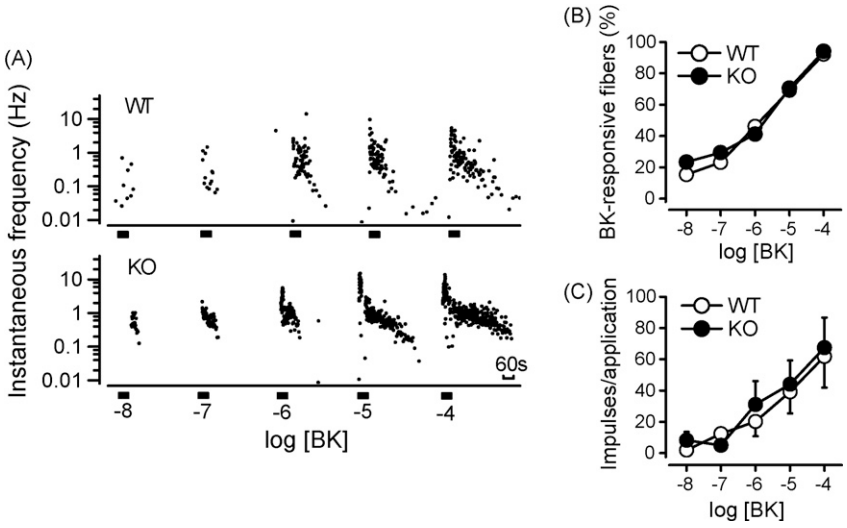

Fig. 2. Concentration-dependent responses of cutaneous mechano-sensitive C-fiber receptors to bradykinin (BK). (A) A representative single unit is shown for each wild-type (WT, *upper panel*) and TRPV1-deficient (KO, *lower panel*) mice. Each dot indicates a single-action potential discharge; its instantaneous frequency (Hz, in logarithmic scale) is plotted against a time scale. Series of 10-fold graduated concentrations of BK (10^{-8} to 10^{-4} M) were applied for 1 min (black bars) at >7-min intervals each. (B and C) Summary of BK-evoked responses of the C-fibers. WT, *open circles*; KO, *solid circles*. (B) Incidences of BK-responsive C-fibers. WT, $n = 13$; KO, $n = 17$. (C) Net numbers of impulses evoked by BK for 5 min after each exposure. Background spontaneous discharges were subtracted from raw records to yield net BK-evoked impulses (see Section 2). Results were shown as means ± S.E.M. WT, $n = 7$–10; KO, $n = 14$. The number of units examined is fewer than in B, because we failed to discriminate spike forms in some units.

responses to BK (10^{-8} to 10^{-4} M). The responses to higher concentrations of BK typically outlasted the BK application period as previously reported in rats (Banik et al., 2001). All the BK-evoked responses of C-fibers recorded from WT and KO animals were summarized and compared (Fig. 2B and C). Incidences of BK-responsive fibers increased concentration-dependently, and showed no difference between WT and KO animals at any BK concentration (Fig. 2B). Similarly, the concentration-dependent relationship of the net numbers of impulses evoked by BK, which represent the amplitude of the responses, were indistinguishable between WT and KO mice (Fig. 2C). These results undoubtedly demonstrated that most of the C-fibers of mice retain their BK-sensitivities even in the absence of TRPV1.

3.3. Calcium response of cultured DRG neurons was evoked by BK without TRPV1 and TRPA1

To investigate the requirement of TRPV1 for a BK-evoked excitation of the sensory neurons in a much larger population, we observed calcium responses in the primary cultures of dissociated DRG neurons using the calcium imaging technique. BK-evoked intracellular Ca^{2+} increases (calcium responses) showed considerable variation in their response patterns (latency, duration, magnitude and desensitization) in both genotype neurons (Fig. 3A). Despite such a wide variety, the responses of KO neurons were totally indistinguishable from those of WT. However, the incidence of chemical sensitivities clearly differed (Fig. 3B). BK (10 μM) evoked a calcium response in 18.6% (90/483 neurons) of WT DRG neurons, and 8.3% (41/495 neurons) of the KO neurons, in which CAP-sensitivity was completely lost (Fig. 3B). In addition, the BK-sensitive neurons of WT animals did not always respond to CAP at a saturating concentration (10 μM) (Fig. 5G). These results indicate that TRPV1 contributes to BK-evoked excitation of DRG neurons, but not in any essential way.

Besides the BK-evoked Ca^{2+} influx, BK causes inositol 1,4,5-triphospate (IP_3)-dependent Ca^{2+} releases from internal Ca^{2+} stores (Thayer et al., 1988) via the $G_{q/11}$/PLCβ signaling pathway coupled to B2 receptors. Therefore, we examined the contribution of the internal Ca^{2+} sources to BK-evoked Ca^{2+}-response in KO neurons. In the absence of extracellular Ca^{2+}, BK application resulted in a

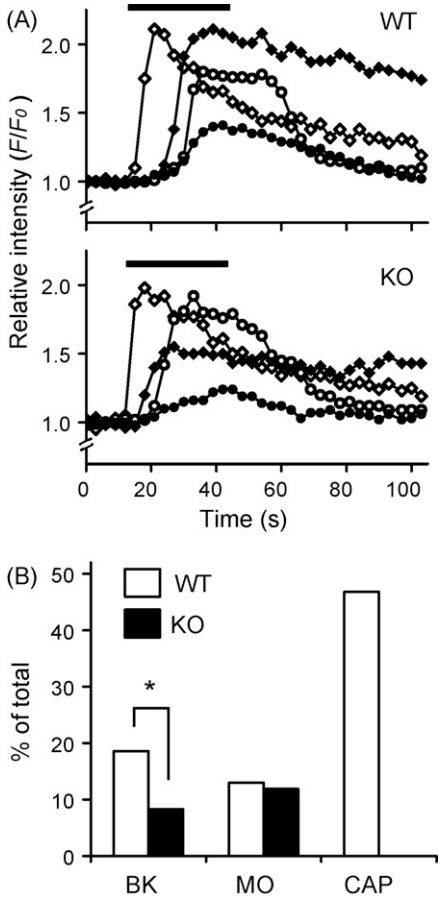

Fig. 3. Bradykinin (BK)-evoked calcium responses in cultured dorsal root ganglion (DRG) neurons. (A) Sample plots of relative fluorescence (F/F_0) changes by BK application (10 μM, 30 s, *black bars*). Four typical responses of DRG neurons were shown each for wild-type (WT, *upper panel*) and TRPV1-KO (KO, *lower panel*). (B) Summary of chemical sensitivities of cultured DRG neurons isolated from WT (*open columns*) and KO (*closed columns*) mice. Incidences of DRG neurons responding to bradykinin (BK, 10 μM), mustard oil (MO, 100 μM) and capsaicin (CAP, 10 μM) are shown. An asterisk denotes significant difference ($P < 0.05$, χ^2-test). Total number of neurons: WT, $n = 483$; KO, $n = 495$.

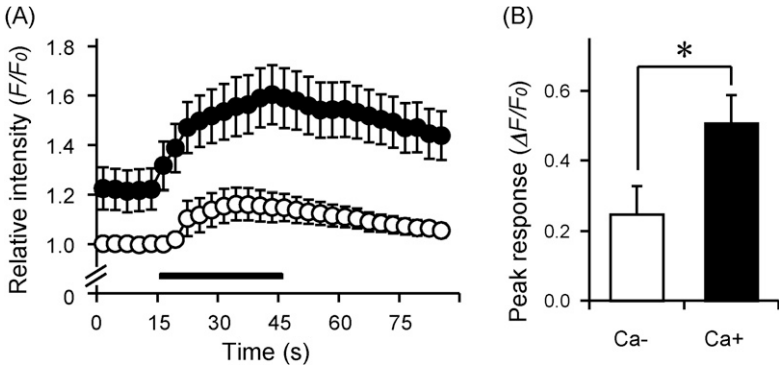

Fig. 4. Calcium source increased by bradykinin (BK) in cultured dorsal root ganglion (DRG) neurons of TRPV1-deficient (KO) mice. (A) BK-evoked calcium responses with (*closed circles*) or without (*open circles*) extracellular Ca^{2+} are shown superimposed. Cells were exposed to BK (*black bar*, 10 μM, 30 s) first in Ca^{2+}-free bath solution, which was then replaced by the Ca^{2+}-plus solution for 15 min, after which the second BK-responses were recorded. Fluorescence level (F) was given as a relative value to basal level under Ca^{2+}-free condition (F_0) and averaged (means ± S.E.M., n = 20). Basal fluorescence level was elevated by replacement with Ca^{2+}-free solution with Ca^{2+}-plus one. (B) Comparison of peak increments of BK-evoked fluorescence changes in (A) between Ca^{2+}-free (*open column*) and Ca^{2+}-plus (*closed column*) conditions. Increments of fluorescence (ΔF) from each pre-exposure level under Ca^{2+}-free and Ca^{2+}-plus conditions, respectively, was measured and normalized by F_0. The results were shown as means + S.E.M. (n = 20). An asterisk denotes significant difference (P = 0.037 by paired t-test).

subtle increase in intracellular Ca^{2+} in KO neurons (Fig. 4A). These cells showed stronger responses to BK after adding Ca^{2+} to the superfusate (Fig. 4A), in which the peak fluorescent change from the pre-exposure level was about double that in the Ca^{2+}-free condition (Fig. 4B). These results indicate that a substantial part of the Ca^{2+} increase in KO neurons, as well as normal DRG neurons (Linhart et al., 2003), originated from extracellular sources, suggesting that BK opened ion channels other than TRPV1 to depolarize and/or excite DRG neurons.

BK-evoked excitation of DRG neurons from TRPV1-KO mice might be explained by the presence of TRPA1. Therefore, to examine whether BK-sensitive neurons might overlap with functional expressions of TRPA1, a specific agonist for TRPA1, mustard oil (MO), was subsequently applied to DRG neurons. Nearly the same proportion of neurons in WT and KO cultures responded to MO (Fig. 3B; WT, 13.0%; KO, 11.9%). However, as shown in the sample images in Fig. 5A–F, BK-sensitive TRPV1-KO DRG neurons did not always respond to MO (100 μM). In this field, two BK-sensitive neurons (*arrowheads* in Fig. 5B) responded to a depolarizing stimulus with high K^+ (Fig. 5C), but not to either MO (Fig. 5D) or CAP (Fig. 5E). Thus, a considerable portion of the BK-sensitive neurons isolated from KO mice, i.e. CAP-insensitive neurons, was not MO-sensitive, and only about one-third (14/41) responded to MO (Fig. 5G), suggesting the presence of TRPV1/A1-independent mechanisms. In WT mice, a large part of BK-sensitive DRG neurons responded to CAP and/or MO (see below). About 40% of them responded to both CAP and MO, agreeing with previous studies showing that TRPA1 was co-expressed in a sub-population of TRPV1-positive neurons (Story et al., 2003; Kobayashi et al., 2005). However, no additive or synergistic effect of co-expression of TRPV1 and A1 was observed on their BK-evoked Ca^{2+}-responses (Fig. 5H). These data suggested that TRPV1 and A1 can mediate the BK-evoked responses of DRG neurons independently.

Finally, an analysis of cell sizes (data not shown) has revealed that the BK-sensitive neurons mainly belong to small (area $<$500 μm^2) or medium (500–1250 μm^2) size group in both WT and KO animals (WT, 62 small and 21 medium cells out of 90 BK-sensitive neurons; KO, 24 small and 15 medium cells out of 41 BK-sensitive neurons). These sizes of cell bodies are comparable with those of nociceptors. A few large cells ($\geq$1250 μm^2) also responded to BK, which is consistent with previous findings using electrophysiological recording of cutaneous afferent fibers (Fjallbrant and Iggo, 1961; Beck and Handwerker, 1974) in both genotypes (WT, 7/90; KO, 2/41 of BK-sensitive cells). However, no clear difference was observed in the size distribution of the BK-sensitive neurons between WT and KO mice (mean area ± S.E.M.; WT, 471.6 ± 20.1 μm^2, n = 90; KO, 504.7 ± 36.2 μm^2, n = 41; P = 0.55 by Student's t-test).

4. Discussion

TRPV1 and TRPA1 have been proposed as possible target ion channels indirectly activated by BK through the multiple B2R-signaling pathways in nociceptors. Here, using TRPV1-KO mice, we showed that TRPV1 plays some role in BK-evoked nociception, and that TRPV1-independent mechanisms also contribute to BK-evoked pain responses, which might include TRPA1 and other ion channels.

Our behavioral assay demonstrated that TRPV1-KO mice had a reduced sensitivity to BK compared to WT mice, though at the highest dose there was no difference. These results indicate that TRPV1 is partially involved in BK-evoked nociception, and that TRPV1-independent mechanisms also contribute to BK-evoked nociception, especially at higher dose. The difference in dose requirement for BK-evoked nocifencive behavior between KO and WT mice may reflect that the TRPV1-dependent pathway might possess higher BK-sensitivity than the TRPV1-independent ones. As the later pathway, TRPA1 might be involved in the BK-evoked acute pain, because TRPA1-mediated current of DRG neurons is sensitized to allyl isothiocyanate, a TRPA1-activating chemical, by BK at 1 μM, but not activated by BK itself at the same dose (Wang et al., 2008), suggesting that much higher concentrations of BK might be required to activate TRPA1 in DRG neurons.

The mice exhibited the obvious nocifensive paw-licking behavior upon an injection of 100–1000 pmol BK, i.e. 5 μl of 2×10^{-5} and 2×10^{-4} M solution, respectively. The reported BK concentrations in experimentally injured or inflamed human tissue have ranged from 10^{-10} to 10^{-7} M (Kumakura et al., 1988; Langberg et al., 2002; Blunk et al., 2003; Shah et al., 2005). Considering the diffusion and the short half-life of BK *in vivo* (Decarie et al., 1996), the actual local concentration must be higher than the reported values, yet it could not be as high as the concentration used in the present experiment. Thus, in *in vivo* tissues, where a comparatively low level of BK is produced by inflammation, the role of the TRPV1-dependent nocifensive pathway might be more important than that of TRPV1-independent mechanism, because its relatively high sensitivity to BK, as discussed above.

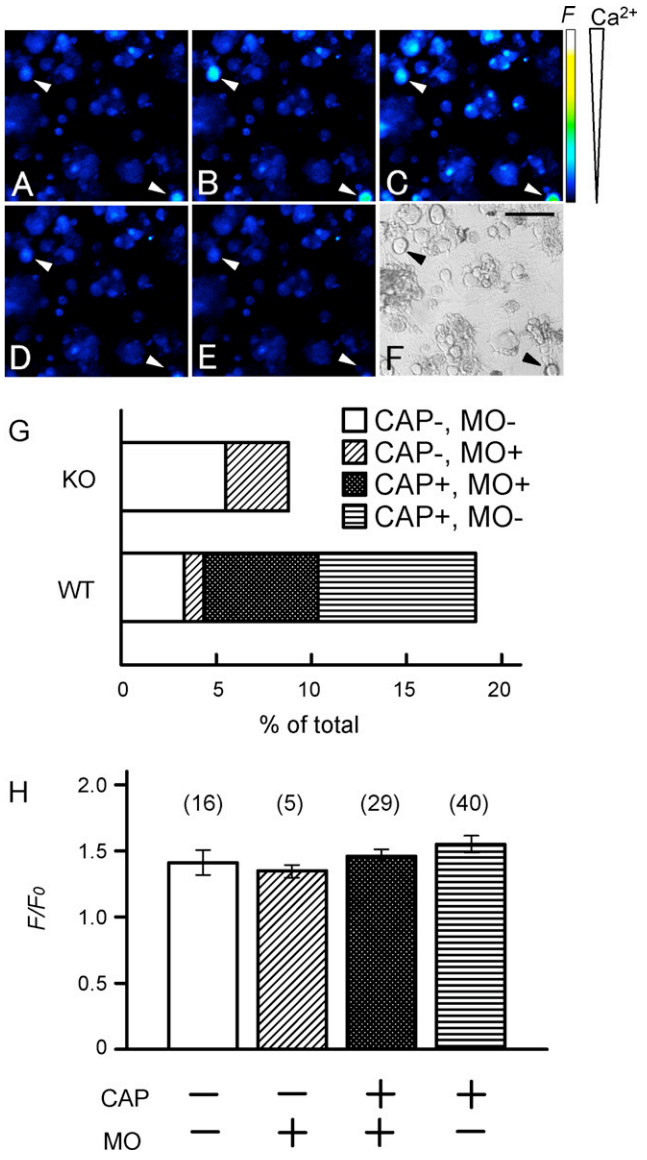

Fig. 5. Chemical sensitivities of bradykinin (BK)-sensitive DRG neurons isolated from wild-type (WT) and TRPV1-deficient (KO) mice. (A–F) Representative images of calcium responses of DRG neurons from TRPV1-KO mice. Pseudo color images of the fluorescence intensities at basal level (A), 15 s after exposure to 10 μM BK (B), 45 mM high K^+ (C), 100 μM mustard oil (MO) (D), and 10 μM capsaicin (CAP) (E). In these images, two neurons (*arrowheads*) clearly responded to BK (B) and high K^+ (C), but neither to MO (D) nor CAP (E). A color scale is shown at right, where higher calcium level is indicated by yellowish and whitish colors. (F) A bright-field image of A–E. Scale bar, 50 μm. (G) Profiles for MO- and CAP-sensitivities of BK-sensitive DRG neurons isolated from WT and TRPV1-KO mice. MO- or CAP-sensitive fractions are indicated as hatched areas in the respective columns of the BK-sensitive populations. A horizontal axis means percentage in the total neurons. CAP-sensitive population was completely lost in the neurons from KO mice. (H) Comparison of peak intensities (F/F_0) of BK-evoked Ca^{2+}-responses among the four populations of WT DRG neurons in G (mean ± S.E.M., numbers of cells were indicated on each column). The types of hatches in the columns are the same as in G.

Regarding the excitation mechanism for nociceptors by BK, Reeh and Pethö (2000) have hypothesized that BK excites nociceptors by lowering their threshold temperatures for activation, whereby the body temperature itself becomes an adequate stimulus for nociceptors. This hypothesis has been supported by Sugiura et al. (2002), who showed that BK lowers the threshold temperature of the heat-induced current (presumed to be mediated by TRPV1) below the normal skin temperature (<32 °C) in the capsaicin-sensitive small DRG neurons. In the present study, the BK concentrations that evoked nocifensive behavior in mice are high enough to reduce the threshold of TRPV1 below skin temperature (Sugiura et al., 2002). Therefore, our results support the contention that the above-mentioned mechanism works for the BK-evoked nociception in living animals, at least partially.

The result of our single-fiber recording showed a discrepancy from the result of behavioral tests with regard to the requirement of TRPV1. While a partial decrease of BK-evoked nociception was observed in TRPV1-KO mice, most of the C-fiber afferents in KO mice responded to BK. Additionally, there was no apparent difference in the stimulus-response relation in C-fiber afferents excitation by BK between WT and KO mice. One possible reason for this discrepancy might be that we recorded only the mechano-sensitive C-fibers. Our experience suggests that the majority of these fibers are also sensitive to heat, and they compose the largest population in nociceptors. However, we cannot exclude a possibility that other types of nociceptors such as the mechanically insensitive C-fibers and A-δ mechano-heat receptors that are also sensitive to bradykinin (Khan et al., 1992; Schmelz et al., 2003), may also have contributed to nocifensive behavior. BK-evoked responses similar to ours have been reported in visceral afferents of TRPV1-KO mice (Kollarik and Undem, 2004; Rong et al., 2004). The C-fibers in the KO mice might have its gene deficit compensated for by expressing alternative molecules, and thus they could exhibit normal BK responses. However, an over-expression of TRPA1 seems less likely, since the percentage of MO-sensitive DRG neurons did not differ between TRPV1-KO and WT mice. Conflicting with our results, several studies have suggested that TRPV1 is essential to the BK-evoked responses (Shin et al., 2002; Ferreira et al., 2004). In these studies, capsazepine, an antagonist for TRPV1, completely blocked BK-evoked discharges of C-fiber afferents in a normal rat skin-nerve preparation (Shin et al., 2002) and BK-evoked nociception (Ferreira et al., 2004). The different effects of the antagonist from those of the gene deficiency might be due to non-specific effects of capsazepine (Docherty et al., 1997). However, all the reports using TRPV1-KO mice including ours imply that mice and their sensory fibers, at least partially, retain the BK-sensitivity even in the absence of TRPV1 (Kollarik and Undem, 2004; Rong et al., 2004).

Consistent with the results of the behavioral test, our Ca^{2+}-imaging studies showed that incidence of BK-sensitive DRG neurons in TRPV1-KO mice was reduced to half of that in WT. TRPA1 is a plausible candidate responsible for the remaining BK-sensitivities of DRG neurons in TRPV1-KO mice (Bautista et al., 2006). However, a certain part of BK-responsive neurons were sensitive to neither MO nor CAP. Since the concentration of MO used here was sufficiently high (100 μM) to evoke saturating responses to cloned TRPA1 (EC_{50} = ~10–20 μM, Jordt et al., 2004; Bandell et al., 2004), the percentage of MO-sensitive cells seems not to be underestimated in KO mice. The members of TRPC subfamily (TRPC1–7) could be other candidate ion channels for the BK-response, because they are activated downstream of $G_{q/11}$/PLC pathways (Hardie, 2007), and also expressed in DRG neurons (Elg et al., 2007). Interestingly, TRPC3 is exclusively expressed in TRPV1-negative small neurons (Elg et al., 2007), suggesting its cell type-specific roles. However, our observation that the BK-responses remained in TRPV1-KO neurons even in the presence of ruthenium red, a non-selective inhibitor of TRP families (data not shown), implies that mechanisms independent of TRPs are also involved in the BK-evoked excitation of DRG neurons. In support of this, BK has been reported to induce the multimodal actions of many types of current in trigeminal ganglion neurons (Kitakoga and Kuba, 1993). We often observed delays of the BK-evoked

responses in C-fiber recording and Ca^{2+}-imaging experiments, that would probably reflect the time required for diffusion of the reagent into tissues and various threshold levels among individual neurons. One cannot exclude another possibility of indirect involvement of the surrounding non-neuronal cells, such as fibroblasts and satellite glial cells, because they could respond to BK and then transmit the signal to co-cultured neurons *in vitro* (Estacion, 1991; Parpura et al., 1995; England et al., 2001; Heblich et al., 2001). Such a cellular environment surrounding the peripheral terminals of sensory neurons might contribute to BK-responses *in vivo* or in the skin-nerve preparation.

Recently, Bautista et al. (2006) reported that BK-evoked Ca^{2+}-influx in DRG neurons was diminished in both TRPV1-KO and TRPA1-KO neurons, indicating that these ion channels have synergistic effects on the BK-response. Based on the correlation of chemical sensitivities, the authors have proposed the sequential involvement of TRPV1 and TRPA1 in BK-evoked excitation, in which TRPA1 would serve as a main determinant of the BK response, and would be enhanced by the presence of TRPV1. However, in our Ca^{2+}-imaging study, a considerable part of BK-sensitive neurons responded either CAP or MO alone, suggesting BK-response of DRG neurons may not always need co-expression and synergism of TRPV1 and A1. Furthermore, TRPA1 does not seem to be essential for BK nociception, because BK-evoked acute nociception in TRPA1-KO mice was diminished, but not eliminated (Kwan et al., 2006). Considering all these results, the presence of parallel routes TRPV1 and/or A1-dependent and TRPV1/A1-indepentdent are suggested for BK-evoked nociception. A double knockout of TRPV1 and TRPA1 would provide a good system for evaluating the *in vivo* role of such TRPV1/A1-indepentdent mechanisms.

In conclusion, the present study demonstrated that TRPV1 plays some roles, though not essential, in BK-evoked nociception, and that TRPA1 is not sufficient to explain the rest part of the BK responses. Other ion channels may be involved in the underlying mechanism of BK-evoked pain responses.

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