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Contribution of vanilloid receptors to the overt nociception induced by B_2 kinin receptor activation in mice

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1 The vanilloid receptor (TRPV1) is viewed as a molecular integrator of several nociceptive stimuli. In the present study, we have investigated the role played by TRPV1 in the nociceptive response induced by the peripheral activation of kinin B_2 receptor in mice.

2 The intraplantar (i.pl.) administration of bradykinin (BK) and the selective B_2 agonist Tyr^8-BK, or the vanilloid agonists resiniferatoxin and capsaicin, into the mouse paw induced a dose-related overt nociception of short duration. The B_2 receptor antagonist Hoe 140 inhibited BK-induced, but not capsaicin-induced, nociceptive response. On the other hand, the TRPV1 antagonist capsazepine inhibited both capsaicin- and BK-mediated nociception.

3 Repeated injections of BK or capsaicin produced desensitization to their nociceptive response. Capsaicin desensitization greatly reduced BK-induced nociception, but in contrast, the desensitization to BK increased the capsaicin response.

4 Administration of low doses of capsaicin or acidified saline did not produce nociception when administered alone, but caused a pronounced effect when administered in association with a subthreshold dose of BK. Moreover, the degeneration of the subset of primary afferent fibers, sensitive to capsaicin, abolished both capsaicin- and BK-induced nociception.

5 The inhibition of phospholipase C (PLC), protein kinase C or phospholipase A_2 markedly decreased the nociception caused by BK, but not that of capsaicin. BK administration increased leukotriene B_4 levels in the injected paw. Likewise, BK-induced overt nociception was decreased by lipoxygenase (LOX) inhibition.

6 These results demonstrate that BK produces overt nociception mediated by TRPV1 receptor stimulation, *via* PLC pathway activation and LOX product formation.
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Keywords: B_2 receptor; vanilloid receptor; nociception; bradykinin; capsaicin; mice

Abbreviations: BK, bradykinin; ipl, intraplantar; LTB_4, leukotriene B_4; LOX, lipoxygenase; PBS, phosphate-buffered saline; PIP_2, phosphatidylinositol-4,5-bisphosphate; PLA_2, phospholipase A_2; PLC, phospholipase C; PKC, protein kinase C; TRPV1, vanilloid receptor

Introduction

Pain is initiated when noxious thermal, mechanical, or chemical stimuli excite the peripheral terminals of specialized primary afferent neurons (C and Aδ fibres) called 'nociceptors'. Tissue damage associated with infection, inflammation, or ischemia produces an array of chemical mediators that activate or sensitize nociceptor terminals to elicit pain at the site of injury (Julius & Basbaum, 2001). Vanilloid receptor 1 (TRPV1, formerly called VR1) (Montell *et al*., 2002) is expressed by a subset of peripheral pain-sensing neurons and may be gated by several different painful stimuli, including protons, heat, lipid mediators (such as anandamide, *N*-arachidonoyl-dopamine or lipoxygenase (LOX) products), and vanilloid compounds (such as capsaicin, the pungent principle of chili peppers) (Caterina *et al*., 1997; Zygmunt *et al*., 1999; Hwang *et al*., 2000; Huang *et al*., 2002). When such neurons are exposed to tissue-damaging stimuli, TRPV1 receptors become permeable to Na^+ and Ca^{2+} ions, causing in turn neuronal depolarization. Sensory neuron firing transmits these pain signals towards the central nervous system, evoking at the same time a variety of local tissue responses (Szallasi & Blumberg, 1999; Caterina & Julius, 2001; Piomelli, 2001).

The role of endogenous TRPV1 ligands in pain processes has been affirmed on the basis that inflammation-induced nociceptive responses may be inhibited by the TRPV1 antagonist capsazepine (Santos & Calixto, 1997; Kwak *et al*., 1998) or following TRPV1 gene deletion (Caterina *et al*., 2000; Davis *et al*., 2000). Such findings suggest that an endogenous capsaicin-like substance seems to be produced by peripheral tissues and acts during inflammation. These and other findings indicate that TRPV1 might act as a target for the signaling pathways stimulated by inflammatory mediators, such as prostaglandins, ATP, serotonin and bradykinin (BK).

BK produces primary sensory nerve terminal depolarization and sensitization to other noxious or even innocuous stimuli

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through the activation of kinin B_2 receptors (Calixto *et al*., 2000; 2001). Interestingly, there are several similarities between BK- and capsaicin-induced responses. For example, the depolarization of sensory neurons caused by BK or capsaicin is highly correlated (Martin *et al*., 1987) and the thermal hyperalgesia produced by BK in wild-type mice is absent in mice lacking TRPV1 (Chuang *et al*., 2001). Therefore, in the present study, we sought to investigate the role played by TRPV1 receptors in the nociceptive response induced by peripheral activation of kinin B_2 receptor *in vivo*.

Methods

Animals

Male Swiss mice weighing 30–35 g, maintained at $22\pm2^{\circ}C$ with free access to water and food, under a 12 : 12 h light : dark cycle, were used. Animals were acclimatized to the laboratory for at least 2 h before testing and were used once throughout the experiments. All experiments were carried out in accordance with current guidelines for the care of laboratory animals and ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983). The number of animals and the nociceptive stimulus were the minimum necessary to demonstrate the consistent effects of drug treatments.

Algogen-induced overt nociception in mice

The procedure used was similar to that described previously (Sakurada *et al*., 1992; De Campos *et al*., 1999). Volumes of 20 μl of BK (0.3–60 nmol paw^{-1}), Tyr^8-BK (1–60 nmol paw^{-1}), des-Arg^9-BK (3–30 nmol paw^{-1}), or the vanilloids resiniferatoxin (0.005–50 fmol paw^{-1}) or capsaicin (0.03–5.2 nmol paw^{-1}) were injected intraplantarly (i.pl.) under the surface of the right hind paw. Separate groups of animals received an i.pl. injection of the appropriated vehicle (phosphate-buffered saline (PBS) for peptides or PBS plus ethanol 0.5% for vanilloids). Other groups of animals received an i.pl. injection of lactate (0.01 M) acidified saline (0.9% of NaCl, pH 5.5). Animals were placed individually in chambers (transparent glass cylinders of 20 cm in diameter) and were adapted for 20 min before algogen or vehicle injection. After challenge, mice were observed individually for 10 min. The amount of time spent licking the injected paw was timed with a chronometer and was considered as indicative of nociception.

Effect of treatment with drugs

To assess the involvement of kinin receptors or TRPV1 in the algogen responses induced by BK or capsaicin, animals received the selective B_1 kinin receptor antagonist des-Arg^9-Leu^8-BK (10 nmol paw^{-1}), the B_2 kinin receptor antagonist Hoe 140 (1–10 nmol paw^{-1}), or the TRPV1 antagonist capsazepine (1–30 nmol paw^{-1}). To test whether the PLC–PKC or PLA_2 pathways were involved in BK- or capsaicin-induced licking, we evaluated the effect of the co-administration of PLC (U73122, 1 pmol paw^{-1}, Bleasdale *et al*., 1990), PKC (GF 109203X; 1 nmol paw^{-1}, Toullec *et al*., 1991), PLA_2 ($PACOCF_3$; 1 nmol paw^{-1}, Ackermann *et al*., 1995), LOX (baicalein, 3 nmol paw^{-1}, Sekiya & Okuda, 1982) or cyclooxygenase (ibuprofen, 100 nmol paw^{-1}) inhibitors in association with BK or capsaicin. Control animals received a similar volume of vehicle (20 μl paw^{-1}).

Desensitization protocol

Repeated administration of capsaicin or BK results in a marked desensitization of their responses on sensory neurons *in vitro* (Mizumura *et al*., 1987; Dray *et al*., 1989). To test this hypothesis *in vivo*, mice received two repeated injections of the vehicle (20 μl paw^{-1}), BK (10 nmol paw^{-1}), or capsaicin (5.2 nmol paw^{-1}) with a 30 min of interval between the injections. At 30 min after the last administration, vehicle, BK, or capsaicin were injected again in the previously treated mice.

Neonatal capsaicin treatment

To further explore the role of capsaicin-sensitive fibers in the nociceptive effect induced by the algogen substances, neonatal mice (on day 2 of life) were anesthetized with ether and received capsaicin (50 mg kg^{-1}, subcutaneously) or the vehicle alone (10% ethanol, 10% Tween-80 and 80% PBS), as described previously (Ferreira *et al*., 1999). Animals were used at 6–7 weeks after the administration of capsaicin or vehicle (used as control).

Skin temperature measurement

BK injection produces classical inflammatory signs, including local temperature increase. Since heat is one of the TRPV1 activators, the temperature of the BK-injected paw skin was also measured using a surface radiation thermometer (Pro Check, Italy).

Leukotriene B_4 (LTB_4) level measurement

Separated groups of mice were killed by cervical dislocation 5 min after BK, capsaicin or vehicle i.pl. injection. The injected paw was coaxially perfused as previously described (Rocha e Silva & Antonio, 1960). A double polyethylene tube was inserted into the subcutaneous space of the paw and 400 μl of PBS was perfused at a rate of 200 μl min^{-1} through the inner tube, after which the perfusate was collected through the outer tube. LTB_4 was measured in the collected perfusate using an Enzyme Immuno Assay kit. The assays were carried out in accordance with the manufacturer's instructions (Amersham, U.K.).

Drugs

The following drugs were used: BK, Tyr^8-BK, des-Arg^9-BK, des-Arg^9-Leu^8-BK, resiniferatoxin, baicalein, lactic acid, PBS tablets (all from Sigma Chemical Company, St Louis, MO, U.S.A.), GF109203X, U73122, $PACOCF_3$ (Tocris, Ellisville, MO, U.S.A.), capsaicin and capsazepine (RBI, Natick, MA, U.S.A.). Hoe 140 (Icatibant) was kindly provided by Aventis (Frankfurt am Main, Germany). The stock solutions of the drugs were prepared in PBS in siliconized plastic tubes, maintained at $-20^{\circ}C$ and diluted to the desired concentration just before use. Capsaicin, capsazepine, baicalein, GF109203X, U73122, and $PACOCF_3$ stock solutions were prepared in

absolute ethanol. For i.pl. drug administration, the final concentration of ethanol did not exceed 0.5% and did not cause any detectable effect *per se*.

Statistical analysis

The results are presented as mean ± s.e.m., except the ED_{50} or ID_{50} values (i.e. the dose of agonist necessary to produce 50% of the pain response relative to the maximum effect, or the dose of antagonists necessary to reduce agonist response by 50% relative to the control value, respectively), which are reported as geometric means accompanied by their respective 95% confidence limits. The ED_{50} and ID_{50} values were calculated using at least three doses of each drug, between the minimum and the maximum effect, using linear regression for individual experiments with the GraphPad Prism software. In order to obtain data purely derived by the treatments in algogen-induced nociception, the maximal inhibition (MI) values were represented as the difference between the licking times of the vehicle-treated and algogen-treated animals. The statistical significance between the groups was assessed by means of one-way ANOVA followed by Dunnett's or Student–Newmann–Keuls' test, as appropriate. *P*-values of less than 0.05 were considered as indicative of significance.

Results

Intraplantar administration of BK or of the more selective B_2 receptor agonist Tyr^8-BK in mice (1–60 nmol paw^{-1}) resulted in a dose-related overt nociceptive behavior (Figure 1). The nociception occurred quickly and did not last more than 10 min. The calculated mean ED_{50} values (and the 95% confidence limits) for BK- or Tyr^8-BK-induced licking were 5.75 (4.85–6.82) and 7.03 (5.89–8.39) nmol paw^{-1}, respectively. The maximal licking responses caused by BK- and Tyr^8-BK were 53.9 ± 6.5 and 41.5 ± 8.0 s. However, the i.pl. administration of the selective B_1 receptor agonist des-Arg^9-BK (3–30 nmol paw^{-1}) did not produce any detectable nociceptive behavior (Figure 1).

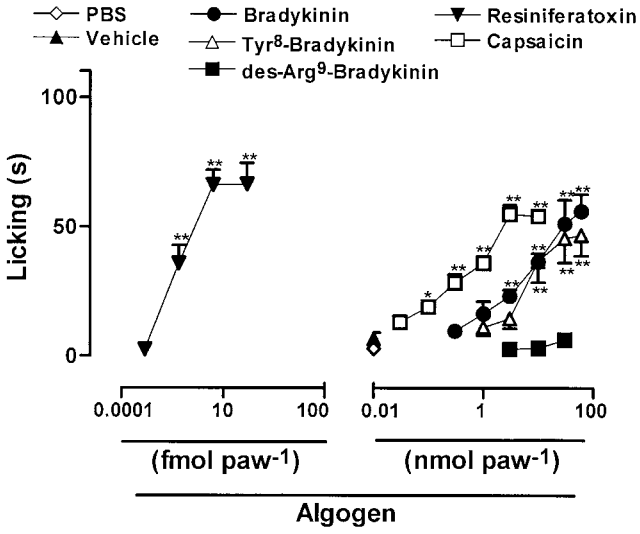

Figure 1 Dose–response curves for the overt nociception caused by i.pl. injection of BK, Tyr^8-BK, des-Arg^9-BK, resiniferatoxin, or capsaicin in mice. The effects of the drugs are expressed as licking time (s). Each point on the curve represents the mean of 4–6 animals and vertical lines show the s.e.m. Asterisks denote the significance levels in comparison with control groups (PBS for kinins or vehicle for vanilloids; one-way ANOVA followed by Dunnett's test): $^*P<0.05$, $^{**}P<0.01$.

Similar to BK, i.pl. administration of the vanilloid agonists capsaicin (0.03–5.2 nmol paw^{-1}) or resiniferatoxin (0.005–50 fmol paw^{-1}) resulted in a quick and dose-related nociceptive behavior (Figure 1). The calculated mean ED_{50} values (and the 95% confidence limits) for these effects were 0.61 (0.32–1.14) nmol paw^{-1} and 0.0175 (0.0058–0.0295) fmol paw^{-1}, and the maximal licking times were 50.3 ± 3.6 and 63.2 ± 8.4 s for capsaicin and resiniferatoxin, respectively. The ultrapotent TRPV1 agonist resiniferatoxin was about 300 million-fold more potent than capsaicin in the induction of nociception according to the analysis at the ED_{50} level. Comparing kinin- and vanilloid-induced nociceptive responses, capsaicin was about nine-fold more potent than BK in inducing nociception, although BK was as efficacious as capsaicin in the induction of licking response.

The i.pl. coadministration of the selective B_2 receptor antagonist Hoe 140 (1–10 nmol paw^{-1}) produced a dose-related inhibition of BK-induced nociception (10 nmol paw^{-1}, Figure 2a). The calculated mean ID_{50} value for this effect (and its respective 95 % confidence limit) was 2.1 (2.0–2.2) nmol paw^{-1} and the maximal inhibition was 88 ± 13%. On the other hand, the selective B_1 receptor antagonist des-Arg^9-Leu^8-BK (25 nmol paw^{-1}) completely failed to inhibit BK-induced nociception (results not shown), demonstrating that B_2 receptor activation mediates BK effect. Moreover, Hoe 140 (10 nmol paw^{-1}) was not able to affect the nociception produced by capsaicin (1 nmol paw^{-1}, results not shown). As previously described (Santos & Calixto, 1997), the i.pl. co-administration of the selective TRPV1 antagonist capsazepine (1 nmol paw^{-1}) completely blocked capsaicin-induced nociception (results not shown). Interestingly, the i.pl. co-administration of capsazepine (1–30 nmol paw^{-1}) produced a dose-related and almost complete inhibition of BK-induced nociception (Figure 2b). The calculated mean ID_{50} value for this effect (and its respective 95% confidence limit) was 6.45 (5.09–7.81) nmol paw^{-1} and the maximal inhibition was 91.7 ± 1.3%. These data suggest that the activation of TRPV1s is an important mechanism underlying BK-induced nociception in the mouse paw.

The results obtained with antagonists were confirmed using *in vivo* desensitization procedures. An initial i.pl. injection of BK (10 nmol paw^{-1}) or capsaicin (5.2 nmol paw^{-1}) produced a marked nociceptive response, as described above. However, a great reduction of the nociception was observed after a second challenge with both substances (Figure 3a). Capsaicin produced desensitization only when used at the highest dose (5 nmol paw^{-1}), but not at a submaximal dose (1 nmol paw^{-1}) (results not shown). Importantly, the desensitization of the paw injected with capsaicin completely abrogated the BK-induced nociceptive response. In contrast, desensitization to BK significantly increased capsaicin-induced nociceptive response (Figure 3a). These results suggest again that BK stimulates TRPV1 to produce overt nociception.

The results in Figure 3b suggest that BK sensitizes TRPV1 to agonist activation, as the i.pl. injection of low doses of BK (0.3 nmol paw^{-1}), capsaicin (0.03 nmol paw^{-1}), or acidified saline (pH 5.5) did not produce nociception when compared

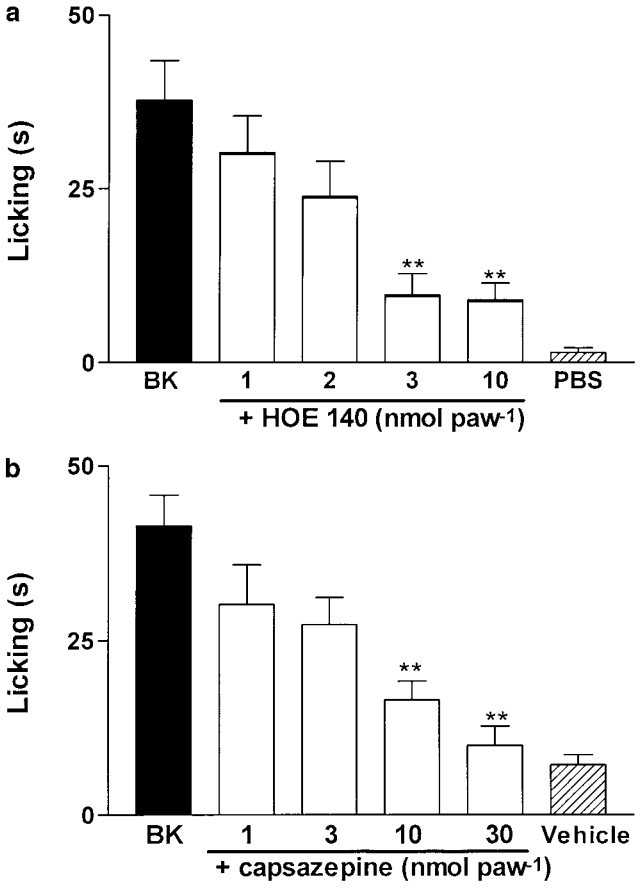

Figure 2 Effect of i.pl. treatment with the selective B_2 receptor antagonist Hoe 140 (a) or with the TRPV1 antagonist capsazepine (b) on BK-induced nociception. Each column represents the mean$\pm$s.e.m. of 4–6 mice. The asterisks denote the significance levels. $^{**}P<0.01$, compared with BK-treated mice (one-way ANOVA followed by Dunnett's test).

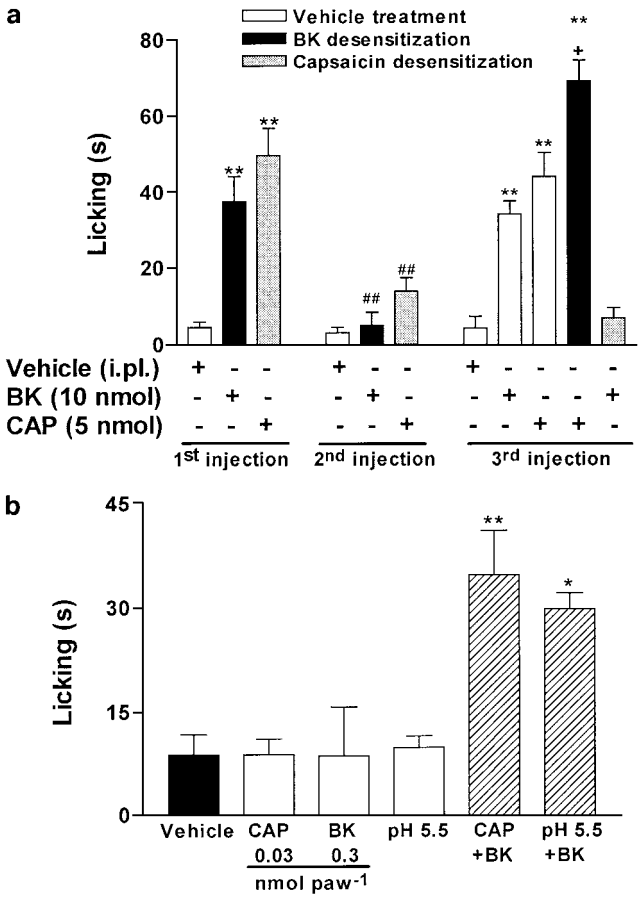

Figure 3 BK-induced overt nociception is dependent on TRPV1 activation. (a) Desensitization to the nociceptive effect caused by the i.pl. injection of capsaicin (CPS, 5.2 nmol paw^{-1}) or BK (10 nmol paw^{-1}). (b) Nociceptive effect caused by the coinjection of low doses of BK (0.3 nmol paw^{-1}) with capsaicin (CAP, 0.03 nmol paw^{-1}) or acidified saline (pH 5.5). Each column represents the mean$\pm$s.e.m. of 4–6 mice. The asterisks denote the significance levels. (a) $^{**}P<0.01$, compared with the first PBS injected mice; $^{\#\#}P<0.01$, compared with the first BK or CPS injected mice; $^{+}P<0.01$, compared with the third PBS injected mice (one-way ANOVA followed by Student–Newmann–Keuls' test). (b) $^{*}P<0.05$; $^{**}P<0.01$, compared with the vehicle injected mice (one-way ANOVA followed by Dunnett's test).

with the vehicle-treated animals. However, capsaicin or acidified saline administration in conjunction with a low dose of BK resulted in a significant nociceptive response (Figure 3b). Moreover, i.pl. injection of BK significantly increased the paw skin temperature (from a baseline of 27.5$\pm$0.3 to 28.1$\pm$0.2 and 28.7$\pm$0.4°C, 5 and 10 min after injection, respectively, $P<0.05$). Intraplantar injection of PBS did not alter the paw skin temperature (results not shown).

To investigate the role played by capsaicin-sensitive fibers in BK-mediated nociception, the animals were treated during the neonatal period with capsaicin (50 mg kg^{-1}, subcutaneously), a method known to produce degeneration of the thin primary afferent fibers (Holzer, 1991) and to decrease the expression of TRPV1 at the dorsal root ganglion (Rashid *et al*., 2003). Present results demonstrate that the neonatal treatment with capsaicin abolished both capsaicin- and BK-induced nociception (Figure 4a and b).

We next investigated some of the possible pathways involved in the B_2 receptor-mediated activation of TRPV1. The i.pl. coadministration of the selective inhibitors of PLC U73122 (1 pmol paw^{-1}), PLA_2 $PACOCF_3$ (0.1 nmol paw^{-1}), or the PKC inhibitor GF 109203X (1 nmol paw^{-1}) significantly reduced BK-induced nociception (Figure 5a). In marked contrast, these inhibitors failed to affect capsaicin-induced nociception (results not shown).

Several studies suggest that some TRPV1 ligands may be generated by the degradation of the main PLA_2 derivate product arachidonic acid, especially those from LOX. This pathway seems to be activated *in vivo* since the LOX inhibitor baicalein (3 nmol paw^{-1}) significantly reduced BK-induced nociception (Figure 5a). Furthermore, BK was also able to increase about two times the levels of the LOX product LTB_4 in the injected paw, an effect that was completely prevented by the injection of baicalein (Figure 5b). On the other hand, the cyclooxygenase inhibitor ibuprofen (100 nmol paw^{-1}) was not able to alter BK-induced nociception (licking time of 39$\pm$4.3 and 36$\pm$5.2 s for BK or BK plus ibuprofen, respectively).

Discussion

The present study demonstrates that BK injection into the mouse paw produces a rapid and marked overt nociception. This effect seems to be related to B_2 kinin receptor activation,

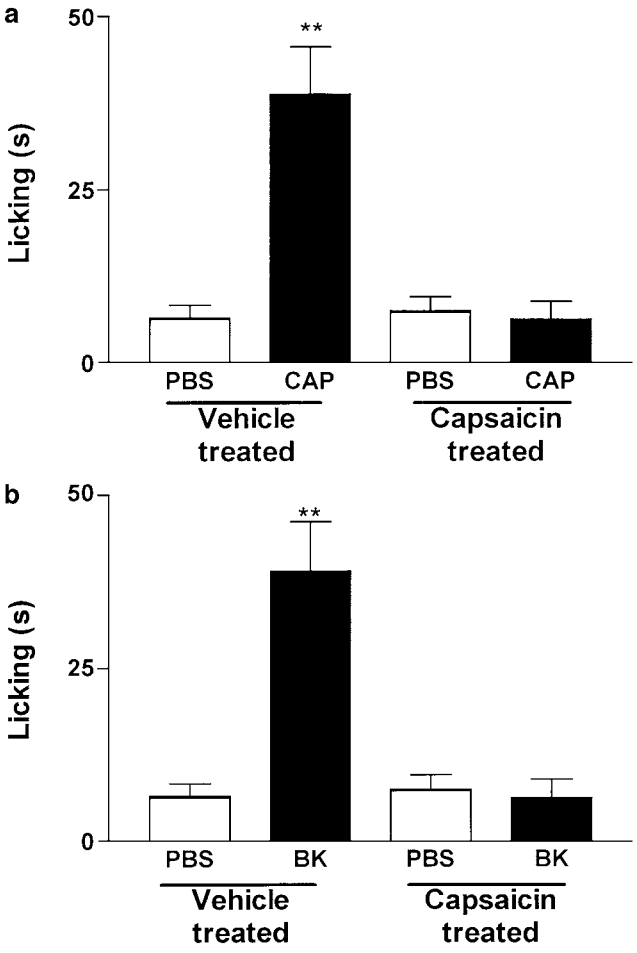

Figure 4 Disruption of the nociceptive effect caused by the i.pl. injection of (a) capsaicin (CPS, 1 nmol paw^{-1}) or (b) BK (10 nmol paw^{-1}) produced by treatment of neonate mice with capsaicin (50 mg kg^{-1}, s.c.). Each column represents the mean ± s.e.m. of 4–6 mice. The asterisks denote the significance levels. $^{**}P<0.01$, compared with PBS injected mice (one-way ANOVA followed by Dunnett's test).

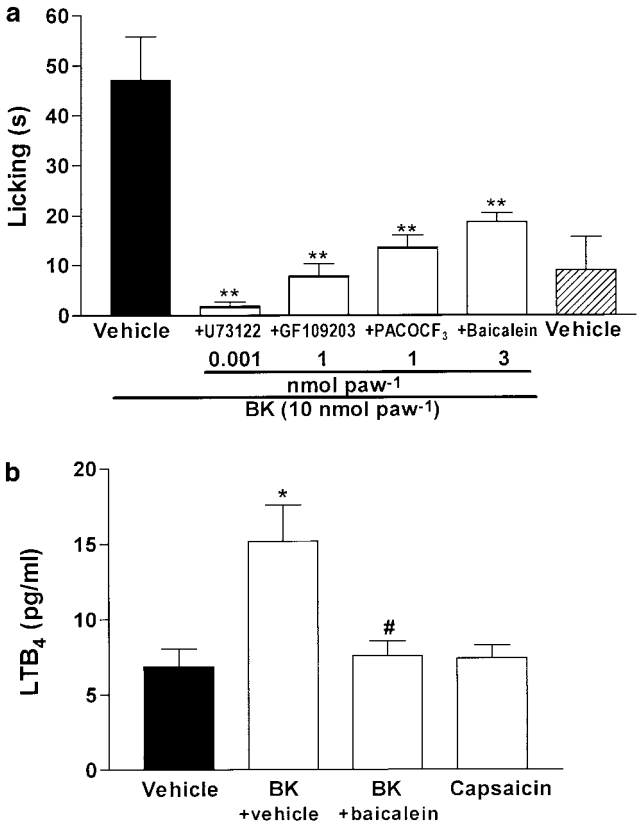

Figure 5 Mechanisms involved in the BK-induced nociception. (a) Effect of i.pl. treatment with selective inhibitors of phospholipase C (PLC) U73122 (1 pmol paw^{-1}), protein kinase C (PKC) GF109203X (1 nmol paw^{-1}), PLA_2 $PACOCF_3$ (0.1 nmol paw^{-1}) or LOX baicalein (3 nmol paw^{-1}) on BK-induced nociception. (b) Levels of LTB_4 in paw perfusate 5 min after i.pl. injection of vehicle, BK (10 nmol paw^{-1}) or capsaicin (1 nmol paw^{-1}). Each column represents the mean ± s.e.m. of 4–6 mice. The asterisks denote the significance levels. (a) $^{**}P<0.01$, compared with BK-treated mice. (b) $^{*}P<0.05$, compared with vehicle-treated mice, $^{\#}P<0.05$, compared with BK-treated mice (one-way ANOVA followed by Dunnett's test).

as the selective B_2 receptor agonist Tyr^8-BK mimicked its response. Also, BK-induced nociception was almost abolished by the selective B_2 receptor antagonist. The possible participation of kinin B_1 receptor in BK action was discarded since the B_1 receptor antagonist was not able to reduce BK-induced nociceptive behavior, and the injection of the B_1 receptor agonist did not produce any detectable overt nociceptive response. BK-induced overt nociception was mediated, at least in part, by TRPV1 stimulation. The coadministration of the selective capsaicin receptor antagonist capsazepine with BK, at a range of dose in which it markedly inhibited capsaicin-mediated nociception, dose-dependently and completely prevented BK-mediated nociceptive response.

Another interesting and also conclusive piece of evidence indicating the involvement of TRPV1 activation in the nociception caused by BK was the complete cross-desensitization observed between capsaicin and BK. In marked contrast, the paws desensitized by repeated injection of BK exhibited a significantly higher nociceptive response to capsaicin. Similar results have been reported using an *in vitro* preparation of neonatal rat spinal cord with attached tail indicating that a supramaximal concentration of capsaicin to the tail reduced BK-induced ventral root depolarization (Dray *et al*., 1990). On the other hand, prolonged exposure to BK did not reduce capsaicin-induced depolarization (Rueff *et al*., 1994). TRPV1 desensitization is a complex process that seems to be mediated by calcineurin activation (Docherty *et al*., 1996) and by calmodulin binding (Numazaki *et al*., 2003). Different ligands, such as capsaicin, resiniferatoxin, and protons, produce TRPV1 desensitization with different profiles, including extracellular Ca^{2+}-dependence and degree of efficacy (Acs *et al*., 1997; Mohapatra *et al*., 2003; Numazaki *et al*., 2003). Moreover, a recent study has shown that only the TRPV1 antagonist capsazepine, but not the TRPV1 channel blocker ruthenium red, inhibit capsaicin-induced desensitization to its nociceptive effect *in vivo* (Sakurada *et al*., 2003). Interestingly, TRPV1 may be resensitized since its desensitization may be reversed by PKA phosphorylation (Bhave *et al*., 2002). Further studies are required to answer as to why BK is able to activate, but not to desensitize, TRPV1-mediated nociceptive response.

Furthermore, the neonatal treatment of animals with capsaicin, which has been widely used to disrupt capsaicin-sensitive fibers (Holzer, 1991), also completely abrogated BK-

mediated nociception. These findings provide evidence indicating that BK and capsaicin activate the same subpopulation of sensory fibers to produce overt nociception.

Recent observations furnish compelling evidence indicating that BK, acting *via* B_2 receptors on sensory neurons, has the ability to sensitize the TRPV1 to noxious stimuli through the activation of PLC–PKC signaling pathways (Julius & Basbaum, 2001). The data shown in the present study confirm and also extend these previous *in vitro* observations by demonstrating that the coadministration of selective PLC antagonist, at a dose where it had no significant effect on capsaicin-mediated nociception, completely abolished BK-induced nociception. Such results suggest that also *in vivo* the activation of PLC pathway plays a critical role in inducing BK nociceptive action. *In vitro* studies carried out with native or heterologously expressed TRPV1 receptors have demonstrated that B_2 kinin receptor-PLC activation might modulate TRPV1 through PKC-dependent and -independent mechanisms. The direct PLC-mediated phosphatidylinositol-4,5-bisphosphate (PIP_2) hydrolysis may release TRPV1 from PIP_2-mediated inhibition (Chuang *et al*., 2001). Moreover, TRPV1 activity may also be modulated by PKCε phosphorylation, increasing the gating of TRPV1 by capsaicin, anandamide, protons, and heat (Premkumar & Ahern, 2000; Vellani *et al*., 2001; Numazaki *et al*., 2002). In fact, BK has the ability to sensitize the heat response through a mechanism involving the activation of PKCε (Cesare *et al*., 1999; Sugiura *et al*., 2002). Our findings are in perfect agreement with such a hypothesis since they demonstrate that the coadministration of the selective PKC inhibitor GF109203X, at a dose where it failed to interfere with capsaicin-mediated algesic response, completely abolished BK-induced nociception. Results of the present study confirm and also extend these previous findings, as BK, at a dose where it did not cause nociception by itself, consistently potentiated the pronociceptive responses produced by capsaicin and low pH *in vivo*.

We have shown that BK produces a discrete, but significant, increase in the injected paw temperature. Added to other stimulus, this variation of temperature might contribute to TRPV1 stimulation. Under normal conditions, TRPV1 is activated by temperatures greater than 43°C (Caterina *et al*., 1997). However, sensitization of TRPV1 produced by phosphorylation may decrease the temperature threshold to below 35°C (Tominaga *et al*., 2001). Thus, when the conditions are appropriate, normal body temperature is able to act as a primary stimulus to thermoceptors (Reeh & Petho, 2000). BK action may therefore be relevant for TRPV1 activation at normal body temperature, an event that could be involved in the manifestation of burning pain or in some of the acute and chronic painful pathologies. In fact, mice lacking TRPV1 receptor have a marked reduction in thermal hyperalgesia in a model of inflammatory pain (Caterina *et al*., 2000; Davis *et al*., 2000), thus further confirming the critical role played by TRPV1 receptor in the inflammatory pain states.

Furthermore, BK may also exert indirect effects on the pain process, such as generating PLA_2 lipid mediators, for example, prostaglandins and leukotrienes (Calixto *et al*., 2000; 2001; Shin *et al*., 2002). Capsaicin is a hydrophobic molecule that binds to the intracellular site of TRPV1 (Jung *et al*., 1999), between the second and the third transmembrane domains (Jordt & Julius, 2002). Taken together, such results suggest that TRPV1 endogenous ligand might be a lipid intracellular activator. In fact, several mediators, such as LOX products, anandamide and *N*-arachidonoyl-dopamine, are able to activate TRPV1 (Zygmunt *et al*, 1999; Hwang *et al*., 2000; Huang *et al*., 2002). There is recent *in vitro* evidence suggesting that BK, acting at B_2 receptors, excites sensory nerve endings *via* activation of TRPV1 receptor through production of LOX metabolites (Hwang *et al*., 2000; Shin *et al*., 2002). The excitation of cultured sensory neurons or sensory nerve fibers of the adult rat skin by BK is greatly restricted by inhibitors of PLA_2–LOX–TRPV1 pathway (McGuirk & Dolphin, 1992; Shin *et al*., 2002). In fact, BK stimulates LOX product formation by sensory neurones *in vitro* and by skin *in vivo* (Gammon *et al*., 1989; Shin *et al*., 2002; Wang *et al*., 1999). It has also been reported that BK-induced thermal hyperalgesia is blocked by LOX inhibition (Shin *et al*., 2002). Our results extend these previous observations and show that the PLA_2 and LOX inhibitor reduce BK-induced overt nociception without interfering with capsaicin-induced nociception. Furthermore, BK injection increases the tissue levels of the 5-LOX metabolite LTB_4.

Alternatively, the possibility that a cyclooxygenase-derived metabolite might also be involved in BK-mediated activation of TRPV1 channel in sensory neurons cannot be fully discarded. In fact, Pethö *et al*. (2001) demonstrated that BK produces both excitation and sensitization of rat C-fibres. Moreover, the BK-induced excitatory effect, in contrast to the heat sensitization, is subject to strong tachyphylaxis and is insensitive to cyclooxygenase inhibition. To explore the relationship between the *in vitro* and *in vivo* BK nociceptive effect, we have performed experiments with cyclooxygenase blocking and found that i.pl. injection of ibuprofen was not able to reduce BK-induced nociception. This result suggests

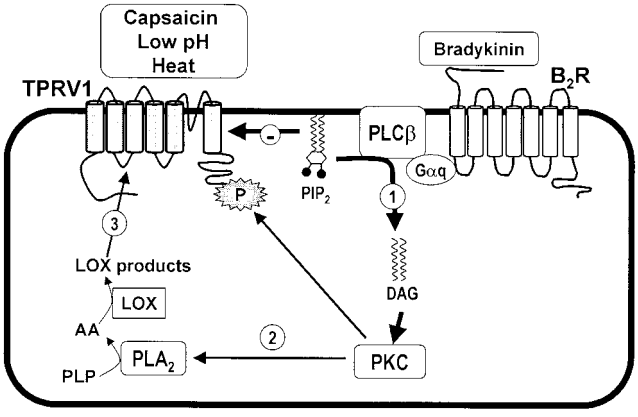

Figure 6 Proposed mechanisms involved in BK-induced activation of TRPV1. As discussed, the current results provide consistent *in vivo* experimental evidence supporting the concept that, at least in part, the nociception caused by i.pl. injection of BK into the mouse paw is indirectly mediated by both activation and sensitization of TRPV1 receptor. BK, by acting at protein Gq coupled B_2 receptors, is able to stimulate PLCβ that in turn produces hydrolysis of the plasma membrane phospholipid PIP_2, exerting an inhibitory action on TRPV1 channel function (step 1). Furthermore, after stimulation of neurons with B_2 receptor agonists such as BK, a rapid formation of diacylglycerol (DAG) occurs that in turn induces phosphorylation of key targets through PKC-mediated mechanisms (step 2). The third proposed mechanism involves the ability of BK to induce the activation of PLA_2 and generation of endogenous LOX-derived products that act directly to stimulate the TRPV1 receptor (step 3).

that cyclooxygenase products do not seem to mediate this BK action. However, it has been reported that PGE_2 causes hyperalgesia *via* enhancement of cAMP and activation of PKA-dependent phosphorylation of TRPV1 (Bhave *et al*., 2002; Hu *et al*., 2002). Since TRPV1 desensitization may be reversed by PKA phosphorylation (Bhave *et al*., 2002), prostaglandins could be involved in the lack of TRPV1 desensitization after BK repeated injections.

In summary, these results and those discussed above provide convincing evidence that BK produces nociceptive effects through its ability to sensitize the primary afferent neurons by stimulating the production of lipid-derived second messengers that in turn sensitize TRPV1 receptor (Figure 6). The nociceptive action of BK could be mediated not only by direct activation of sensory neurons but also by indirect release of lipid mediators from cells surrounding nerve terminals. Thus, following tissue injury or during certain inflammatory processes, the release of proinflammatory mediators, namely BK and arachidonic acid metabolites, causes a reduction of threshold to noxious stimuli, leading to the development of hypersensitivity states.

The current results give new insights into the *in vivo* mechanisms through which BK causes nociception, but additionally provide promising targets for the development of new peripherally acting analgesic drugs.

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