Cellular localization of kinin B₁ receptor in the spinal cord of streptozotocin-diabetic rats with a fluorescent [Nα-Bodipy]-des-Arg⁹-bradykinin
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Abstract

Background: The kinin B₁ receptor (B₁R) is upregulated by pro-inflammatory cytokines, bacterial endotoxins and hyperglycaemia-induced oxidative stress. In animal models of diabetes, it contributes to pain polyneuropathy. This study aims at defining the cellular localization of B₁R in thoracic spinal cord of type 1 diabetic rats by confocal microscopy with the use of a fluorescent agonist, [Nα-Bodipy]-des-Arg⁹-BK (BdABK) and selective antibodies.

Methods: Diabetes was induced by streptozotocin (STZ; 65 mg/kg, i.p.). Four days post-STZ treatment, B₁R expression was confirmed by quantitative real-time PCR and autoradiography. The B₁R selectivity of BdABK was determined by assessing its ability to displace B₁R [¹²⁵I]-HPP-desArg⁹-Hoe140 and B₂R [¹²⁵I]-HPP-Hoe140 radioligands. The in vivo activity of BdABK was also evaluated on thermal hyperalgesia.

Results: B₁R was increased by 18-fold (mRNA) and 2.7-fold (binding sites) in the thoracic spinal cord of STZ-treated rats when compared to control. BdABK failed to displace the B₂R radioligand but displaced the B₁R radioligand (IC₅₀ = 5.3 nM). In comparison, IC₅₀ values of B₁R selective antagonist R-715 and B₁R agonist des-Arg⁹-BK were 4.3 nM and 19 nM, respectively. Intraperitoneal BdABK and des-Arg⁹-BK elicited dose-dependent thermal hyperalgesia in STZ-treated rats but not in control rats. The B₁R fluorescent agonist was co-localized with immunomarkers of microglia, astrocytes and sensory C fibers in the spinal cord of STZ-treated rats.

Conclusion: The induction and up-regulation of B₁R in glial and sensory cells of the spinal cord in STZ-diabetic rats reinforce the idea that kinin B₁R is an important target for drug development in pain processes.
Background

Kinins are vasoactive peptides and central mediators acting through the activation of two G-protein-coupled receptors (R) denoted as B₁ and B₂ [1, 2]. The B₂R is widely and constitutively expressed in central and peripheral tissues and is activated by its preferential agonists bradykinin (BK) and Lys-BK. The B₁R is activated by the active metabolites des-Arg⁹-BK and Lys-des-Arg⁹-BK and has a low level of expression in healthy tissues. The latter receptor is upregulated after exposure to pro-inflammatory cytokines, bacterial endotoxins, and hyperglycaemia-induced oxidative stress [3-7].

An important role for kinin B₁R has been postulated in nociception and pain [8-10]. B₁R knock out mice are less sensitive to pro-inflammatory pain stimuli and to spinal sensitization [11-13]. B₁R partakes to mechanical and/or thermal hyperalgesia induced by cytokines [14, 15] through peripheral protein kinase C activation [16] and in the formalin test [17, 18]. It also contributes to neuropathic pain after peripheral nerve injury [18-23] or after the induction of type 1 diabetes with streptozotocin (STZ) [24-27] and type 2 diabetes with high glucose feeding [7, 28, 29]. Thermal hyperalgesia was evoked by intraspinral stimulation of B₁R in STZ-diabetic rats [9].

Basal expression of B₁R was reported in the rat and human spinal cord dorsal horn as well as in rat dorsal root ganglion and small caliber primary sensory neurons [30-32]. Autoradiographic B₁R binding sites are increased and distributed all over the grey matter of the spinal cord after peripheral nerve injury [22] and in models of diabetes [7, 29, 33]. This spatial distribution of B₁R binding sites suggests that this receptor is not limited to primary sensory afferents but could also be present on spinal cord microglia and astrocytes.

To consolidate the role of B₁R in pain polyneuropathy, its cellular distribution was investigated in the spinal cord of STZ-induced B₁R with a newly developed fluorescent agonist named [Nα-Bodipy]-des-Arg⁹-BK (BdABK). The B₁R selectivity of BdABK was determined by assessing its ability to displace B₁R ([¹²⁵I]-HPP-desArg¹⁰-Hoe 140) and B₂R ([¹²⁵I]-HPP-Hoe 140) radioligands by autoradiography. Moreover, the displacement of BdABK fluorescent labeling by B₁R antagonists (R-715 and SSR240612) was assessed by confocal microscopy. We also investigated the in vivo activity of BdABK in comparison with its native agonist on thermal hyperalgesia in both STZ-treated and control rats. Appropriate selective antibodies were used in confocal microscopy to co-localize B₁R on astrocytes, microglia and sensory C fibers in STZ-diabetic rats. The induction and overexpression of B₁R in the spinal cord of STZ-diabetic rats was confirmed by qPCR and autoradiography. Experiments were achieved 4 days after STZ administration because previous studies showed that spinal cord B₁R was maximally up-regulated and engaged in thermal hyperalgesia 2 days after STZ treatment [9, 33].

Methods

Animals and treatments

All research procedures and the care of the animals were in compliance with the guiding principles for animal experimentation as enunciated by the Canadian Council on Animal Care and were approved by the Animal Care Committee of our University. Male Sprague-Dawley rats (200–225 g, Charles River, St-Constant, Que., Canada) were housed two per cage, under controlled conditions of temperature (23°C) and humidity (50%), on a 12 h light-dark cycle and allowed free access to normal chow diet (Charles River Rodent) and tap water.

STZ treatment

Rats were used 5 days after their arrival and injected under low light with freshly prepared STZ (65 mg/kg; i.p.; Sigma-Aldrich, Oakville, ON, Canada). Age-matched controls were injected with vehicle (sterile saline 0.9%, pH. 7.0) [33]. Glucose concentrations were measured, with a commercial blood glucose-monitoring kit (Accusoft; Roche Diagnostics, Laval, Que., Canada), in blood samples obtained from the tail vein, in non-fasting animals, before STZ injection, and 4 days after treatment. Only STZ-treated rats whose blood glucose concentration was higher than 20 mM were considered as diabetic.

Synthesis of [Nα-Bodipy]-des-Arg⁹-BK

BdABK was synthesized using 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid succinimidy ester (BODIPY® FL SE, Molecular Probes/Invitrogen Canada Inc, Burlington, ON; emission 510 nm) and des-Arg⁹-BK (Bachem Bioscience inc., King of Prussia, PA, USA). Des-Arg⁹-BK was solubilized in 100 mM NaHCO₃ 0.1 M (pH 8.4), at a concentration of 1 mg/ml and two equivalents of BODIPY® FL SE, solubilized in degassed dimethyl sulfoxide, at a concentration of 5 mg/ml was added. Completion of the reaction was achieved in 2 h, at ambient temperature, under continuous agitation. The fluorescent peptide was lyophilized and purified by C18 reverse-phase HPLC as previously described [34, 35]. The purity of the peptide was ≥ 98% as assessed by analytical HPLC (UV and fluorescence detection).

Tissue preparation for autoradiography and microscopy

Four days after injection of STZ, rats were anaesthetized with CO₂ inhalation and then decapitated. Upper thoracic spinal cord (T3-T17) was removed and frozen in 2-methylbutane (cooled at -40°C following exposure to liquid nitrogen) and stored at -80°C. Few days later, spinal cords were mounted in a gelatin block and serially cut into 20-μm thick coronal sections with a cryostat. Thus the sec-
tions were thaw-mounted on 0.2% gelatin-0.033% chromium potassium sulfate-coated slides and kept at -80°C for 1 month to allow the adhesion of sections to the coverslip glasses.

**Confocal microscopy**

**Slides preparation**

On the day of experiments, sections were thawed at room temperature for 10 min to enhance sections adhesion. They were pre-incubated for 10 min in 25 mM PIPES-NH₄OH buffer (pH 7.4) to allow degradation of endogenous kinins which could occupy receptors. Sections were exposed for 90 min to 50 μM BdABK. Thereafter, slides were washed twice (1 min) in PIPES and fixed with 4% para-formaldehyde [36]. Slides were washed three times (5 min) and then exposed to 1 M of glycine for 90 min to eliminate autofluorescence from aldehyde-fixed tissue. Tissues were permeabilized for 45 min with 0.1% Triton X-100.

**Immunolabeling protocol**

Slides were incubated with a blocking buffer (25 mM PIPES buffer supplemented with 3% bovine serum albumin (BSA) and 3% donkey serum) to prevent non-specific labeling. Antibodies were diluted in blocking buffer. A direct marker of DNA (TOPRO-3; Molecular Probes, Eugene, OR) was used at concentration of 1:500. Rabbit anti-Ionized calcium binding adapter molecule 1 (anti-IBA-1, Wako, Richmond, VA) at a concentration of 2 μg/ml was used to label microglia [37-39]. Chicken anti-Glial fibrillary acidic protein (anti-GFAP, Chemicon, Hornby, ON) at a concentration of 1:500 was used as a specific marker of astrocytes [40]. Rabbit anti-calcitonin-gene-related peptide (CGRP) (Chemicon, Hornby, ON) at a concentration of 1:500 was used as a specific marker of capsaicin receptor expressed on primary afferents [42]. Secondary antibodies were rhodamine anti-mouse (Chemicon, Hornby, ON) 1:500; cy5 anti-chicken (Chemicon, Hornby, ON) 1:500 and rhodamine anti-rabbit (Chemicon, Hornby, ON) 1:500.

**Coverslip and microscopy**

Slides were washed 3 times (5 min), mounted with coverslip, fixed with mowiol (12 h at room temperature) and stored at -4°C for 1 month or used in confocal microscopy.

**SYBR green-based quantitative RT-PCR**

Four days after injection of STZ, rats were anaesthetized with CO₂ inhalation and then decapitated. The thoracic spinal cord (T1-T2) was isolated and approximately 10 mg of tissue were put in RNA later stabilization reagent (QIAGEN, Valencia, CA, USA). Total RNA was extracted from tissue according to the manufacturer's instructions. First-strand cDNA synthesized from 400 ng total RNA with random hexamer primers was used as template for each reaction with the QuantiTect Rev Transcription Kit (QIAGEN). SYBR Green-based real-time quantitative PCR using Mx3000p device for signal detection (Stratagene, La Jolla, CA, USA) was performed as described [43]. PCR was performed in SYBR Green Master mix (QIAGEN) with 300 nM of each primer. For standardization and quantification, rat 18S was amplified simultaneously. The primer pairs were designed by Vector NTI software and used [6] (Table 1).

PCR conditions were as follows: 95°C for 15 min, followed by 46 cycles at 94°C for 15 s, 60°C for 30 s and 72°C for 30 s. The cycle threshold (Ct) value represents the cycle number at which a fluorescent signal rises statistically above background [44]. The relative quantification of gene expression was analyzed by the 2⁻ΔΔCt method [45].

**Quantitative autoradiography**

**Specific binding sites of [125I]-HPP-desArg₁₀-Hoe 140 and [125I]-HPP-Hoe 140**

The radioligands for kinin B₁R, HPP-desArg₁₀-Hoe140 (3-(4-hydroxyphenyl) propionyl-desArg⁹-D-Arg⁰[Hyp³, Thr⁵, D-Tic⁷, Oic⁸]Bradykinin) and kinin B₂R, HPP-Hoe140 (3-(4 hydroxyphenyl) propionyl-D-Arg⁰[Hyp³, Thr⁵, D-Tic⁷, Oic⁸]Bradykinin) were synthesized and kindly provided by Dr Witold Neugebauer (Dept Pharmacology, University of Sherbrooke, Sherbrooke, Que., Canada). They were iodinated by the chloramine T method [46]. On the day of experiments, sections were incubated at room temperature for 90 min in 25 mM PIPES-NH₄OH buffer (pH 7.4) containing: 1 mM 1,10-phenanthroline, 1 mM dithiothreitol, 0.014% bacitracin, 0.1 mM captopril, 0.2% bovine

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**Table 1: PCR primer pairs used in this study**

| Sequences | Position | Gen Bank |
|-----------|----------|----------|
| 18S Forward | 5’ TCA ACT TTC GAT GGT AGT CGC CGT 3’ | 363 – 386 | X01117 |
| 18S Reverse | 5’ TCC TTG GAT GTG GTA GCC GTT TCT 3’ | 470 - 447 |
| B₁ receptor Forward | 5’ GCA GCG CTT AAC CAT AGC GGA AAT 3’ | 367 – 391 | NM_030851 |
| B₁ receptor Reverse | 5’ CCA GTT GAA ACG GTT CCC GAT GTT 3’ | 478 - 454 |

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serum albumin (protease free) and 7.5 mM magnesium chloride in the presence of 200 pM of [125I]-HPP-desArg10-Hoe 140 or [125I]-HPP-Hoe 140 (specific activity: 2000 cpm/fmol or 1212 Ci/mmol) [29,33]. Non-specific binding was determined in the presence of 1 μM of unlabeled B1R antagonist: R-715 (AcLys [D-βNal7, lle8]des-Arg9-BK) [1] or 1 μM of unlabeled B1R antagonist: Hoe 140 (Icatibant or JE 049, Jerini AG, Berlin, Germany) [47]. At the end of the incubation period, slides were transferred sequentially through four rinses of 4 min each in 25 mM PIPES (pH 7.4; 4°C) dipped for 15s in distilled water (4°C) to remove the excess of salts, and then air-dried. Kodak Scientific Imaging Films BIOMAX™ MR® (Amersham Pharmacia Biotech Canada) were juxtaposed onto the slides in the presence of [125I]-microscales and exposed at room temperature for 7 days. The films were developed (GBX developer) and fixed (GBX fixer). Autoradiograms were quantified by densitometry using an MCID™ image analysis system (Imaging Research, St. Catharines, ON, Canada). A standard curve from [125I]-microscales was used to convert density levels into femtomoles per milligram of protein [48]. Specific binding was determined by subtracting values of nonspecific binding from that of total binding.

**Specificity of BdABK**

To assess the specificity of BdABK for B1R, competition curves were performed in autoradiography by incubating 200 pM of [125I]-HPP-desArg10-Hoe 140 with increasing concentrations (10^-10 to 10^-6 M) of R-715 (selective B1R antagonist, kindly provided by Dr Domenico Regoli, Pharmacology, University of Ferrara, Italy), des-Arg9-BK (dABK, selective B1R agonist, Bachem Bioscience inc., King of Prussia, PA, USA) and BdABK. Moreover, competition curves were performed by incubating 200 pM of [125I]-HPP-Hoe 140 with increasing concentrations (10^-10 to 10^-6 M) of Hoe 140 (selective B1R antagonist) and BdABK. Each concentration of each competitor was tested on 4 sections per rat from 7 different rats. Those sections were exposed to the film, and total binding was calculated as described above. Moreover, the specificity of BdABK was determined in confocal microscopy by the displacement of fluorescent labeling with the addition of 10^-5 M R-715 or SSR240612 [(2R)-2-[(3R)-3-{1,3-benzodioxol-5-yl}-3-[[6-methoxy-2-naphthyl]sulfonyl]amino]propanoyl]amino]-3-(4-[2R, 6S]-2,6 dimethylpiperidinyl)methyl]phenyl)-N-isopropyl-N-ethylpropanamide hydrochloride] (kindly provided by Dr Pierre Carayon, Sanofi-Aventis, Montpellier, France) [18] to the incubation medium.

**Microglial cell culture**

**Primary cell culture method**

Mixed glial cultures were prepared following the protocol of McCarthy and de Veullis [49] with some modifications. Briefly, forebrains were dissected out from one litter of 2-day-old Sprague-Dawley rat pups and the meninges were stripped off before enzymatic and mechanical dissociation. For enzymatic dissociation, HBSS containing 0.25% trypsin (Gibco 15090-046) was used. The tissue-trypsin suspension was incubated for 20 min at 37°C in a water bath with intermittent shaking. After the waiting time for the trypsin digestion is over we added to the tissue-trypsin suspension a mixture of prewarmed DMEM/Dnase 1 (Sigma DN-25, Dnase I final concentration 0.25 mg/ml) followed by an incubation for 4 min at 37°C. The resulting suspension was dispersed by a mild mechanical trituration which consisted in the passage through 18-, 22- and 25- gauge needles. This cell suspension was then filtered through 70 μm strainer (BD Falcon 352350). After extensive washes in prewarmed HBSS, these dissociated cells were resuspended and plated in 75-cm² Falcon tissue-culture flasks (BD Biosciences) previously coated with 10 μg/ml poly-D-lysine (PDL). These mixed cells were growing at 37°C and 5% CO₂ in DMEM (Gibco) supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 mg/ml). The media was changed every 2 or 3 days thereafter.

At 10 days- in vitro, a confluent monolayer of astrocytes was apparent, on top of which oligodendrocyte precursor cells and a loosely attached layer of phase-bright microglia was obtained. Microglia were collected by shaking the flasks for 1 h at 200 rpm at 37°C and 5% CO₂. Dislodged cells were resuspended and grown in culture medium for microglia (RPMI medium 1640 (Gibco) supplemented with 10% FBS, L-glutamine (1 mM), sodium pyruvate (1 mM), penicillin (100 units/ml), and streptomycin (100 mg/ml)). The cells were allowed to adhere to the surface of PDL-coated coverslips (30 min at 37°C and 5% CO₂), and nonadherent cells were rinsed off.

**Microglia cells preparation for confocal microscopy**

Briefly, confluent cells were exposed to 500 nM of BK for 24 h to induce B1R [50,51]. Control cells were exposed to vehicle. After incubation with BdABK, cell were washed, then fixed and permeabilized with 100% methanol previously stored at -20°C. The fixed cells were then processed as described for immunostaining.

**Thermal hyperalgesia**

Thermal hyperalgesia was assessed according to the method described by Hargreaves et al., 1988 [52] with minor modifications. Briefly, rats were placed (unrestrained) within a Plexiglass enclosure on a transparent glass floor and allowed to acclimatize for 20–30 min. An infrared beam that constitutes the noxious heat stimulus (Plantar test, Ugo Basile, Italy) was moved beneath the plantar surface of the hind paw. Thermal nociceptive threshold was defined as the latency (seconds) between the heat stimulus (46°C) onset and the paw withdrawal using a feedback-controlled shut-down unit. A cut-off time of 33
s was used to avoid tissue damage. Each paw was tested three times alternatively at minimum intervals of 3 min between stimulation to avoid sensitization of the hind paw. The rats were trained on several days prior to testing B1R agonists. Thereafter, the thermal nociceptive threshold was assessed on 3 consecutive days as follows: day 1: baseline, saline and the first dose of des-Arg9-BK and BdABK (22.5 μg/kg); day 2: des-Arg9-BK and BdABK (225 μg/kg); day 3: des-Arg9-BK and BdABK (2250 μg/kg). Agonists were injected intraperitoneally at 1 h apart. This series of experiments was conducted in 3 control and 3 STZ-diabetic rats because the quantity of BdABK available for in vivo study was restricted. Thermal hyperalgesia was calculated as a percentage of the maximum possible effect (% MPE) according to the following formula: % MPE = (100 × (drug latency minus baseline latency)/(cut-off time minus baseline latency)) [9]. The baseline latency corresponds to the average of the first three measurements.

### Statistical analyses

All data were expressed as means ± S.E.M. obtained from n rats. Statistical significance was determined with Student’s t-test for unpaired samples or a one-way analysis of variance (ANOVA) followed by post-hoc Dunnett test for multiple comparisons. IC50 values were calculated by Graph Pad Prism 4.0 (GraphPad software, USA). Only probability (P) values less than 0.05 were considered to be statistically significant.

## Results

### B1R fluorescent labeling and selectivity of BdABK

Figure 1 illustrates B1R labeling with BdABK from low (i) to high magnification (V) in dorsal horn of thoracic spinal cord of STZ-treated rats. As depicted in Figure 2, BdABK showed no labeling in control thoracic spinal cord (A), while the labeling of B1R was apparent in thoracic spinal cord of STZ-treated rats as revealed by green dots (B). Selectivity and specificity of the labeling were demonstrated by the absence of BdABK labeling in STZ-spinal cord sections when the B1R antagonists SSR240612 (D) and R-715 (E) were added at 10⁻⁵M. BdABK mediated in vivo thermal hyperalgesia

The in vivo effect of BdABK on pain behavior was assessed by determining its ability to induce thermal hyperalgesia upon intraperitoneal injection in STZ-treated rats. As
expected, BdABK and des-Arg^9^-BK had no significant effect on the nociceptive threshold in control rats, yet both agonists caused thermal hyperalgesia in STZ-diabetic rats at 0.225 and 2.25 mg/kg. These effects were dose-dependent and significant when compared to saline or control (Fig. 5). BdABK was however slightly but significantly less potent than des-Arg^9^-BK to induce hyperalgesia at the highest dose. As exemplified by des-Arg^9^-BK, this response peaked at 15 min post-injection and was reversible after 30 min (Fig. 6).

B^1R mRNA expression assessed by qPCR
A low basal expression of kinin B^1R mRNA was detected in the spinal cord of control rats (Fig. 7). This expression was significantly increased (18-fold) in the spinal cord of STZ-diabetic rats.

Density of B^1R binding sites assessed by quantitative autoradiography
As presented in Figure 8, quantitative in vitro autoradiography showed an increase density of specific B^1R binding sites throughout the grey matter of the thoracic spinal cord in STZ-treated rats when compared to age-matched control spinal cord. B^1R binding sites (2.4 fmol/mg protein) in spinal cord of STZ-treated rats were 2.7-fold greater than those measured in control rats (0.9 fmol/mg protein).

B^1R colocalized on microglial cells in thoracic spinal cord
Figure 9 shows the colocalization of BdABK, TOPRO-3 and anti-IBA-1 in STZ thoracic spinal cord. Data suggest that B^1R is present on spinal microglial cells in STZ-diabetic rats.

B^1R colocalized in primary cultured microglial cells
Figure 10 shows the colocalization of BdABK, TOPRO-3 and anti-IBA-1 in primary microglial cell culture. B^1R was induced by a pre-treatment with 300 nM BK. About 95 ± 2% of the primary cell culture showed a positive labeling.
with anti-IBA-1 confirming cell purity. Data suggest that B1R can be induced in vitro on microglial cells.

**B1R colocalized on sensory C fibers in thoracic spinal cord**

Figure 11 shows the colocalization of BdABK, anti-TRPV1 and anti-CGRP in the thoracic spinal cord of STZ-treated rats. Data suggest that B1R and TRPV1 are co-localized on sensory C fibers in STZ-diabetic rats.

**B1R colocalized on astrocytes in thoracic spinal cord**

Figure 12 shows the colocalization of BdABK and anti-GFAP in the spinal cord of STZ-treated rats. Data suggest that B1R is also present on spinal astrocyte cells in STZ-diabetic rats.

**Discussion**

This study is using a newly developed selective and high affinity fluorescent ligand enabling the cellular localization of B1R on unfixed tissue. It provides the first evidence that B1R is localized on microglial cells, astrocytes and sensory C fibers in the thoracic spinal cord of STZ-diabetic rats. This study also highlights the early upregulation of B1R (mRNA and binding sites) in the thoracic spinal cord of hyperglycaemic STZ-diabetic rats.

**Diabetes induces B1R expression**

STZ-diabetic rats provide an accessible model for studying the expression, the pharmacology and physiopathology of the B1R in the central nervous system. Pharmacological data showed that functional B1R was expressed in spinal cord of STZ-treated rats; its spinal activation led to sympathetically mediated increases of blood pressure and heart rate [53] and to thermal hyperalgesia [9]. Further autoradiographic and functional evidence for B1R induction was demonstrated in the lung [54], spinal cord [33], retina [6,55] and brain [56] of STZ-diabetic rats. However, this is the first report on mRNA expression in thoracic spinal cord of STZ-diabetic rats by qPCR. Hyperglycaemia associated with type 1 diabetes can activate NF-κB [57] which is known to induce B1R [2,3,58]. Moreover, oxidative stress associated with diabetes was reported to be involved in the induction of B1R [6,7,29,59].

**[Nα-Bodipy]-des-Arg^9^BK selectivity for B1R**

Experiments by autoradiography confirm that BdABK is highly selective for B1R and does not bind to B2R. Indeed, BdABK failed to displace the B2R radioligand [125I]-HPP-Hoe-140 while it displaced the B1R radioligand, [125I]-

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**Figure 6**

Time-course effect of des-Arg^9^BK (2.25 mg/kg, i.p.) on the nociceptive threshold in STZ-treated rats. Data are means ± SEM of 3 rats. Statistical comparison to time 0 (*) is indicated by ** P < 0.01.

**Figure 7**

B1R mRNA expression in thoracic spinal cord. Data are means ± SEM of (3 to 6) rats. Statistical comparison with control is indicated by * P < 0.05.

**Figure 8**

B1R binding sites in STZ-treated and control thoracic spinal cords were measured by quantitative autoradiography. Specific density of B1R binding sites are means ± SEM of (7 to 8) rats. Statistical comparison with control is indicated by *** P < 0.001.
HPP-desArg\textsuperscript{10}-Hoe 140, with an IC\textsubscript{50} of 5.3 ± 0.1 nM in thoracic spinal cord of STZ-treated rats. Results also evidenced that B\textsubscript{1}R binding sites were displaced by the selective antagonist, Hoe 140, with an IC\textsubscript{50} value of 1.3 ± 0.1 nM while B\textsubscript{1}R binding sites were displaced by the natural B\textsubscript{1}R agonist, des-Arg\textsuperscript{8}-BK (IC\textsubscript{50} = 19 ± 0.2 nM) and by R-715, a selective B\textsubscript{1}R peptide antagonist (IC\textsubscript{50} = 4.3 ± 0.2 nM). Comparison of IC\textsubscript{50} values suggests that the affinity of the B\textsubscript{1}R agonist is increased by the addition of the Bodipy molecule. The stabilization of the N-terminus part of the peptide may contribute to prevent its degradation.

The reason for using 50 \( \mu \)M BdABK was based on preliminary study. The concentration of fluorescent probe needed to get a consistent labeling was higher than the IC\textsubscript{50} value most likely because BdABK binds to B\textsubscript{1}R non-covalently and can be eliminated during the washout period of tissue sections. Signal amplification with radioactivity is also expected to be greater than that achieved with a fluorescent probe. BdABK showed no labeling in thoracic spinal cord of control rats which is in accordance with the inducible character of the B\textsubscript{1}R and its virtual absence in healthy tissues. The elimination of B\textsubscript{1}R labeling with BdABK after co-incubation with R-715 or SSR240612 confirms the specificity of the B\textsubscript{1}R fluorescent ligand.

Interestingly, BdABK maintained its biological activity as B\textsubscript{1}R agonist \textit{in vivo}. Data obtained on the Hargreaves test revealed that BdABK was only slightly less potent than des-Arg\textsuperscript{8}-BK to cause thermal hyperalgesia upon peripheral administration. This is consistent with the transient thermal hyperalgesia previously reported in the tail-flick test after intrathecal injection of des-Arg\textsuperscript{8}-BK in rats made diabetics with STZ 24 h earlier [9]. Likewise, Gabra and Sirois [24] showed that intraperitoneal administration of des-Arg\textsuperscript{8}-BK (400 \( \mu \)g/kg) in STZ-treated rats significantly reduced the paw withdraw threshold in the hot plate and tail-flick test.

**Localization of B\textsubscript{1}R**

**B\textsubscript{1}R on microglial cells**

Previous work by Noda and coworkers [50,51] showed that B\textsubscript{1}R can be expressed in cultured rat microglia exposed to...
BK. We confirmed this result by using our fluorescent ligand and in the same condition, thus providing additional evidence of its ability to bind B1R in a pure rat microglia model. BK acting via B2 receptors induces elevation of intracellular calcium leading to the phosphorylation and activation of NF-κB by protein kinase C [60]. NF-κB upregulates B1R upon binding to its nuclear promoter [2].

A recent study has demonstrated that B1R is involved in microglial migration toward rat brain lesion sites [61]. The presence of B1R on spinal microglial cells is in keeping with a recent study suggesting that activated dorsal horn microglia is a crucial component of STZ-induced tactile allodynia, mediated in part, by extracellular signal-regulated protein kinase signaling [62]. Importantly, the development of tactile and cold allodynia in a rat model of insulin-resistance was blocked by the B1R antagonist SSR240612 [28] and by two antioxidants (N-acetyl-L-cysteine and alpha-lipoic acid) known to prevent the induction of B1R [7,29]. Taken together, these results suggest a critical role for microglial B1R in generation of tactile allodynia, a manifestation of pain polyneuropathy. It is possible that microglial B1R is also involved in STZ-induced thermal hyperalgesia as this response was abolished by B1R antagonists [5,24,27] and was absent in B1R knockout mice treated with STZ [26].

B1R on astrocytes
In addition, the present study provides the first evidence that thoracic spinal cord astrocytes bear the B1R in STZ-diabetic rats. Astrocyte B1R may represent another target for neuropathic or chronic pain. Emerging evidence suggests a critical role for astrocytes in the passage from acute to chronic and neuropathic pain. It seems that intracellular calcium level oscillation in astrocytes could spread through astrocytal network and thereby facilitate the formation of new synapses. These new synapses could establish neuronal contacts for maintaining and spreading pain sensation [63]. Moreover, astrocytes are known to release various inflammatory mediators that promote neuroimmune activation and can sensitize primary afferent sensory neurons contributing to development of neuropathic pain [64].

B1R on sensory C fibers
Immunohistochemical data showed the presence of B1R in DRG and superficial laminae of spinal cord dorsal horn
Basal B\textsubscript{1}R expression in control rats

Authors failed to observe specific fluorescent labelling for B\textsubscript{1}R in normal rats which is rather consistent with the negligible level of B\textsubscript{1}R mRNA and binding sites. Moreover, intrathecal injection of B\textsubscript{1}R agonists or antagonists failed to cause behavioural, cardiovascular or nociceptive responses in control rats, suggesting that the basal expression of B\textsubscript{1}R is not functional in naive rats [9,53]. Thus the function of the B\textsubscript{1}R detected by immunohistochemistry in the spinal cord of rodents and human remains elusive. It is feasible that B\textsubscript{1}R in control animals is uncoupled to G protein as demonstrated for other G-protein-coupled receptors [68,69]. Although it is possible that the immunological approach is more sensitive, we have evidence (unpublished data) showing that the commercially available B\textsubscript{1}R antibodies (M-19) from SantaCruz Biotechnologies (Santa Cruz, CA, USA) are not specific for immunohistochemical detection since B\textsubscript{1}R labeling persists in spinal cord isolated from B\textsubscript{1}R knockout mice. The latter B\textsubscript{1}R antibodies remain however suitable for Western blot analysis, suggesting that immunohistochemical studies reported with B\textsubscript{1}R antibodies remain to be validated with the appropriate controls in mutant mice.

Conclusion

[\textalpha\textsubscript{N\textalpha\textbeta\textgamma\textdelta\textepsilon\textnomicity}]-des-Arg\textsuperscript{3}-BK was found selective for B\textsubscript{1}R with an IC\textsubscript{50} value of 5.3 ± 0.1 nM in the rat spinal cord. Furthermore, BdABK maintains its biological activity as agonist as evidenced by its ability to induce thermal hyperalgesia in STZ-treated rats. This new fluorescent ligand enabled the detection of B\textsubscript{1}R in primary microglial cell culture and on microglial cells, astrocytes and sensory C fibers in the thoracic spinal cord of STZ-diabetic rats. Because all these cells have been implicated in neuropathic pain, the induction and up-regulation of the B\textsubscript{1}R on these elements consolidate the idea that kinin B\textsubscript{1}R is an important target for drug development in pain processes.

List of abbreviations

B\textsubscript{1}R: kinin B\textsubscript{1} receptor; STZ: streptozotocin; qPCR: quantitative real-time PCR; BK: Bradykinin; BdABK: [\textalpha\textbeta\textgamma\textdelta\textepsilon\textnomicity]-des-Arg\textsuperscript{3}-BK; BSA: bovine serum albumin; anti-IBA-1: anti-ionized calcium binding adapter molecule 1; anti-GFAP: anti-Glial fibrillary acidic protein; anti-CGRP: anti-calcitonin-gene-related peptide; anti-TRPV1: anti-transient receptor potential vanilloid 1.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

ST performed animal treatments, Hargreaves test, real-time PCR analysis, confocal microscopy experiments and draft the manuscript. PTT helped designed the confocal microscopy protocol. DL performed in vitro microglia experiments. JS made cryostat tissue sections and autoradiography experiments. PG synthesized the fluorescent agonist. RC designed the study and revised the manuscript.

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