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RESEARCH PAPER

The role of kinin B_1 and B_2 receptors in the scratching behaviour induced by proteinase-activated receptor-2 agonists in mice

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Background and purpose: Activation of the proteinase-activated receptor-2 (PAR-2) induces scratching behaviour in mice. Here, we have investigated the role of kinin B_1 and B_2 receptors in the pruritogenic response elicited by activators of PAR-2.
Experimental approach: Scratching was induced by an intradermal (i.d.) injection of trypsin or the selective PAR-2 activating peptide SLIGRL-NH_2 at the back of the mouse neck. The animals were observed for 40 min and their scratching response was quantified.
Key results: I.d. injection of trypsin or SLIGRL-NH_2 evoked a scratching behaviour, dependent on PAR-2 activation. Mice genetically deficient in kinin B_1 or B_2 receptors exhibited reduced scratching behaviour after i.d. injection of trypsin or SLIGRL-NH_2. Treatment (i.p.) with the non-peptide B_1 or B_2 receptor antagonists SSR240612 and FR173657, respectively, prevented the scratching behaviour caused by trypsin or SLIGRL-NH_2. Nonetheless, only treatment i.p. with the peptide B_2 receptor antagonist, Hoe 140, but not the B_1 receptor antagonist (DALBK), inhibited the pruritogenic response to trypsin. Hoe 140 was also effective against SLIGRL-NH_2-induced scratching behaviour when injected by i.d. or intrathecal (i.t.) routes. Also, the response to SLIGRL-NH_2 was inhibited by i.t. (but not by i.d.) treatment with DALBK. Conversely, neither Hoe 140 nor DALBK were able to inhibit SLIGRL-NH_2-induced scratching behaviour when given intracerebroventricularly (i.c.v.).
Conclusions and implications: The present results demonstrated that kinins acting on both B_1 and B_2 receptors played a crucial role in controlling the pruriceptive signalling triggered by PAR-2 activation in mice.
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Keywords: PAR-2; scratching behaviour; kinins; kinin B_2 and B_1 receptors

Abbreviations: PAR-2, proteinase-activated receptor-2

Introduction

Kinins are endogenous peptides exerting a critical role in controlling physiological and pathological processes, particularly during nociceptive and inflammatory conditions. Once formed from their precursors by the action of kallikrein enzymes, kinins are released and exert their actions via activation of two subtypes of G-protein-coupled receptors, named kinin B_1 and B_2 receptors (nomenclature follows Alexander *et al.*, 2007). B_2 receptors are usually expressed in a constitutive manner throughout the central and peripheral tissues, and mediate most of the physiological effects of kinins, presenting higher affinity for bradykinin (BK) and kallidin peptides. On the other hand, B_1 receptors displays high affinity for the kinin metabolites des-Arg^9-BK and Lys-des-Arg^9-BK, and they are generally absent under physiological conditions, being rapidly expressed after tissue trauma and in certain inflammatory states (Calixto *et al.*, 2000; 2001; 2004; Marceau and Regoli, 2004). Nevertheless, the constitutive expression of B_1 receptors in the sensory neurons has been previously reported (Ma and Heavens, 2000; Ma, 2001; Wothersponn and Winter, 2000).

During the last two decades, it has become clear that both B_1 and B_2 receptors are involved in the onset and maintenance of several inflammatory and nociceptive conditions (Calixto *et al.*, 2000; 2001; 2004). There is some evidence that kinins and their receptors might play a role as effectors of pruriceptive signalling. For instance, intradermal application of BK into the injured skin of patients with atopic dermatitis evoked a very intense itch sensation (Hosogi *et al.*, 2006).

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Interestingly, it has also been verified that itching behaviour induced by sodium deoxycholic acid in mice is mediated by the kallikrein-kinin system, essentially through the activation of B_2 receptors (Hayashi and Majima, 1999). In a recent study, we have inferred the possible roles of such systems in the scratching behaviour in mice caused by trypsin, a non-selective agonist of proteinase-activated receptors (PAR)-2 (Costa *et al.*, 2008). In line with these findings, earlier works have demonstrated that the activation of BK B_2 receptors is a downstream event of PAR-2 activation in pain (Kawabata *et al.*, 2006; Paszcuk *et al.*, 2008).

The understanding of the pathophysiological basis of itch was greatly advanced by the discovery of PAR-2 involvement in pruritus (Steinhoff *et al.*, 2003). PARs comprise a family of four G-protein-coupled receptors, named PAR-1 to -4, that are activated by proteolytic cleavage of their extracellular terminal sequence, which exposes a new NH_2-terminus. This new portion acts as a tethered ligand, activating the cleaved receptor molecule (Ramachandran and Hollenberg, 2008; Vergnolle, 2009). PAR-2 is highly expressed in the skin (Steinhoff *et al.*, 1999), and can be activated by both tryptase (from mast cell degranulation) and trypsin (from pancreatic and/or extra-pancreatic sources) (Corvera *et al.*, 1997; Cottrell *et al.*, 2004). Furthermore, this receptor is located throughout the sensory system and it has been indicated as a potent effector of nociceptive and pruriginous processes in both humans and animals (Steinhoff *et al.*, 2003; Vergnolle *et al.*, 2003; 2001; Shimada *et al.*, 2006; Ui *et al.*, 2006). Activation of PAR-2 evoked itching behaviour when intradermally injected at the back of the mouse neck (Shimada *et al.*, 2006; Ui *et al.*, 2006; Costa *et al.*, 2008) and this has been used as a reproducible model to evaluate pruritus in rodents (Sun and Chen, 2007; Tsujii *et al.*, 2008; 2009; Akiyama *et al.*, 2009).

In the present study, in order to provide new evidence for the relevance of both kinin B_1 and B_2 receptors in itch, we sought to analyse, by the use of mice genetically deficient in kinin B_1 ($B_1R^{-/-}$) or B_2 receptors ($B_2R^{-/-}$), the contribution of these receptors to the scratching behaviour induced by different pruriginous agents. Particular attention was given to evaluate the anti-pruriceptive effects of selective kinin B_1 or B_2 receptor antagonists in the scratching behaviour elicited by PAR-2 activators.

Methods

Animals

All animal care and experimental procedures were carried out in accordance with the National Institutes of Health Animal Care Guidelines (NIH publications No. 80-23), and were approved by the Ethics Committee of the Universidade Federal de Santa Catarina (protocol number PP00032). Male adult Swiss mice (8–10 weeks) kept in controlled room temperature (22 ± 2°C) and humidity (around 60–80%) under a 12:12 h light–dark cycle (lights on 06:00 am) were used in this study. Food and water were provided *ad libitum* except during the experiments. In some experiments, C57BL/6 wild-type and kinin B_1 or B_2 receptor knockout ($B_1R^{-/-}$ and $B_2R^{-/-}$ respectively) mice were also used. Wild-type and knockout mice were originally obtained from the Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia, from the Universidade Federal de São Paulo (São Paulo, Brazil). Deletion of the entire coding sequence for kinin B_1 and B_2 receptors was as described by Pesquero *et al.* (2000) and Rupniak *et al.* (1997) respectively.

Induction of scratching behaviour

The experiments were carried out as described by Hayashi and Majima (1999), with minor modifications (Costa *et al.*, 2008). Two days before the experiments, the hair at the back of the mouse neck was shaved. On the day of the experiments, the animals were individually placed into glass cylinders 20 cm in diameter, for at least 30 min, in order to acclimatize them to the experimental environment. After this period, each mouse was briefly removed from the cylinder and given an intradermal (i.d.) injection of saline (50 µL) containing the non-selective PAR-2 receptor agonist trypsin (200 µg·site^{-1}), the selective peptide PAR-2 receptor agonist SLIGRL-NH_2 (25 to 200 µg·site^{-1}), sodium deoxycholic acid (100 µg·site^{-1}), chloroquine (200 µg·site^{-1}) or compound 48/80 (10 µg·site^{-1}). Immediately after pruritic stimulus administration, the animals were returned to their chambers. The animals were observed for 40 min, and their scratching behaviour was quantified by counting the number of scratches with fore- and hindpaws close to the injected site. Scratching behind the ears, but not on the face, was also counted. When a mouse scratched continuously for about 1 s without stopping, and repeated it more than once, this episode of scratching was counted as one. The results were expressed as the number of scratches in 40 min. Saline-treated animals (50 µL·site^{-1}) were used as control.

Trypsin-induced overt nociception

Initially, the animals were separately placed into glass cylinders 20 cm in diameter, for at least 30 min, in order to acclimatize them to the experimental environment. After the adaptation period, each mouse received an intraplantar (i.pl.) injection of saline (20 µL) containing trypsin (300 µg·paw^{-1}) into the right hindpaw. Control animals received saline (20 µL) by i.pl. route. The mice were observed individually for 10 min following trypsin injection. The amount of time (in seconds) spent licking the injected paw, was recorded with a stopwatch and was considered as an index of overt nociception (Paszcuk *et al.*, 2008).

Intrathecal and intracerebroventricular drug injections

Intrathecal (i.t.) drug injections were performed in accordance with the method described by Hylden and Wilcox (1980), with minor modifications (Ferreira *et al.*, 2002). The animals were lightly anaesthetized with isoflurane and a needle connected to a microsyringe by a polyethylene tubing was introduced through the skin. Subsequently, a volume of 5 µL of saline solution (0.9% NaCl) alone (control) or containing the drugs was injected between the L5 and L6 vertebral spaces. For intracerebroventricular (i.c.v.) injections, the animals were lightly anesthetized with isoflurane and a volume of 5 µL of sterile saline containing the drugs was

injected directly into the lateral ventricle (coordinates from bregma: 1 mm lateral; 1 mm rostral; 3 mm vertical), as described previously by Laursen and Belknap (1986). Control animals received the same volume of saline.

Pharmacological treatments

In order to confirm the involvement of PAR-2 in the scratching behaviour elicited by SLIGRL-NH_2 (100 μg·site^{-1}), mice were treated with the selective peptide PAR-2 receptor antagonist FSLLRY-NH_2 (100 μg·site^{-1}). The sensitivity of the pruriceptive effects produced by trypsin (200 μg·site^{-1}) or SLIGRL-NH_2 (100 μg·site^{-1}) to clinically used anti-pruritic treatments was assessed by systemic pretreatment with the corticoid dexamethasone (0.5 mg·kg^{-1}, s.c., 4h) or the selective histamine H_1 receptor antagonist pyrilamine (10 mg·kg^{-1}, s.c., 30 min).

To assess the involvement of kinin B_1 receptors in the scratching behaviour induced by either trypsin or SLIGRL-NH_2, animals were treated with the selective peptide or non-peptide B_1 receptor antagonists, des-Arg^9-Leu^8-bradykinin (DALBK) and SSR240612, respectively, by different pathways of administration. Firstly, DALBK (150 nmol·kg^{-1}) or SSR240612 (1 mg·kg^{-1}) were given by i.p. injection, 30 min before the injection of PAR-2 activators. In other experimental groups, the local effect of DALBK (0.3 nmol·site^{-1}) was tested by i.d. co-injection with the PAR-2 activator. In another set of experiments, the animals received DALBK (25 pmol·site^{-1}) by i.t. or i.c.v. routes, 15 min before SLIGRL-NH_2 administration.

The contribution of kinin B_2 receptors to the scratching behaviour induced by either trypsin (200 μg·site^{-1}) or SLIGRL-NH_2 (100 μg·site^{-1}) was analysed by the treatment with the selective peptide or non-peptide B_2 receptor antagonists Hoe 140 and FR173657, respectively, by different pathways of administration. Initially, Hoe 140 (50 nmol·kg^{-1}) or FR173657 (30 mg·kg^{-1}) were administered i.p., 30 min before PAR-2 activators. The local effect of Hoe 140 (3 nmol·site^{-1}) was tested by i.d. co-injection with PAR-2 activators. In another set of experiments, the animals received Hoe 140 (100 pmol·site^{-1}) by i.t. or i.c.v. routes, 15 min before SLIGRL-NH_2.

The protocols of all tested drugs (doses and time of injection) were chosen on the basis of pilot studies (data not shown), or in accordance with previous publications (Ferreira *et al*., 2002; 2004; 2008; Costa *et al*., 2006; 2008; Ui *et al*., 2006; Paszcuk *et al*., 2008; Quintão *et al*., 2008).

Data analysis

The results are presented as the mean ± SEM of six to 10 animals. Statistical comparison of the data was performed by one way analysis of variance (ANOVA) followed by Dunnett's or Newman-Keuls tests, as appropriate. *P* values less than 0.05 were considered significant. The inhibition of scratching behaviour are given as the difference (in percentage) between the mean of the responses in drug-treated group (or $B_1R^{-/-}$ or $B_2R^{-/-}$ group) in relation to vehicle-treated group (or wild-type group).

Materials

The following drugs were used: trypsin (from porcine pancreas), compound 48/80, dexamethasone, sodium deoxycholic acid, chloroquine, pyrilamine and des-Arg^9-[Leu^8]-bradykinin (DALBK) all from Sigma Chemical Company (St. Louis, MO, USA). Hoe 140, FR173657 and SSR240612 were kindly donated by Sanofi-Aventis (Bridgewater, NJ, USA), Fujisawa Pharmaceutical Co. (Osaka, Japan) and Sanofi Recherché (Moontpellier, France) respectively. The peptide fragments FSLLRY-NH_2 (Phe-Ser-Leu-Leu-Arg-Tyr-NH_2), SLIGRL-NH_2 (Ser-Leu-Ile-Gly-Arg-Leu-NH_2) and LRGILS-NH_2 (Leu-Arg-Gly-Ile-Leu-Ser-NH_2) were synthesized by Dr Luis Juliano (UNIFESP, São Paulo, Brazil).

Results

Scratching behaviour induced by PAR-2 agonists in mice

As described earlier (Costa *et al*., 2008), trypsin evoked a scratching behaviour when injected at the back of the mouse neck in different mouse strains (Swiss and C57BL/6) compared with saline-treated group (Figures 1, 2 and 4). This effect is dependent on the trypsin serine-proteolytic activity, and is mediated by PAR-2 receptor activation (Costa *et al*., 2008). As shown in the Figure 1A, the i.d. injection of the selective PAR-2 activating peptide SLIGRL-NH_2 (25 to 200 μg·site^{-1}) into the back of the mouse neck also displayed a marked and dose-related scratching behaviour response. The dose of 100 μg·site^{-1} was chosen for the following experiments, as this was the dose capable of inducing reproducible effects with less variability. Moreover, this dose was equivalent to the effects induced by the positive control compound 48/80 (10 μg·site^{-1}) (Figure 1A). Unlike the PAR-2 agonist, the i.d. injection of the reverse sequence LRGILS-NH_2 (100 μg·site^{-1}) did not cause any significant scratching response in comparison with the saline-treated group (Figure 1A). As expected, co-treatment with the selective peptide PAR-2 receptor antagonist FSLLRY-NH_2 (100 μg·site^{-1}) markedly inhibited SLIGRL-NH_2-induced scratching behaviour (88 ± 10% of inhibition) (Figure 1B).

Next, we assessed the sensitivity of the pruriceptive effect produced by trypsin or SLIGRL-NH_2 to some clinically used anti-pruritic treatments. The systemic administration of the corticoid dexamethasone (0.5 mg·kg^{-1}, s.c., 4 h) abolished both trypsin- and SLIGRL-NH_2-induced itching (107 ± 11% and 76 ± 13% of inhibition respectively) (Figure 1C and D). Nonetheless, the treatment with the selective histamine H_1 receptor antagonist pyrilamine (10 mg·kg^{-1}, s.c., 30 min), at a dose effective against compound 48/80-induced scratching (data not shown), was not able to inhibit the itching induced by both PAR-2 agonists (Figure 1C and D), suggesting that PAR-2 activation-elicited scratching behaviour was not dependent on histamine release from mast cells.

Scratching behaviour induced by PAR-2 agonists in kinin B_1 receptor and B_2 receptor knockout mice

To check the relevance of both kinin B_1 and B_2 receptors to the scratching behaviour induced by PAR-2 activators, we have employed kinin B_1 ($B_1R^{-/-}$) and B_2 receptor ($B_2R^{-/-}$) genetically deficient mice and the corresponding wild-type littermates (C57BL/6 strain). As shown in Figure 2, trypsin (200 μg·site^{-1}) or SLIGRL-NH_2 (100 μg·site^{-1}) i.d. injections provoked

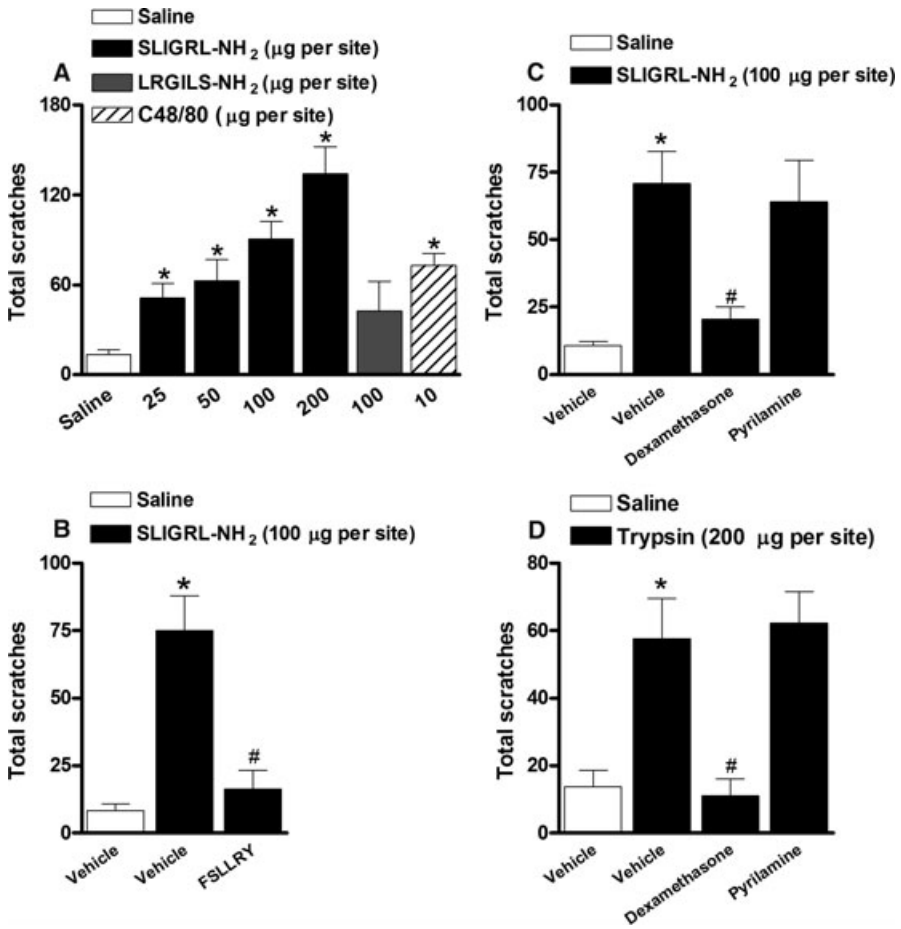

Figure 1 (A) Scratching behaviour induced by the selective PAR-2 activating peptide SLIGRL-NH_2 (25 to 200 μg·site^{-1}, i.d.), the inactive control peptide LRGILS-NH_2 (100 μg·site^{-1}, i.d.) or compound 48/80 (C48/80; 10 μg·site^{-1}, i.d.) in Swiss mice. (B) Effect of the treatment with the selective peptide PAR-2 antagonist FSLLRY-NH_2 (100 μg·site^{-1}, co-injection) on the SLIGRL-NH_2 (100 μg·site^{-1})-induced scratching behaviour in Swiss mice. (C, D) Effect of the treatment with the corticoid dexamethasone (0.5 mg·kg^{-1}, s.c., 4h) or the selective histamine H_1 receptor antagonist pyrilamine (10 mg·kg^{-1}, s.c., 30 min) on the (C) SLIGRL-NH_2 (100 μg·site^{-1})- or (D) trypsin (200 μg·site^{-1})-induced scratching behaviour in Swiss mice. Each column represents the mean of six to 10 animals and the vertical bars represent the SEM. Significantly different when compared with saline group (*$P < 0.05$) and SLIGRL-NH_2- or trypsin-treated group ($^{\#}P < 0.05$). PAR-2, proteinase-activated receptor-2.

significant scratching behaviour in C57BL/6 wild-type mice, when compared with saline-treated group (Figure 2A–D), in a manner essentially similar to that elicited in Swiss mice. Nevertheless, when trypsin (Figure 2A and B) or SLIGRL-NH_2 (Figure 2C and D) were injected in $B_1R^{-/-}$ or $B_2R^{-/-}$ mice, the frequency of scratching bouts was significantly lower than that observed in the wild-type animals. The deletion of B_2 receptors almost abolished the scratching response evoked by PAR-2 activation (83 ± 18% and 84 ± 9% against trypsin and SLIGRL-NH_2 respectively), while the B_1 receptor deficiency only partially inhibited the scratching behaviour (53 ± 9% and 42 ± 14%, for trypsin and SLIGRL-NH_2 models respectively).

Overt nociception induced by trypsin in kinin B_1 receptor and B_2 receptor knockout mice

In order to assess whether kinin receptors may also contribute to pain evoked by PAR-2 activation, we examined the overt nociception induced by the i.pl. injection of trypsin, a non-selective PAR-2 activator, in either B_1 or B_2 receptor knockout mice. As previously described (Paszcuk *et al.*, 2008), trypsin injection into the mouse hindpaw (300 μg·paw^{-1}) evoked a marked nociceptive response (mostly licking behaviour) in C57BL/6 wild-type mice (Figure 2E and F). On the other hand, the responses induced by i.pl. trypsin administration in $B_1R^{-/-}$ or $B_2R^{-/-}$ were significantly diminished when compared with the control animals (Figure 2E and F). In contrast to that observed in the scratching behaviour models, the inhibition of nociceptive response caused by B_1 receptor deficiency was greater than that caused by B_2 receptor deletion (73 ± 8% and 55 ± 15%, for $B_1R^{-/-}$ and $B_2R^{-/-}$ respectively) (Figure 2E and F).

Scratching behaviour induced by different pruritic agents in kinin B_1 receptor and B_2 receptor knockout mice

In this set of experiments, the relevance of kinin receptors for the scratching behaviour induced by i.d. injection of different pruritic stimuli was investigated using both kinin $B_1R^{-/-}$ and $B_2R^{-/-}$ mice. It is possible to observe from Figure 3 that genetic deletion of kinin B_2 receptors abolished the increase in the scratching bouts evoked by sodium deoxycholate acid

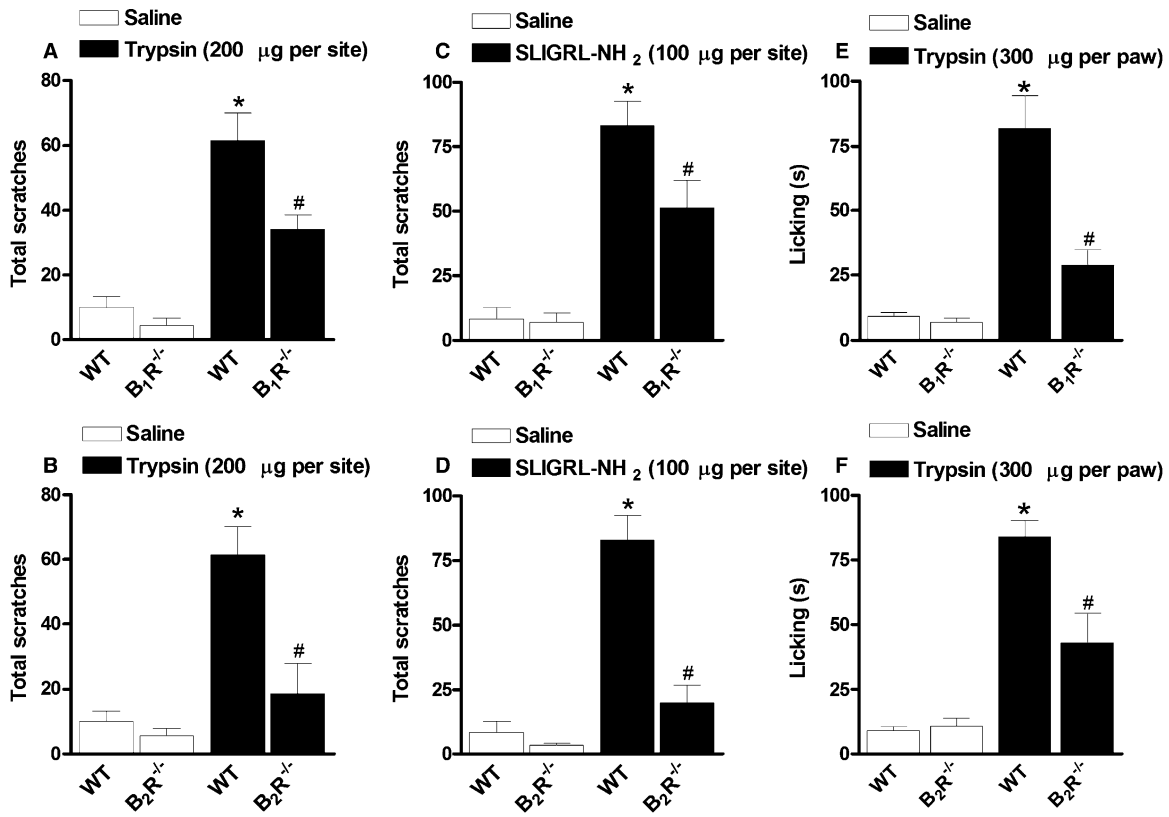

Figure 2 (A, B) Scratching behaviour induced by trypsin (200 μg·site^{-1}, i.d.) in kinin (A) B_1 ($B_1R^{-/-}$) or (B) B_2 ($B_2R^{-/-}$) receptor deficient mice. (C, D) Scratching behaviour induced by SLIGRL-NH_2 (100 μg·site^{-1}, i.d.) in (C) $B_1R^{-/-}$ or (D) $B_2R^{-/-}$ mice. (E, F) Overt nociception (licking) induced by trypsin (300 μg·site^{-1}, i.pl.) in (E) $B_1R^{-/-}$ or (F) $B_2R^{-/-}$ mice. Each column represents the mean of six to 10 animals and the vertical bars represent the SEM. Significantly different when compared with wild-type (WT) saline group (*$P < 0.05$) and WT SLIGRL-NH_2- or trypsin-treated group ($^{\#}P < 0.05$).

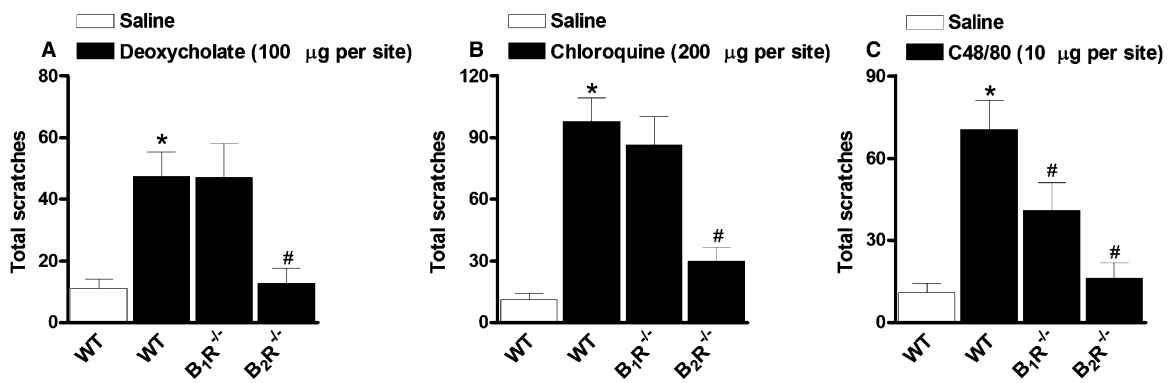

Figure 3 Scratching behaviour induced by (A) deoxycholate (100 μg·site^{-1}, i.d.), (B) chloroquine (200 μg·site^{-1}, i.d.) or (C) compound 48/80 (C48/80; 10 μg·site^{-1}, i.d.) in kinin B_1 ($B_1R^{-/-}$) or B_2 ($B_2R^{-/-}$) receptor deficient mice. Each column represents the mean of six to 10 animals and the vertical bars represent the SEM. Significantly different when compared with wild-type (WT) saline group (*$P < 0.05$) and WT deoxycholate-, chloroquine- or compound 48/80-treated group ($^{\#}P < 0.05$).

(100 μg·site^{-1}), chloroquine (200 μg·site^{-1}) or compound 48/80 (10 μg·site^{-1}), achieving 96 ± 13%, 78 ± 8% and 92 ± 9% of inhibition respectively (Figure 3A–C). On the other hand, in $B_1R^{-/-}$ mice, the scratching response induced by compound 48/80, but not by sodium deoxycholate acid or chloroquine, was significantly inhibited in comparison with wild-type animals (50 ± 17% of inhibition) (Figure 3C).

Effect of the treatment with the selective kinin B_1 or B_2 receptor antagonists on the scratching behaviour induced by PAR-2 agonists in mice

Initially, we sought to assess the effects of systemic kinin B_1 and B_2 receptor antagonists on the scratching behaviour induced by PAR-2 activation in mice. For this purpose, a series of different selective kinin B_1 or B_2 receptor antagonists were

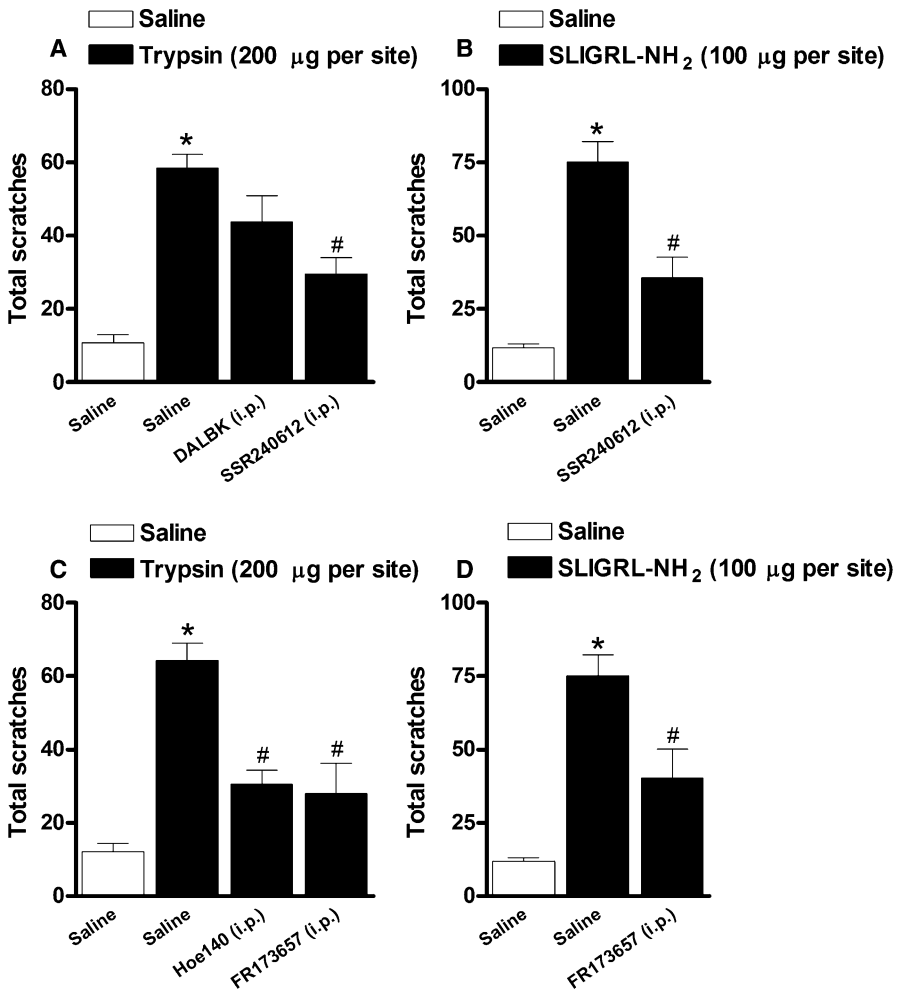

Figure 4 (A, B) Effect of the treatment with the selective kinin B_1 receptor antagonists des-Arg^9-Leu^8-bradykinin (DALBK; 150 nmol·kg^{-1}, i.p., 30 min) or SSR240612 (1 mg·kg^{-1}, i.p., 30 min) on the scratching behaviour induced by (A) trypsin (200 μg·site^{-1}) or (B) SLIGRL-NH_2 (100 μg·site^{-1}) in Swiss mice. (C, D) Effect of the treatment with the selective kinin B_2 receptor antagonists Hoe 140 (50 nmol·kg^{-1}, i.p., 30 min) or FR173657 (30 mg·kg^{-1}, i.p., 30 min) on the scratching behaviour induced by (C) trypsin (200 μg·site^{-1}) or (D) SLIGRL-NH_2 (100 μg·site^{-1}) in Swiss mice. Each column represents the mean of six to 10 animals and the vertical bars represent the SEM. Significantly different when compared with saline group ($^*P < 0.05$) and SLIGRL-NH_2- or trypsin-treated group ($^{\#}P < 0.05$).

systemically tested on the scratching induced either by trypsin or SLIGRL-NH_2. As shown in Figure 4, treatment i.p. of mice with the selective non-peptide kinin B_1 receptor antagonist SSR240612 (1 mg·kg^{-1}, 30 min), but not the selective peptide antagonist DALBK (150 nmol·kg^{-1}, 30 min), produced a considerable inhibition of both trypsin- and SLIGRL-NH_2-induced pruritus (60 ± 9% and 62 ± 11% of inhibition respectively) (Figure 4A and B). As shown in the Figure 4C, the scratching behaviour induced by trypsin was markedly inhibited by i.p. injection of the selective kinin B_2 receptor antagonists Hoe 140 (50 nmol·kg^{-1}, 30 min) or FR173657 (30 mg·kg^{-1}, 30 min) (63 ± 7% and 69 ± 17% respectively). Likewise, FR173657 (30 mg·kg^{-1}, i.p., 30 min) treatment also inhibited SLIGRL-NH_2-induced scratching behaviour (Figure 4D) (54 ± 17%).

Effect of peripheral or central blockade of kinin B_1 or B_2 receptors on the scratching behaviour induced by PAR-2 agonists

In order to investigate the involvement of intradermal kinin receptors in SLIGRL-NH_2-induced pruritus, separate groups of animals were treated locally with the selective kinin B_1 or B_2 receptor antagonists Hoe 140 and DALBK respectively. Figure 5 shows that local co-injection of Hoe 140 (3 nmol·site^{-1}) significantly prevented the scratching behaviour response induced by SLIGRL-NH_2 (51 ± 15% inhibition) (Figure 5D). On the contrary, DALBK (0.3 nmol·site^{-1}, co-injected) treatment was not able to alter this response (Figure 5A). Further, to check the possible involvement of central pathways in the modulatory actions of kinin receptors on the scratching induced by the PAR-2 activator, the animals were treated by i.t. or i.c.v. routes with the selective kinin receptor antagonists. As shown in the Figure 5, the i.t. treatment with the selective kinin B_1 or B_2 receptor antagonists, DALBK (25 pmol·site^{-1}, 15 min) or Hoe140 (100 pmol·site^{-1}, 15 min), reduced the scratching behaviour induced by SLIGRL-NH_2 (54 ± 17% and 76 ± 13% respectively) (Figure 5B and E). Conversely, when injected by i.c.v. route, neither DALBK (25 pmol·site^{-1}, i.c.v.) nor Hoe 140 (100 pmol·site^{-1}, i.c.v.) was effective in inhibiting the pruritus induced by SLIGRL-NH_2 (Figure 5C and F).

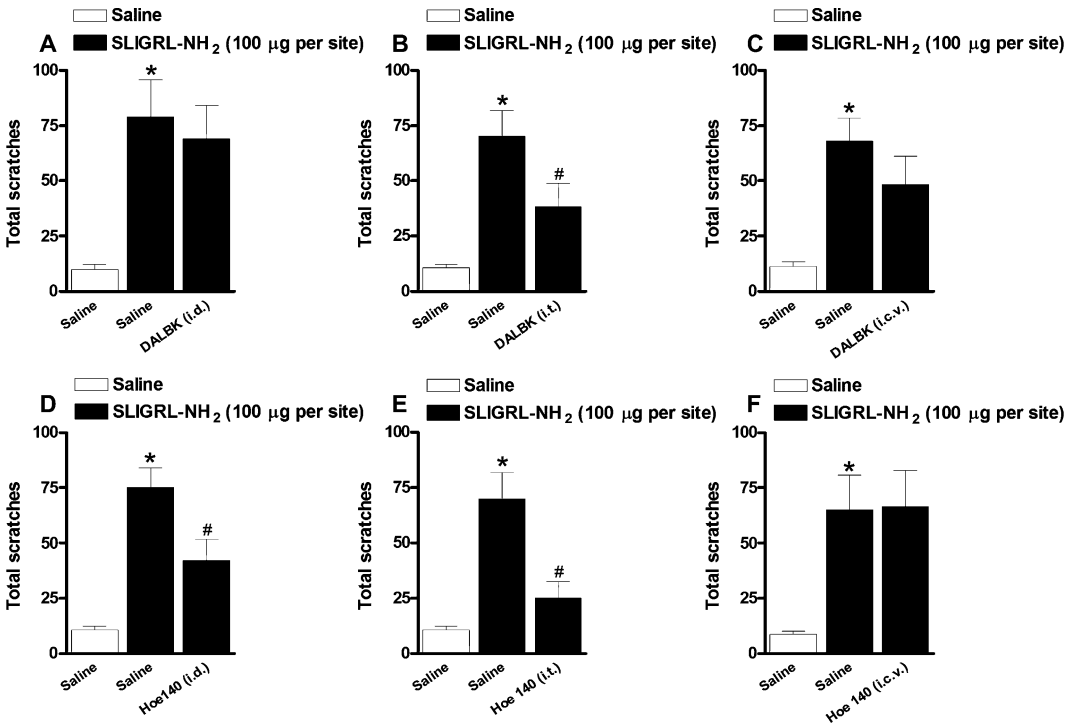

Figure 5 (A, B, C) Effect of (A) intradermal (0.3 nmol·site^{-1}, i.d.), (B) intrathecal (25 pmol·site^{-1}, i.t.) or (C) intracerebroventricular (25 pmol·site^{-1}, i.c.v.) treatment with the selective kinin B_1 receptor antagonist DALBK on the scratching behaviour induced SLIGRL-NH_2 (100 μg·site^{-1}) in Swiss mice. (D, E, F) Effect of (D) intradermal (3 nmol·site^{-1}, i.d.), (E) intrathecal (100 pmol·site^{-1}, i.t.) or (F) intracerebroventricular (100 pmol·site^{-1}, i.c.v.) treatment with the selective kinin B_2 receptor antagonist Hoe 140 on the scratching behaviour induced SLIGRL-NH_2 (100 μg·site^{-1}) in Swiss mice. Each column represents the mean of six to 10 animals and the vertical bars represent the SEM. Significantly different when compared with saline group (*$P < 0.05$) and SLIGRL-NH_2-treated group ($^{\#}P < 0.05$).

Discussion and conclusions

Pruritus, in spite of being a self-defensive mechanism, is a common symptom present in several skin or systemic diseases such as atopic dermatitis, psoriasis, renal failure and cholestasis. In spite of much effort, there are, so far, no available pharmacotherapies for the treatment of itching (Ikoma *et al.*, 2006; Paus *et al.*, 2006; Steinhoff *et al.*, 2006). Thus, the need to understand the mechanisms underlying pruritus neurotransmission has as its main focus the discovery of new molecular targets intended to lead to the development of effective anti-pruritic drugs. In this study, we provide convincing evidence implicating both kinin receptors, especially the B_2 receptor subtype, in the acute scratching behaviour induced by different pruriginous stimuli in mice. We have made the following major findings: (i) kinin B_1 and B_2 receptor deficient mice exhibited lower frequency of scratching bouts after the i.d. administration of different pruriginous agents, when compared with wild-type littermates; and (ii) the pretreatment of mice with the selective kinin B_1 or B_2 receptor antagonists, given by different routes, prevented the scratching behaviour induced by PAR-2 activators.

Recent studies have demosntrated that a single i.d. injection of the selective PAR-2 activating peptide (SLIGRL-NH_2) or the non-selective PAR-2 agonists, tryptase or trypsin, is capable of eliciting scratching behaviour in mice (Shimada *et al.*, 2006; Ui *et al.*, 2006; Costa *et al.*, 2008). Our first set of data (Figure 1) confirmed the previous reports, showing that the i.d. injections of SLIGRL-NH_2 or trypsin were able to evoke scratching behaviour when applied at the back of the mouse neck. Also, we have shown that pruritus induced by SLIGRL-NH_2 was predominantly triggered by PAR-2 activation, as the scratching behaviour was effectively prevented by the selective PAR-2 receptor antagonist FSLLRY-NH_2 (Figure 1B). Previously, we had demonstrated that FSLLRY-NH_2 treatment almost abolished trypsin-induced itching response (Costa *et al.*, 2008). Herein, the scratching response induced by SLIGRL-NH_2 or by trypsin was observed in different mouse strains (Swiss and C57/BL6) allowing us to suggest that i.d. injections of PAR-2 activators can be used as reproducible models to evaluate pruritus in mice.

Our next step was to verify the effects of treatment with a pair of drugs representing the most common classes of clinically used anti-pruritic treatments on the scratching behaviour induced by PAR-2 activators. As noted, the pretreatment with the corticoid dexamethasone greatly reduced both trypsin- and SLIGRL-NH_2-induced itching behaviour in mice. Nonetheless, these pruriceptive responses were resistant to the treatment with the selective histamine H_1 receptor antagonist pyrilamine (Figure 1C and D). Earlier works have demonstrated that PAR-2 activation-induced itching behaviour was not sensitive to anti-histamine treatment (Shimada *et al.*, 2006; Costa *et al.*, 2008; Tsujii *et al.*, 2008). Therefore, one may infer that PAR-2-mediated scratching behaviour might be used as an experimental model of pruritus aiming at the screening of new therapeutic approaches that could be

further applied to the clinical conditions resistant to antihistamine drugs.

The kallikrein-kinin system has consistently been involved in the pathophysiology of itching (Rajakulasingam *et al.*, 1991; Hayashi and Majima, 1999; Schmelz *et al.*, 2003; Hosogi *et al.*, 2006; Costa *et al.*, 2008). Confirming and extending these previous findings, the results of the present study clearly implicate both kinin B_1 and B_2 receptors in the scratching behaviour induced by PAR-2 activators in mice. Accordingly, the scratching behaviour induced by trypsin or SLIGRL-NH_2 was almost completely abolished in $B_2R^{-/-}$ mice, while the genetic deletion of B_1 receptor produced a partial but significant reduction of these responses (Figure 2A–D).

Interestingly, we also verified that the deletion of either kinin B_1 and B_2 receptors significantly reduced the overt nociception caused by i.pl. trypsin injection (Figure 2E–F). B_1 or B_2 receptor deficient mice are known to display hypoalgesia against the acute overt nociception induced by capsaicin, formalin, phorbol 12-myristate 13-acetate or by high intensity heat stimuli (Boyce *et al.*, 1996; Rupniak *et al.*, 1997; Pesquero *et al.*, 2000; Ferreira *et al.*, 2008). An overall analysis of our data suggests a greater involvement of B_1 receptors in the nociceptive response caused by PAR-2 activation, while B_2 receptors seem to be preferentially associated with PAR-2-mediated scratching behaviour. In addition, our data also permit us to propose that distinct mechanisms seem to mediate the pruriceptive and nociceptive behaviours caused by PAR-2 activators in mice. Thus, we might suggest that B_1 receptors and B_2 receptors would differently modulate the nociceptive and pruriceptive pathways. In spite of these suggestions, complementary studies are necessary to make clear the precise mechanisms underlying the actions of kinins in these two models.

The predominant role of the B_2 receptors in pruriceptive responses (in relation to the B_1 receptor involvement) was also confirmed in three additional acute scratching behaviour models. $B_2R^{-/-}$ animals displayed a reduced itching behaviour after the i.d. injection of sodium deoxycholate acid, chloroquine or compound 48/80, when compared with wild-type or $B_1R^{-/-}$ animals (Figure 3). The results presented here are consistent with those in a previous publication showing that sodium deoxycholate acid-evoked scratching behaviour was prevented by the treatment with selective kinin B_2 receptor (Hoe 140 or FR173657) but not B_1 receptor (DALBK) antagonists (Hayashi and Majima, 1999). Although B_1 receptor deletion did not alter the pruriceptive responses generated by sodium deoxycholate acid or chloroquine, it was able to significantly reduce the scratching behaviour triggered by compound 48/80 (Figure 3A–C).

Next, we evaluated the effects of selective kinin B_1 or B_2 receptor antagonists in the scratching behaviour induced by PAR-2 activators in mice. The present study demonstrated that the i.p. treatment with the selective peptide (Hoe 140) or non-peptide (FR173657) kinin B_2 receptor antagonists did inhibit both trypsin- and SLIGRL-NH_2-induced scratching behaviour. Also, the scratching responses induced by these same pruriginous agents were reduced by the selective non-peptide kinin B_1 receptor antagonist SSR240612 (Figure 4A–D). Such results are in accordance with our previous data showing that trypsin-induced itching behaviour was reduced by SSR240612 or FR173657 treatments (Costa *et al.*, 2008). Despite the effectiveness of SSR240612, i.p. treatment with the selective peptide kinin B_1 receptor antagonist DALBK was not able to reduce trypsin-elicited itching (Figure 4A). This fact might be related to the pharmacokinetic properties of DALBK, which is a peptide (Campos *et al.*, 2006).

To examine the involvement of dermal kinin receptors in SLIGRL-NH_2-induced itching, we assessed the local (i.d.) effects of the selective kinin B_1 or B_2 receptor antagonists. The peripheral blockade of B_2 receptors by the i.d. co-treatment with Hoe 140 prevented PAR-2 activating peptide-induced scratching behaviour (Figure 5A), emphasizing the contribution of kinin B_2 receptors located in the skin. Indeed, studies using different methodological approaches have found that BK given i.d. may evoke itching sensation in humans in certain circumstances (Schmelz *et al.*, 2003; Hosogi *et al.*, 2006). Also, i.pl. injections of BK or Tyr^8-BK (preferential and selective B_2 receptor agonists respectively) caused overt nociception in rodents essentially through the activation of constitutively and functionally expressed B_2 receptors in peripheral tissues (Boyce *et al.*, 1996; Griesbacher *et al.*, 1998; Campos *et al.*, 1999; Ferreira *et al.*, 2004). However, it is worth mentioning that i.d. injections of BK did not cause a significant increase in the number of scratching bouts when injected at the back of the mouse neck in our paradigm (data not shown). In this study, i.d. co-treatment with the selective kinin B_1 receptor antagonist DALBK was not able to interfere with the pruriceptive response evoked by SLIGRL-NH_2 (Figure 5B), excluding the involvement of peripherally expressed B_1 receptors. In fact, the peripheral injection of B_1 receptor agonists rarely induces nociception in naïve animals (Perkins and Kelly, 1994; Ganju *et al.*, 2001; Fox *et al.*, 2003; Ferreira *et al.*, 2004); and priming stimuli are needed to elicit their nociceptive actions (Campos *et al.*, 1995; De Campos *et al.*, 1998; Ma, 2001). In spite of these results, both kinin B_1 and B_2 receptors, present in the skin, mediated capsaicin-induced ear oedema in mice, suggesting that they could be useful targets in the treatment of some skin inflammatory diseases (Pietrovski *et al.*, 2009).

As a final goal of this study, we sought to investigate the possible involvement of central nervous system pathways in the modulatory actions of kinin receptors in the SLIGRL-NH_2-induced scratching behaviour in mice by the use of selective antagonists directly injected into central structures. Noticeably, the i.t. administration of the following kinin receptor antagonists, DALBK (B_1 receptor) or Hoe140 (B_2 receptor), reduced the pruriceptive response evoked by SLIGRL-NH_2. Conversely, neither DALBK nor Hoe140 injected by i.c.v route inhibited this behavioural response. Our findings are supported by evidence showing that both kinin B_1 and B_2 receptors are functionally expressed at the level of the spinal cord (Chapman and Dickenson, 1992; Corrêa and Calixto, 1993; Pesquero *et al.*, 2000; Ferreira *et al.*, 2002; 2004; Fox *et al.*, 2003). Of relevance, i.t. injection of des-Arg^9-BK or Tyr^8-BK (selective kinin B_1R and B_2R agonists respectively) causes thermal and mechanical hyperalgesia in mice (Ferreira *et al.*, 2002; Fox *et al.*, 2003). Moreover, i.t. treatment with kinin B_1 or B_2 receptor antagonists was effective against the overt nociception caused by formalin in rodents (Chapman and Dickenson, 1992; Ferreira *et al.*, 2002). Corroborating these data, it

has been shown that $B_1R^{-/-}$ mice show hypoalgesia in chemical models of nociception, probably related with a reduction in dependent activity facilitation (wind-up phenomenon) of spinal nociceptive reflexes (Pesquero *et al*., 2000). As the pruriceptive behaviour induced by PAR-2 activators is very short, it peaked at 10 min and lasted up to 40 min (data not shown), it seems unlikely that B_1 or B_2 receptor expression in the spinal cord depends on *de novo* protein synthesis and the involvement of constitutively expressed kinin receptors is more plausible. Indeed, in the present work, the i.d. injection of SLIGRL-NH_2 was not able to induce an increase in the expression of either kinin B_1 or B_2 receptor protein in the spinal cord, as assessed by Western blot analysis (data not shown).

As a great novelty of this study, we have demonstrated that kinins acting on both receptors, particularly on the B_2 receptor subtype, play a critical role in controlling pruriceptive signalling. Firstly, the deletion of kinin B_1 or B_2 receptors prevented the scratching behaviour triggered by different pruriginous stimuli in mice. Secondly, the treatment of mice with the selective kinin B_1 or B_2 receptor antagonists potently inhibited PAR-2 activators-induced scratching behaviour, when given by systemic, local and i.t. routes. The findings presented here are in accordance with previous studies showing that the activation of BK B_2R is a downstream event of PAR-2 activation in pain (Kawabata *et al*., 2006; Paszcuk *et al*., 2008). These pieces of evidence support the notion that selective kinin receptor antagonists, mainly against the B_2 receptor subtype, might represent new attractive therapeutic options for treating pruriginous conditions, especially those resistant to anti-histamine treatment.

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Conflict of interest

The authors state no conflicts of interest.

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