Original Research

Bradykinin-evoked scratching responses in complete Freund's adjuvant-inflamed skin through activation of B1 receptor

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Abstract

Capsaicin, a potent algogen, induces an itch-related behavior in the presence of inflammation. In this study, we tested whether bradykinin (BK) can evoke a similar response and investigated the potential mechanisms involved in this process. Local inflammation was induced by intradermal injection of complete Freund's adjuvant (CFA) into the back of the neck, left hind foot or left cheek of male C57BL/6J mice. BK was then injected intradermally into the same area on indicated days. Four days after CFA inflammation, BK treatment evoked scratching responses in a time- and dose-dependent manner. For BK receptor antagonist treatment, inflamed-mice were either given an intraperitoneal injection of B_1 receptor (B_1R) or B_2 receptor (B_2R) antagonist 30 min prior to BK administration, or an intradermal co-injection of antagonist and BK into the inflamed area. Our results indicate that B_1R and B_2R act in an opposite fashion during this process, as pretreatment with B_1R antagonist by intraperitoneal injection significantly reduced BK-induced scratching behavior, whereas B_2R antagonist treatment dramatically increased scratching behavior. Moreover, combined injection of BK and B_2R antagonist enhanced BK-induced scratching activity in CFA-inflamed mice. In addition, pretreatment or co-injection with B_2R antagonist dramatically reduced the pain-related licking behavior induced by BK injection. The data suggest that BK-induced scratching responses in CFA-inflamed mouse skin occur via activation of B_1R. Furthermore, B_1 and B_2 receptors play different roles in modulating BK-induced itch-related behavior in CFA-inflamed mice.

Keywords: bradykinin, itch, complete Freund's adjuvant, inflammation, bradykinin B_1 receptor

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Introduction

Itching, or pruritus, is an unpleasant sensory experience that usually accompanies various skin diseases such as atopic dermatitis.[1] Itching typically involves scratching behavior, which is considered as a self-defense mechanism to prevent potential injury.[2] Based on the clinical signs and distinguishing characteristics of skin lesion diseases, itching is generally classified into three categories: itching in inflamed (diseased) skin; itching in non-inflamed (non-diseased) skin; and itching presenting with severe chronic secondary scratch lesions.[3] A variety of stimuli generated within or applied to the skin can induce itching, including inflammatory infections, autoimmune cutaneous diseases, genodermatoses, drug reactions and skin lymphomas.[3] Most importantly, the primary skin disease may be aggravated by secondary scratch lesions. However, the pathophysiological basis of itching is largely unknown.

Various substances have been proven to induce itching, and histamine, which is released from mast cells in an early phase of inflammation, has been recognized as an essential mediator of itching sensations in inflamed human skin.[4] Furthermore, the active peptide bradykinin (BK), which is isolated from plasma globulins treated with either trypsin or a snake venom protease,[5] has been linked to a wide range of biological phenomena such as pain and inflammation.[6] Notably, BK is also known to be one of the most potent endogenous algogenic substances, with the ability to act as a potent histamine-independent pruritogen in the lesional skin of atopic dermatitis.[7] We have reported that capsaicin induced an itch-related response in the presence of inflammation evoked by complete Freund's adjuvant (CFA).[8] It is still unclear whether BK can evoke itch-related scratching behaviors in such situations.

BK exerts its biological effects through the activation of two seven-transmembrane G-protein-coupled receptors, termed bradykinin B_1 receptor (B_1R) and B_2 receptor (B_2R).[6] The upregulation of B_1R has been detected following treatment with CFA, endotoxins and cytokines,[9,10] suggesting a close correlation between BK receptors and inflammation. Moreover, B_1R contributes to acute pain following extraction of third molars.[11] In the present study, we observed BK-induced scratching responses in CFA-inflamed mice and demonstrated that a BK receptor-mediated pathway was involved in this process. Interestingly, we found that B_1R and B_2R play different roles in modulating BK-induced pruritus-related behavior in CFA-inflamed mice.

Materials and methods

Reagents

BK was purchased from Tocris (Bristol, UK). Transient receptor potential vanilloid subfamily 1 (TRPV1) antagonist (capsazepine), CFA, B_1 kinin receptor agonist (des-Arg9-bradykinin) and B_1 kinin receptor antagonist (des-Arg^9-[Leu^8]-bradykinin, DALBK) were obtained from Sigma Chemical Co (St Louis, MO, USA). B_2 kinin receptor antagonist (D-Arg-[Hyp^3, D-Phe^7]-bradykinin) was obtained from Bachem (Bubendorf, Switzerland). Capsazepine was dissolved in dimethylsulfoxide (DMSO) to make a stock solution, and was diluted with normal saline (NS) to obtain a working solution. All other compounds were dissolved in NS.

Animals

Male C57BL/6J mice, weighing 20–22 g, were obtained from the Center for Laboratory Animals, Sun Yat-sen University (Guangzhou, China). The animals were housed at $22 \pm 1^{\circ}C$ on a 12/12-h light/dark cycle, with free access to food and water. The animal use and care protocols were approved by the Committee on Ethical Use of Animals of Guangdong General Hospital (Guangzhou, China), following the National Institutes of Health (NIH) Animal Use and Care Guidelines. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Development of local inflammation

To eliminate the influence of shaving on background scratching, mice were shaved at the rostral part of the back of the neck or the left cheek where intradermal injections were then given one day prior to experiments. Animals were randomly divided into two groups, an inflammation group (CFA-inflamed group) and a normal saline group (NS group), and were routinely given 30 min to acclimate in a small plastic chamber (22 cm × 12 cm × 20 cm) before injection. To induce local inflammation, mice in the inflammation group were removed from the chamber and received an intradermal injection of 50, 10 or 10 μL of CFA into the rostral back area (neck), the left cheek or the left hind foot, respectively, with a 30-gauge needle. In control groups, an identical volume of NS was administrated instead of CFA.

BK-induced scratching behavior with back injection

To evaluate BK-induced scratching behavior, between 0 and 1 mmol/L of BK was injected intradermally into the back of the neck on the indicated days after CFA or NS administration. For TRPV1 antagonist and BK receptor antagonist treatment, animals in the inflammation group were given intraperitoneal injections of capsazepine (4 mg/kg body weight), B_1R antagonist (DALBK; 0.4, 2 or 10 mg/kg body weigh) or B_2R antagonist (D-Arg-Hyp^3, D-Phe^7-bradykinin; 0.4, 2 or 10 mg/kg body weigh) 30 min prior to BK administration. The dose of B_1R and B_2R antagonist was determined based on previous experiments.[12] To determine the effect of an injection of a combination of antagonist and BK on scratching activity, B_1R or B_2R antagonist (5 nmol) and BK (0.5 mmol/L) in a total volume of 50 μL were intradermally co-injected into the back of the neck. To stimulate B_1R activity, mice were given B_1R agonist (des-Arg^9-bradykinin) at a dose of 0.016, 0.08, 0.4 or 2 mmol/L (in 50 μL). Combination treatments consisted of: (1) intraperitoneal injection of B_1R or B_2R antagonist (10 mg/kg body weight) 30 min prior to intradermal injection of B_1R agonist (0.4 mmol/L in 50 μL) into CFA-inflamed mice; (2) co-injection of B_1R or B_2R antagonist (5 nmol) with B_1R agonist (0.4 mmol/L in 50 μL) into CFA-inflamed mice. The pruritus model of neck injection was established as previously described.[13–15] Animals were placed in individual cages and the hind limb scratching behavior directed towards the injected area was recorded for a period of 30 min after the final injection. Such activity was considered to be indicative of an itch-related behavior. One scratch was defined as a lifting of the hind limb towards the injection site and replacement of the limb back on the floor, regardless of the number of scratching strokes that occurred between the two movements.[16]

BK-induced pain-related behavior with paw injection

Four days after intradermal injection of 10 μL CFA or NS into the left hind feet, mice were given NS, BK (1 mmol/L) or B_1R agonist (0.4 mmol/L) in a volume of 10 μL. For BK receptor antagonist treatment, inflamed animals were given an intraperitoneal injection of B_1R or B_2R antagonist (10 mg/kg) 30 min prior to BK (1 mmol/L in 10 μL) administration to the hind feet. The combination treatment consisted of co-injection of B_1R or B_2R antagonist (1 nmol) with BK (1 mmol/L in 10 μL) into the left hind feet of CFA-inflamed mice. Animals were placed in individual cages and the time spent licking the injected hind foot was measured with a chronometer for five minutes after the final injection. Licking represented a response to a painful stimulus.

Cheek injection assessment and behavioral analysis

NS, BK (1 mmol/L) or B_1R agonist (0.4 mmol/L) in a volume of 10 μL was injected intradermally into the left cheek on the indicated days after CFA or NS administration following the method introduced by Shimada and LaMotte.[14] The number of hindpaw scratches directed to

the injected area and the number of ipsilateral forelimb wipes directed to the injected area as indicators of itch and pain were recorded for a period of 30 min after the final injection.

Histological assessment of inflammation

Skin from the back of the neck was harvested on day 4 after the induction of inflammation. Tissue was fixed in 10% neutral-buffered formalin for 48 h. Fixed tissues were dehydrated in a graded series of ethanol, cleared in xylene, infiltrated, then processed using a Tissue-Tek VIP automatic tissue processor (Sakura, Tokyo, Japan) with a standard protocol and embedded into paraffin wax (Tissue-Tek). Tissue blocks were sectioned at 5 μm, and slides were stained with hematoxylin—eosin. Tissue samples were evaluated by an experienced pathologist.

Statistical analysis

All data were plotted as mean $\pm$ SEM. Data were statistically evaluated by analysis of variance (ANOVA) followed by Bonferroni's test or, when only two means were to be compared, unpaired Student's *t*-test. A P value < 0.05 was considered statistically significant.

Results

Scratching response induced by BK in CFA-inflamed skin

Local inflammation was induced in mice by a 50 μL intradermal injection of CFA into the back of the neck. As shown in Figure 1, CFA-induced scratching activity occurred immediately after injection and subsided within 72 h (3 d). Scratching frequency was very low in NS-injected mice (Figure 1, non-inflammation group). To eliminate the influence of CFA on drug-stimulated scratching behavior, drugs were administrated four days after CFA inflammation. We next monitored the BK-stimulated scratching behavior in mice after four days of CFA inflammation. Dose-dependent scratching activity was observed when BK was injected intradermally into the back of the neck (Figure 2a). There was a significant difference ($P < 0.05$) in scratching frequency between animals receiving NS and BK treatments at all doses examined. To determine the correlation between BK-evoked scratching behavior and the duration of CFA-induced inflammation, mice were given 0.5 mmol/L BK via intradermal injection on each indicated day after CFA administration. We found that the scratching frequency induced by BK gradually dropped over time after 14 d of CFA inflammation (Figure 2b). These results demonstrate that BK treatment evoked time- and dose-dependent scratching responses in CFA-inflamed mice.

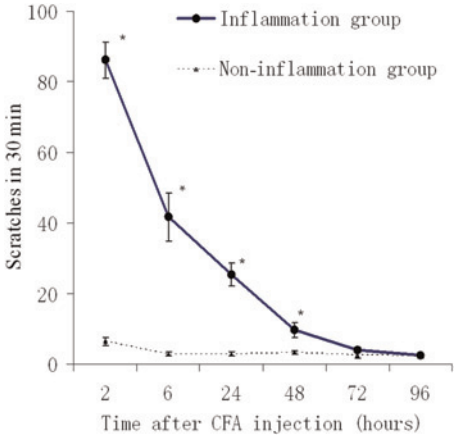

Figure 1 CFA-induced scratching behavior. Mice received an intradermal injection (50 μL) of CFA (inflammation group) or normal saline (non-inflammation group) into the back of the neck. The hind limb scratching behavior directed toward the injected area was recorded for a period of 30 min at 0, 6, 24, 48, 72 or 96 h after injection. Data are expressed as mean $\pm$ SEM ($n = 10$ in each group). $^*P < 0.05$ compared with the NS group. CFA, complete Freund's adjuvant; NS, normal saline

The effects of BK receptor antagonist on BK-induced scratching behavior

To evaluate the effects of BK receptor antagonist on BK-induced scratching activity, B_1R (DALBK) and B_2R antagonists (D-Arg-Hyp^3, D-Phe^7-bradykinin) were used. Four days after CFA injection, mice were given an intraperitoneal injection of either B_1R or B_2R antagonist 30 min prior to BK administration. Notably, treatment with 2 or 10 mg/kg B_1R antagonist significantly reduced BK-induced scratching behavior, whereas treatment with 10 mg/kg B_2R antagonist dramatically increased scratching activity induced by BK (Figure 3a). To evaluate the combination effect of BK receptor antagonists and BK, BK and B_1R or B_2R antagonists were intradermally co-injected four days after CFA injection. Interestingly, a combined injection of BK and B_2R antagonist significantly enhanced BK-induced scratching behavior in CFA-inflamed mice ($P < 0.05$ compared with BK injection alone), while no obvious effect was found after co-injection of BK plus B_1R antagonist (Figure 3b). These findings demonstrate that B_1 and B_2 receptor inhibition have opposing effects on BK-induced scratching behavior.

The effects of capsazepine on BK-induced scratching behavior

Antagonism or gene deletion of B_1R can lead to the abolishment of phorbol myristate acetate (PMA)-induced nociceptive behavior.[17] This behavior is also reduced in TRPV1 gene knockout mice.[18] In addition, BK-induced pain in mice can be dose-dependently prevented by both B_2R antagonist and TRPV1 antagonist.[19] To evaluate the effects of TRPV1 antagonist on BK-induced scratching activity, mice were given an intraperitoneal injection of capsazepine (4 mg/kg in 1% DMSO) or vehicle (1% DMSO) prior to BK administration. Pretreatment with systemically administered capsazepine did not significantly affect the number of scratches induced by BK (Figure 4). The dose of capsazepine was sufficient to inhibit the scratching response induced by capsaicin in CFA-treated mice.[8] These results suggest that activation of B_2R inhibits the scratching response in CFA-inflamed skin, possibly through a

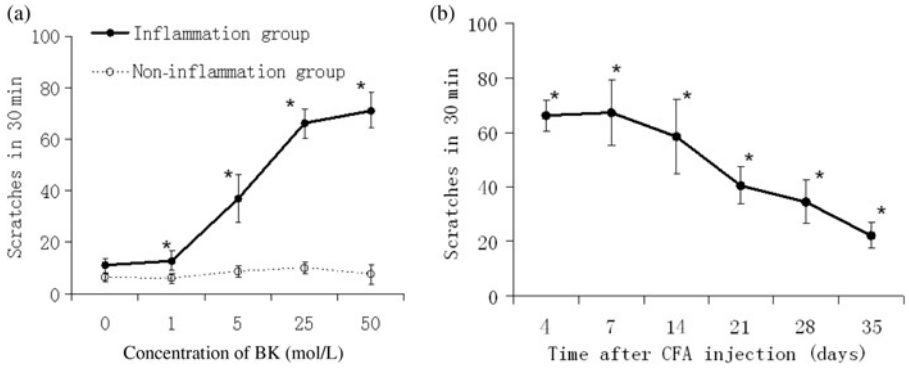

Figure 2 BK-induced scratching behavior. (a) Four days after CFA or normal saline administration, mice were given different doses of BK (from 0 to 1 mmol/L, 50 μL) by intradermal injection into the back of the neck. The scratching activity was recorded for a period of 30 min. Data are expressed as mean $\pm$ SEM ($n = 8$ in each group). $^*P < 0.05$ compared with the non-inflammation group. (b) On each indicated day after CFA administration, mice were given 50 μL of 0.5 mmol/L BK by intradermal injection into the back of the neck. The scratching activity was recorded for a period of 30 min. Data are expressed as mean $\pm$ SEM ($n = 6$ in each group). $^*P < 0.05$ compared with injection of 0.5 mmol/L BK four days after normal saline administration. CFA, complete Freund's adjuvant; BK, bradykinin

non-TRPV1 pathway. However, the underlying mechanism should be elucidated further.

Scratching behavior induced by B_1R agonist and the role of BK receptor antagonists

We further analyzed the potential influence of a combination treatment with selective B_1R agonist and BK receptor antagonists on CFA-inflamed scratching behavior. The optimal concentration of a widely used B_1R agonist (des-Arg^9-bradykinin) was determined via intradermal injection at doses ranging from 0 to 2 mmol/L in CFA-inflamed mice. B_1R agonist-treated mice exhibited elevated scratching behavior at doses over 0.016 mmol/L, with no additional increases observed at 0.4 and 2 mmol/L agonist application (Figure 5a). Therefore, we used the 0.4 mmol/L dose. Either B_1R or B_2R antagonist (10 mg/kg body weight) was injected into CFA-inflamed mice intraperitoneally 30 min prior to intradermal injection of B_1R agonist (0.4 mmol/L), or the receptor antagonists were co-injected with B_1R agonist (0.4 mmol/L) into CFA-inflamed mice. We found that a 30-min pretreatment with B_1R antagonist suppressed the scratching behavior induced by B_1R agonist, whereas no obvious effect was observed upon B_2R antagonist treatment or B_1R antagonist co-injection (Figures 5b and c).

BK receptor modulates BK-induced pain-related behavior in CFA-inflamed mice

Finally, we examined the effects of BK receptor activation on BK-induced pain-related behavior in CFA-inflamed mice. Unilateral inflammation was induced in the left hind feet of mice by CFA injection, and mice were then given NS, BK (1 mmol/L) or B_1R agonist (0.4 mmol/L) in the inflamed area four days later. Licking behavior was monitored immediately after injection for a period of five minutes. Paw licking time was increased in CFA-inflamed mice compared with non-inflamed mice (NS group) with each

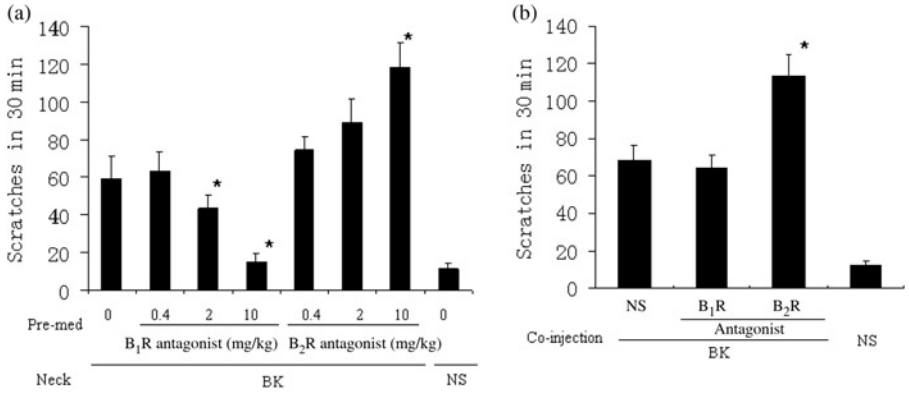

Figure 3 Effects of BK receptor antagonist on BK-induced scratching behavior. (a) Four days after CFA inflammation, mice were given an intraperitoneal injection of B_1R or B_2R antagonist (0.4, 2 or 10 mg/kg body weight) 30 min prior to BK (0.5 mmol/L, 50 μL) or normal saline (50 μL) injection into the back of the neck. The scratching activity was recorded for a period of 30 min. Data are expressed as mean $\pm$ SEM ($n = 8$ in each group). $^*P < 0.05$ compared with BK injection after intraperitoneal injection of normal saline (NS) in CFA-treated mice. (b) After four days of CFA inflammation, B_1R, B_2R antagonist (5 nmol) or NS were intradermally co-injected with BK (0.5 mmol/L) in a total volume of 50 μL into the back of the neck. The scratching activity was recorded for a period of 30 min. Data are expressed as mean $\pm$ SEM ($n = 8$ in each group). $^*P < 0.05$ compared with co-injection of solvent and BK in CFA-treated mice. CFA, complete Freund's adjuvant; BK, bradykinin

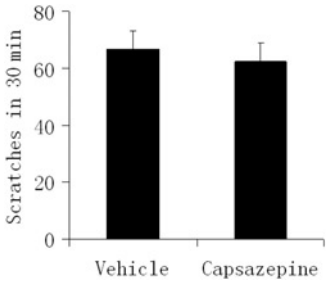

Figure 4 Effects of capsazepine on BK-induced scratching behavior. After four days of CFA inflammation, mice were given an intraperitoneal injection of TRPV1 antagonist (capsazepine, 4 mg/kg body weight in 1% DMSO) or vehicle (1% DMSO) 30 min prior to BK (0.5 mmol/L, 50 μL) injection into the back of the neck. The scratching activity was recorded for a period of 30 min. Data are expressed as mean $\pm$ SEM ($n = 8$ in each group). CFA, complete Freund's adjuvant; BK, bradykinin; DMSO, dimethylsulfoxide

treatment ($P < 0.05$) (Figure 6a). However, pretreatment or co-injection with B_2R antagonist dramatically reduced the elongated licking time induced by BK injection ($P < 0.05$ compared with BK treatment alone) (Figures 6b and c), while B_1R antagonist treatment had no significant effect. This suggests that B_2R plays a critical role in modulating BK-induced pain-related behavior in CFA-inflamed mice, while B_1R does not.

BK-induced scratching and wiping behavior with cheek injection

Local inflammation was induced in mice by a 10 μL intradermal injection of CFA into the left cheek. As shown in Figure 1, CFA-induced scratching and wiping activity occurred immediately after injection ($P < 0.05$ compared with the non-inflammation group; Figure 7). We next monitored the B_1R agonist- and BK-evoked scratching and wiping behavior in mice after four days of CFA inflammation. Scratching activity was observed when B_1R agonist or BK was injected intradermally into the cheek ($P < 0.05$ compared with the non-inflammation group; Figure 8a). Wiping activity was increased in all animals in the inflammation group when compared with the non-inflammation group (Figure 8b). B_1R agonist evoked more scratches than BK; on the contrary, less wipes were evoked by B_1R agonist when compared with BK (Figure 8).

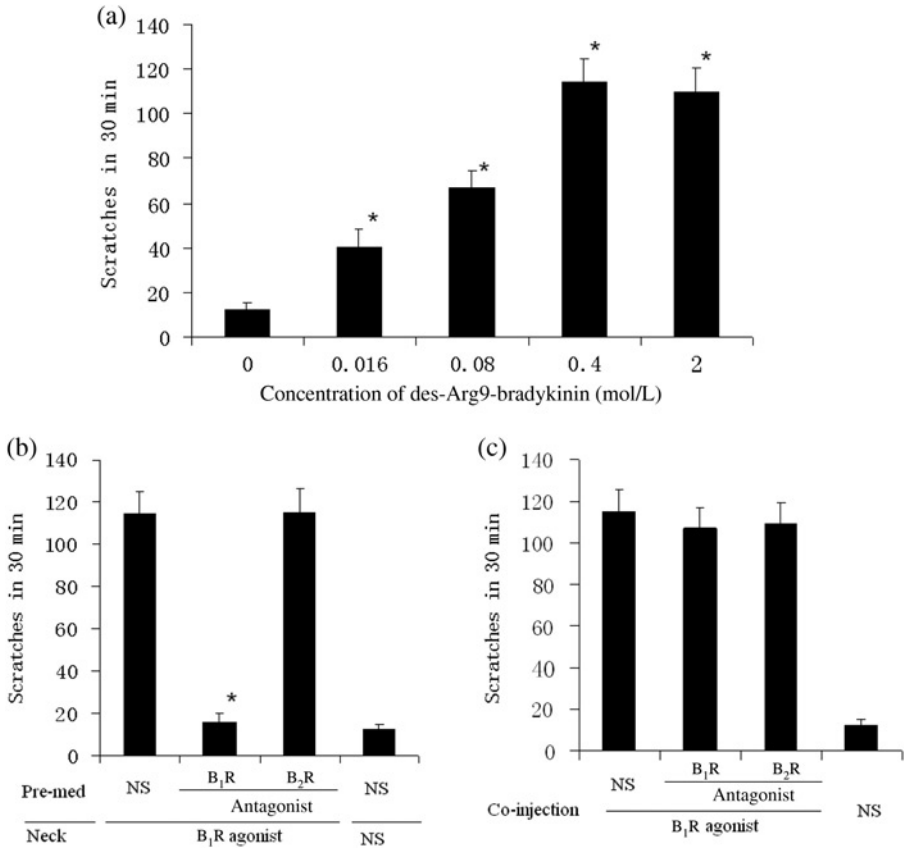

Figure 5 Effects of B_1R agonist and BK receptor antagonist on scratching behavior. (a) After four days of CFA inflammation, mice were given B_1R agonist (des-Arg^9-bradykinin) at a dose of 0, 0.016, 0.08, 0.4 or 2 mmol/L (in 50 μL) by intradermal injection into the back of the neck. Data are expressed as mean $\pm$ SEM ($n = 8$ in each group). $^*P < 0.05$ compared with 0 mmol/L. (b) Four days after CFA inflammation, mice were given an intraperitoneal injection of B_1R or B_2R antagonist (10 mg/kg body weight) 30 min prior to B_1R agonist (0.4 mmol/L in 50 μL) or normal saline (50 μL) injection into the back of the neck. The scratching activity was recorded for a period of 30 min. Data are expressed as mean $\pm$ SEM ($n = 8$ in each group). $^*P < 0.05$ compared with B_1R agonist injection after intraperitoneal injection of normal saline (NS) in CFA-treated mice. (c) After four days of CFA inflammation, B_1R, B_2R antagonist (5 nmol) or NS were intradermally co-injected with B_1R agonist (0.4 mmol/L) in a total volume of 50 μL into the back of the neck. The scratching activity was recorded for a period of 30 min. Data are expressed as mean $\pm$ SEM ($n = 8$ in each group). $^*P < 0.05$ compared with co-injection of solvent and B_1R agonist in CFA-treated mice. CFA, complete Freund's adjuvant; BK, bradykinin

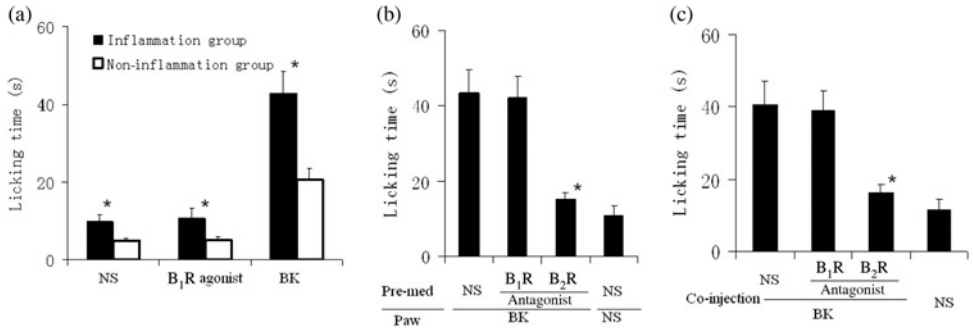

Figure 6 BK receptor modulates BK-induced pain-related behavior in CFA-inflamed mice. (a) Four days after intradermal injection of 10 μL CFA (inflammation group) or NS (non-inflammation group) into the left hind feet, mice were given NS, B_1R agonist (0.4 mmol/L) or BK (1 mmol/L) in a volume of 10 μL. Licking behavior was assayed immediately after injection for a period of five minutes. Data are expressed as mean $\pm$ SEM ($n = 7$ in each group). $^*P < 0.05$ compared with the same treatment in the non-inflammation group. (b) Four days after CFA inflammation, mice were given an intraperitoneal injection of B_1R or B_2R antagonist (10 mg/kg body weight) 30 min prior to BK (1 mmol/L in 10 μL) or normal saline (10 μL) injection into the hindpaw. Licking behavior was assayed immediately after injection for a period of five minutes. Data are expressed as mean $\pm$ SEM ($n = 7$ in each group). $^*P < 0.05$ compared with BK injection after intraperitoneal injection of normal saline (NS) in CFA-treated mice. (c) After four days of CFA inflammation, B_1R, B_2R antagonist (1 nmol) or NS were intradermally co-injected with BK (1 mmol/L) in a total volume of 10 μL into the hindpaw. Licking behavior was assayed immediately after injection for a period of five minutes. Data are expressed as mean $\pm$ SEM ($n = 7$ in each group). $^*P < 0.05$ compared with co-injection of solvent and BK in CFA-treated mice. CFA, complete Freund's adjuvant; BK, bradykinin; NS, normal saline

Histological findings

Diffuse infiltration of inflammatory cells was observed in the subcutaneous area in the CFA-inflammation mice. Scattered necrosis was also found in the subcutaneous area (Figure 9).

Discussion

Peripheral administration of CFA has been widely used to evoke an inflammatory response in mice and rats.[20–22] For instance, acute inflammation after unilateral injection of CFA into the plantar surface of the rat's hindpaw causes intense edema and hyperalgesia within a few hours.[23] Samad *et al.*[24] demonstrated that this effect could be mediated by several pathways, including local nociceptor sensitization; systemic neuronal mechanisms, such as central sensitization; and immune responses, such as increased cytokine serum levels. In the present study, local inflammation was induced by intradermal injection of CFA into the back of the neck, the hind foot or the cheek of the mouse. CFA-induced scratching activity occurred immediately after injection to the back or the cheek. More scratches than wipes were evoked by the cheek injection of CFA. It is interesting that itchness rather than pain was evoked by CFA at the early time points. Many inflammatory mediators and pathways were found to be involved in the process of CFA-induced inflammation. The early inflammation induced by CFA has been shown to be mast cell–dependent.[25] The mechanism of itch induced by CFA in the acute phase should be investigated further. We found that BK dose-dependently evoked scratching responses in the CFA-inflamed area. However, whether or not scratching behavior is a result of itch, pain or some other sensation is difficult to determine in mice. A previous study confirmed that scratching behavior induced by intradermal injection

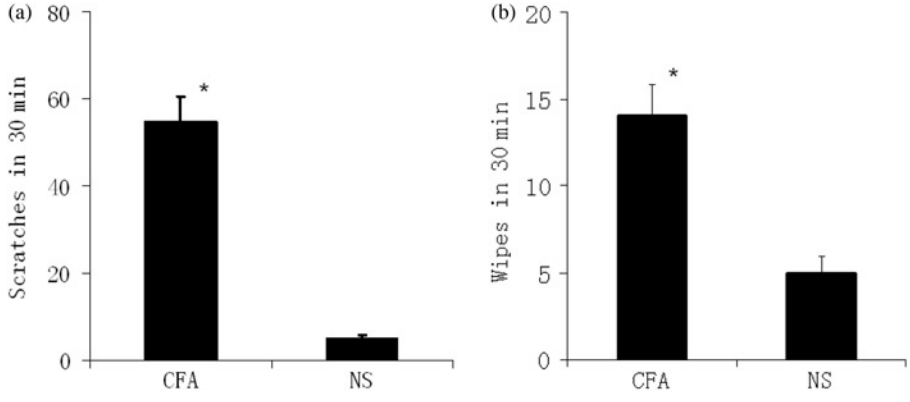

Figure 7 CFA-induced scratching and wiping behavior. Mice received an intradermal injection (10 μL) of CFA (inflammation group) or NS (non-inflammation group) into the left cheek. Data are expressed as mean $\pm$ SEM ($n = 8$ in each group). (a) The hind limb scratching behavior directed toward the injected area was recorded for a period of 30 min after injection of CFA or NS. $^*P < 0.05$ compared with the NS group. (b) The left forelimb wiping behavior directed toward the injected area was recorded for a period of 30 min after injection of CFA or NS. $^*P < 0.05$ compared with the NS group. CFA, complete Freund's adjuvant; NS, normal saline

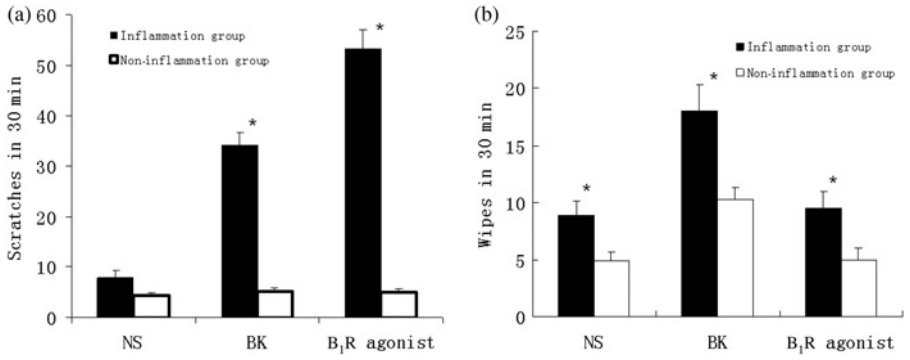

Figure 8 BK- and B_1R agonist-induced scratching and wiping behavior. Four days after intradermal injection of 10 μL CFA (inflammation group) or NS (non-inflammation group) into the left cheek, mice were given NS, B_1R agonist (0.4 mmol/L) or BK (1 mmol/L) in a volume of 10 μL. Data are expressed as mean $\pm$ SEM ($n = 8$ in each group). (a) The hind limb scratching behavior directed toward the injected area was recorded for a period of 30 min after injection. $^*P < 0.05$ compared with the non-inflammation group. (b) The left forelimb wiping behavior directed toward the injected area was recorded for a period of 30 min after injection of CFA or NS. $^*P < 0.05$ compared with the non-inflammation group. CFA, complete Freund's adjuvant; BK, bradykinin; NS, normal saline

of pruritogenic agents into the back of the mouse neck was itch-related.[26] In addition, we have verified that itchiness was induced by BK in inflamed skin by injecting into the cheek. Our results showed that B_2R antagonist enhanced the scratching activity induced by BK while reducing pain-associated licking behavior, suggesting the scratching behavior detected here is in fact an itch-related activity.

Itch, a common skin sensation, is an essential symptom of skin diseases and is usually linked to the desire to scratch.[1] It can be generated by a variety of stimuli, including inflammatory infections. It was reported that a majority of children accidentally injected with the Bacille Calmette-Guérin vaccine will have an itchy, painful skin reaction at the injection site.[27] An unexpectedly high incidence of persistent itching nodules and delayed hypersensitivity to aluminium in children were also reported after the use of adsorbed vaccines from a single manufacturer.[28] In the present study, we established a novel mouse model of itch-related scratching behavior evoked by BK, a pain-producing substance in CFA-inflamed skin. In patients with atopic dermatitis, a spontaneously occurring and frequent skin disease, pain can be induced independently of itch in lesional skin.[7,29] Disease models for atopic dermatitis in rodents include hapten-induced dermatitis in mice, NC/Nga mice and genetically engineered mouse models.[30] However, in this current study, B_2R antagonist greatly enhanced the scratching behavior evoked by BK in inflamed mice, implying that BK might stimulate pain in the inflamed area, and that this pain possibly suppressed the itch sensation. However, the current rodent model is different from atopic dermatitis, in which the pain stimuli are perceived as itch but do not suppress the itch sensation.

Our results demonstrate that BK can induce scratching responses in CFA-inflamed mouse skin via activation of B_1R. Additionally, B_1 and B_2 receptors appear to play different roles in modulating BK-induced itch-related behavior in CFA-inflamed mice. BK has been linked to a wide range of biological phenomena such as pain, inflammation and itch,[6,31] and exerts its effects through the activation of B_1 and B_2 receptors.[6] B_1R activation has been shown to contribute to inflammatory pain and hyperalgesia.[12,32] Recently, B_1R and B_2R have been shown to play a crucial role in controlling the pruriceptive signaling triggered by proteinase-activated receptor 2 (PAR2) activation in mice.[33] In this study, we find that pretreatment with B_1R antagonist dramatically reduced BK-induced scratching behavior, whereas B_2R antagonist pretreatment promoted the scratching activity induced by BK. Furthermore,

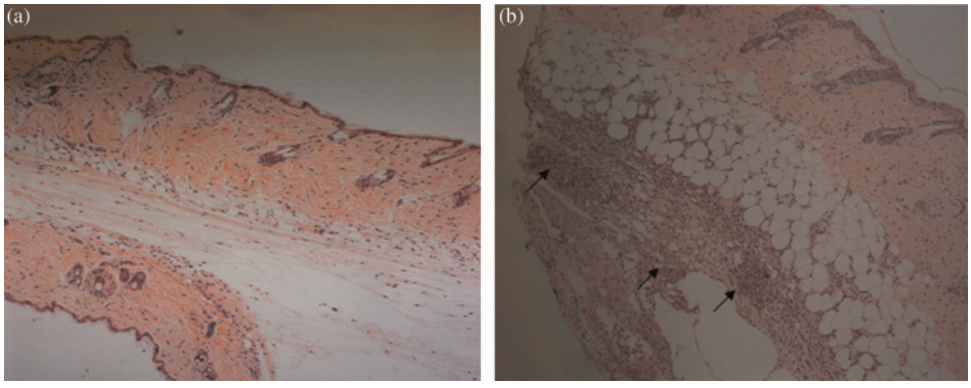

Figure 9 Histological features of CFA-inflammation skin. Mouse skin of the back of the neck was harvested on day 4 after the induction of inflammation. Tissue was fixed and stained with hematoxylin—eosin. (a) Section of skin treated with NS ($n = 4$). (b) Section of skin treated with CFA ($n = 4$). Diffuse infiltration of inflammatory cells was observed in the subcutaneous area in the CFA-inflammation mice. Scattered necrosis was found in the subcutaneous area. Arrows indicate the area of necrosis. Original magnification $\times$100. CFA, complete Freund's adjuvant; NS, normal saline

pretreatment with B_1R antagonist remarkably suppressed the scratching behavior induced by B_1R agonist. These findings strongly suggest that both BK receptors are involved in and contribute to BK-induced scratching behavior in CFA-inflamed mice, but their effects depend on the treatment paradigm. However, B_1R and B_2R clearly play opposite roles in this process. Systemic, but not local injection of B_1R antagonist DALBK reduced scratches evoked by BK or selective B_1R agonist. Co-injection of DALBK was also not able to reduce scratches evoked by SLIGRL-NH_2,[33] while peripheral injection of DALBK abolished the nociceptive behavior induced by intraplantar injection of PMA.[17] These studies, together with our results, suggest that peripheral B_1R was less likely to be involved in this scratching response than was a lack of potent B_1R antagonism, as the dose of DALBK used in this study was larger than that used previously.[17]

Abnormal expression or activity of serine proteases and PAR2 has been associated with several inflammatory skin disorders involving barrier abnormalities, including atopic dermatitis, Netherton syndrome and psoriasis.[34] The endogenous PAR-2 agonist tryptase was increased in atopic dermatitis patients,[35] and PAR2 agonists induce pruritus in patients and animals.[16,33,35] Both B_1R and B_2R antagonists were found to be effective in reducing the scratch response induced by PAR2 activation in mice.[33] B_2R is constitutively expressed in many cell types and in most tissues, and modulates physiological functions under normal conditions.[36] In contrast, B_1R is almost absent in normal tissues and is usually induced during inflammation.[37] Upregulated B_1R expression has been observed following inflammation induced by a number of stimuli.[17,38,39] It will be important to explore the role of B_1R in pathophysiological inflammatory itch conditions, as B_1R antagonists might be effective antipruritic drugs for some chronic itchiness.

With regard to BK-induced pain-related behavior in CFA-inflamed mice, pretreatment or co-injection with B_2R antagonist dramatically reduced the elongated licking time induced by BK injection, while no obvious effect was observed upon B_1R antagonist treatment, suggesting that B_2R plays a critical role in modulating BK-induced pain-related behavior in CFA-inflamed mice. These observations were consistent with previous reports showing that CFA inflammation increased sensitivity of C-fiber nociceptors to BK through B_2R, and B_2R antagonism significantly inhibited CFA-induced ipsilateral mechanical hyperalgesia in mice.[21,40] However, the opposite result has been obtained by other research groups, which have shown that B_1R, but not B_2R, is involved in inflammatory pain. For instance, accumulating evidence suggests that upregulation of B_1R protein levels contributes to inflammatory pain and hyperalgesia.[12,32] In addition, B_1R antagonist inhibited carrageenin-induced inflammatory hypernociception, as well as tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) release in lipopolysaccharide-primed mice, suggesting that B_1R mediates hypernociception through a mechanism dependent on TNF-α and IL-1β.[12] B_2R antagonist had no such effect. Furthermore, p38 stress-activated protein kinase has been demonstrated to contribute to the development of inflammatory hyperalgesia in a rat model by promoting B_1R activity.[12] The discrepancy between these results may be due to differences in the applied experimental paradigms. For instance, local injection of B_1R or B_2R antagonist could inhibit spontaneous pain by reducing licking time in mice inoculated with melanoma, whereas only B_2R antagonist attenuated allodynia in mice with a melanoma-bearing paw.[41] Therefore, it is possible that BK receptors can act through different mediators or in concert, depending on the context. The stimulation of B_1R also contributes to the acute inflammatory pain process, as B_1R antagonist or B_1R gene deletion can lead to the abolishment of PMA-induced nociceptive behavior.[17] Several signaling mechanisms related to PMA-evoked nociception act via protein kinase C.[42] On the other hand, acute and chronic inflammatory pain models may lead to different results.

In summary, this study demonstrated that BK induces itch-related response in CFA-inflamed mouse skin via activation of B_1R, while activation of B_2R inhibits the itch-related response.

Author contributions: All authors participated in the design, study interpretation, data analysis and review of the manuscript. JL and WJ conducted the experiments and wrote the manuscript. YH and WJ revised the manuscript.

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REFERENCES

1 Yosipovitch G, Greaves MW, Schmelz M. Itch. *Lancet* 2003;**361**:690–4
2 Schmelz M. Itch and pain. *Neurosci Biobehav Rev* 2010;**34**:171–6
3 Stander S, Weisshaar E, Mettang T, Szepietowski JC, Carstens E, Ikoma A, Bergasa NV, Gieler U, Misery L, Wallengren J, Darsow U, Streit M, Metze D, Luger TA, Greaves MW, Schmelz M, Yosipovitch G, Bernhard JD. Clinical classification of itch: a position paper of the International Forum for the Study of Itch. *Acta Derm Venereol* 2007;**87**:291–4
4 Fjellner B, Hagermark O. Studies on pruritogenic and histamine-releasing effects of some putative peptide neurotransmitters. *Acta Derm Venereol* 1981;**61**:245–50
5 Blais C Jr., Marceau F, Rouleau JL, Adam A. The kallikrein-kininogen-kinin system: lessons from the quantification of endogenous kinins. *Peptides* 2000;**21**:1903–40
6 Marceau F, Regoli D. Bradykinin receptor ligands: therapeutic perspectives. *Nat Rev Drug Discov* 2004;**3**:845–52
7 Hosogi M, Schmelz M, Miyachi Y, Ikoma A. Bradykinin is a potent pruritogen in atopic dermatitis: a switch from pain to itch. *Pain* 2006;**126**:16–23
8 Liang J, Xiao G, Ji W. Capsaicin induces reflex scratching in inflamed skin. *Pharmacology* 2011;**88**:82–7
9 Marceau F, Bachvarov DR. Kinin receptors. *Clin Rev Allergy Immunol* 1998;**16**:385–401
10 Calixto JB, Cabrini DA, Ferreira J, Campos MM. Kinins in pain and inflammation. *Pain* 2000;**87**:1–5
11 Hamza M, Wang XM, Adam A, Brahim JS, Rowan JS, Carmona GN, Dionne RA. Kinin B_1 receptors contributes to acute pain following minor surgery in humans. *Mol Pain* 2010;**6**:12

12 Cunha TM, Verri WA Jr, Fukada SY, Guerrero AT, Santodomingo-Garzon T, Poole S, Parada CA, Ferreira SH, Cunha FQ. TNF-α and IL-1β mediate inflammatory hypernociception in mice triggered by B_1 but not B_2 kinin receptor. *Eur J Pharmacol* 2007;**573**:221–9
13 Liang J, Kawamata T, Ji W. Molecular signaling of pruritus induced by endothelin-1 in mice. *Exp Biol Med (Maywood)* 2010;**235**:1300–5
14 Shimada SG, LaMotte RH. Behavioral differentiation between itch and pain in mouse. *Pain* 2008;**139**:681–7
15 Liang J, Ji Q, Ji W. Role of transient receptor potential ankyrin subfamily member 1 in pruritus induced by endothelin-1. *Neurosci Lett* 2011;**492**:175–8
16 Shimada SG, Shimada KA, Collins JG. Scratching behavior in mice induced by the proteinase-activated receptor-2 agonist, SLIGRL-NH2. *Eur J Pharmacol* 2006;**530**:281–3
17 Ferreira J, Triches KM, Medeiros R, Cabrini DA, Mori MA, Pesquero JB, Bader M, Calixto JB. The role of kinin B_1 receptors in the nociception produced by peripheral protein kinase C activation in mice. *Neuropharmacology* 2008;**54**:597–604
18 Bölcskei K, Helyes Z, Szabó A, Sándor K, Elekes K, Németh J, Almási R, Pintér E, Petho G, Szolcsányi J. Investigation of the role of TRPV1 receptors in acute and chronic nociceptive processes using gene-deficient mice. *Pain* 2005;**117**:368–76
19 Ferreira J, Da Silva GL, Calixto JB. Contribution of vanilloid receptors to the overt nociception induced by B_2 kinin receptor activation in mice. *Br J Pharmacol* 2004;**141**:787–94
20 Andrew D, Greenspan JD. Mechanical and heat sensitization of cutaneous nociceptors after peripheral inflammation in the rat. *J Neurophysiol* 1999;**82**:2649–56
21 Ferreira J, Campos MM, Pesquero JB, Araujo RC, Bader M, Calixto JB. Evidence for the participation of kinins in Freund's adjuvant-induced inflammatory and nociceptive responses in kinin B_1 and B_2 receptor knockout mice. *Neuropharmacology* 2001;**41**:1006–12
22 Imamachi N, Park GH, Lee H, Anderson DJ, Simon MI, Basbaum AI, Han SK. TRPV1-expressing primary afferents generate behavioral responses to pruritogens via multiple mechanisms. *Proc Natl Acad Sci USA* 2009;**106**:11330–5
23 Hylden JL, Nahin RL, Traub RJ, Dubner R. Expansion of receptive fields of spinal lamina I projection neurons in rats with unilateral adjuvant-induced inflammation: the contribution of dorsal horn mechanisms. *Pain* 1989;**37**:229–43
24 Samad TA, Moore KA, Sapirstein A, Billet S, Allchorne A, Poole S, Bonventre JV, Woolf CJ. Interleukin-1beta-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. *Nature* 2001;**410**:471–475
25 Bergstrand H, Andersson I, Pauwels R, Bazin H. Modulatory effects of Freund's adjuvant treatment on mast cell histamine release and homocytotropic antibody synthesis. *Int Arch Allergy Appl Immunol* 1985;**78**:118–31
26 Kuraishi Y, Nagasawa T, Hayashi K, Satoh M. Scratching behavior induced by pruritogenic but not algesiogenic agents in mice. *Eur J Pharmacol* 1995;**275**:229–33
27 Gross S, Kahan E, Vesterman L, Havkin O, Leventhal A. Adverse reactions to accidental forearm injection of Bacille Calmette-Guerin vaccine in schoolchildren: 12-month cohort follow-up. *Clin Infect Dis* 2004;**38**:1495–7
28 Bergfors E, Trollfors B, Inerot A. Unexpectedly high incidence of persistent itching nodules and delayed hypersensitivity to aluminium in children after the use of adsorbed vaccines from a single manufacturer. *Vaccine* 2003;**22**:64–9
29 Ikoma A, Fartasch M, Heyer G, Miyachi Y, Handwerker H, Schmelz M. Painful stimuli evoke itch in patients with chronic pruritus: central sensitization for itch. *Neurology* 2004;**62**:212–7
30 Petersen TK. *In vivo* pharmacological disease models for psoriasis and atopic dermatitis in drug discovery. *Basic Clin Pharmacol Toxicol* 2006;**99**:104–15
31 Hayashi I, Majima M. Reduction of sodium deoxycholic acid-induced scratching behaviour by bradykinin B2 receptor antagonists. *Br J Pharmacol* 1999;**126**:197–204
32 Khasar SG, Miao FJ, Levine JD. Inflammation modulates the contribution of receptor-subtypes to bradykinin-induced hyperalgesia in the rat. *Neuroscience* 1995;**69**:685–90
33 Costa R, Manjavachi MN, Motta EM, Marotta DM, Juliano L, Torres HA, Pesquero JB, Calixto JB. The role of kinin B_1 and B_2 receptors in the scratching behaviour induced by proteinase-activated receptor-2 agonists in mice. *Br J Pharmacol* 2010;**159**:888–97
34 Lee SE, Jeong SK, Lee SH. Protease and protease-activated receptor-2 signaling in the pathogenesis of atopic dermatitis. *Yonsei Med J* 2010;**51**:808–22
35 Steinhoff M, Neisius U, Ikoma A, Fartasch M, Heyer G, Skov PS, Luger TA, Schmelz M. Proteinase-activated receptor-2 mediates itch: a novel pathway for pruritus in human skin. *J Neurosci* 2003;**23**:6176–80
36 McEachern AE, Shelton ER, Bhakta S, Obernolte R, Bach C, Zuppan P, Fujisaki J, Aldrich RW, Jarnagin K. Expression cloning of a rat B_2 bradykinin receptor. *Proc Natl Acad Sci USA* 1991;**88**:7724–8
37 Menke JG, Borkowski JA, Bierilo KK, MacNeil T, Derrick AW, Schneck KA, Ransom RW, Strader CD, Linemeyer DL, Hess JF. Expression cloning of a human B_1 bradykinin receptor. *J Biol Chem* 1994;**269**:21583–6
38 Davis AJ, Perkins MN. Induction of B1 receptors *in vivo* in a model of persistent inflammatory mechanical hyperalgesia in the rat. *Neuropharmacology* 1994;**33**:127–33
39 Farmer SG, McMillan BA, Meeker SN, Burch RM. Induction of vascular smooth muscle bradykinin B_1 receptors *in vivo* during antigen arthritis. *Agents Actions* 1991;**34**:191–3
40 Banik RK, Kozaki Y, Sato J, Gera L, Mizumura K. B_2 receptor-mediated enhanced bradykinin sensitivity of rat cutaneous C-fiber nociceptors during persistent inflammation. *J Neurophysiol* 2001;**86**:2727–35
41 Ganju P, Davis A, Patel S, Nunez X, Fox A. p38 stress-activated protein kinase inhibitor reverses bradykinin B_1 receptor-mediated component of inflammatory hyperalgesia. *Eur J Pharmacol* 2001;**421**:191–9
42 Ferreira J, Triches KM, Medeiros R, Calixto JB. Mechanisms involved in the nociception produced by peripheral protein kinase c activation in mice. *Pain* 2005;**117**:171–81

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