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RESEARCH PAPER

Bradykinin and B_2 receptor antagonism in rat and human articular chondrocytes

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BACKGROUND AND PURPOSE
In osteoarthritis (OA), bradykinin (BK) is known to contribute to pain and synovitis, but not to cartilage degradation. Here, we investigated effects of BK and its antagonists on chondrocytes, cells involved in cartilage homeostasis.

EXPERIMENTAL APPROACH
BK receptor density and affinities of BK, its analogues and antagonists were measured in cultured human and rat chondrocytes by radioligand binding. Effects of BK were assessed by accumulation of inositol phosphates (IP) and release of interleukin (IL)-6 and IL-8.

KEY RESULTS
Density of [^{3}H]-BK binding sites was higher (13–30-fold) and BK evoked a greater (48-fold) IP production, in human than in rat chondrocytes. The BK B_2 receptor antagonists MEN16132 and icatibant displayed similar binding affinity. MEN16132 was 40-fold more potent than icatibant in the IP assay. In human chondrocytes, BK increased release (over 24 h) of IL-6 and IL-8, effects blocked by MEN16132 but not by the B_1 receptor antagonist Lys-[Leu^8][desArg^9]BK. BK-induced release of IL-6, but not of IL-8, was partially inhibited by indomethacin (10 μM) and nordihydroguaiaretic acid (10 μM). Antagonists for the prostanoid EP receptors (AH6809 10 μM; L-798 196, 200 nM; L-161 982, 1 μM) were ineffective. Dexamethasone (100 nM) partially inhibited release of both IL-6 and IL-8. Inhibitors of intracellular downstream signalling pathways (SB203580 10 μM; PD98059, 30 μM; SP600125, 30 μM; BAY-117085, 5 μM) indicated the involvement of p38 MAPK and the activation of NF-κB.

CONCLUSION AND IMPLICATIONS
BK mediated inflammatory changes and cartilage degradation and B_2 receptor blockade would, therefore, be a potential treatment for OA.

Abbreviations
BK, bradykinin; NDGA, nordihydroguaiaretic acid; COX, cyclooxygenase; LOX, lipoxygenase; PLC, phospholipase C; IL-6, interleukin 6; IL-8, interleukin 8; OA, osteoarthritis; IP, inositol phosphates; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; NSAIDs, nonsteroidal anti-inflammatory drugs

Introduction

Bradykinin (BK, H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH) is a peptide known to have potent pro-inflammatory effects and is one of the most potent endogenous peripheral mediators of pain. BK is enzymically formed from kininogen precursors both in the circulating plasma and interstitial fluids, and exerts its actions by selectively activating the BK B_2

receptor, a seven transmembrane protein receptor (receptor nomenclature follows Alexander *et al.*, 2009). The B_2 receptor couples to Gq/11 proteins, with triggering of phospholipase C (PLC) and consequent intracellular release of inositol 3-phosphate, diacylglycerol and intracellular Ca^{2+}, followed by activation of protein kinase C, phospholipase A_2 (PLA_2), and the corresponding intracellular signalling cascades (Regoli and Barabè, 1980; Leeb-Lundberg *et al.*, 2005).

Osteoarthritis (OA) is a degenerative joint disease that progressively causes loss of joint function and is a major source of physical disability and impaired quality of life in industrialized nations. The pathological changes which occur during OA involve all the joint structures, i.e. synovium, cartilage and bone tissues, but the main hallmark of this disease is the degradation of cartilage (Goldring *et al.*, 2008; Bondeson *et al.*, 2010).

We have recently reviewed the possible involvement of BK in the pathophysiology of knee OA and studies showing the effectiveness of B_2 receptor antagonists in different models of inflammatory articular pain (Meini and Maggi, 2008). The two main actions of BK that may indicate the B_2 receptor blockade as a therapeutically relevant target for local treatment of OA are its algogenic action by activating nociceptors which innervate the capsule and the synovium, and its inflammatory effects, actions that produce the characteristic pain and synovitis in knee OA, respectively (Meini and Maggi, 2008). Moreover, we have recently shown a prolonged release of BK during cartilage degeneration produced by the intra-articular injection of monosodium iodoacetate in the rat knee, and the long-lasting antinociceptive effect produced by B_2 receptor antagonism in this OA model (Cialdai *et al.*, 2009).

One of the main features of OA pathology is the imbalance of metabolic and degradative signals, driven by cytokine cascades, and the production of inflammatory mediators. Chondrocytes are the cells responsible for the homeostasis of the extracellular matrix, but are also capable of inducing increased levels of inflammatory cytokines, which in turn decrease anabolic collagen synthesis and increase the release of catabolic and other inflammatory mediators such as inflammatory cytokines and chemokines, prostanoids, and nitric oxide (Goldring and Berenbaum, 2004; Goldring and Goldring, 2004).

Cytokines and chemokines, such as interleukin (IL)-6 and IL-8, have been described as being involved in OA pathophysiology, as they are present in the knee synovial fluid of OA patients (Kaneko *et al.*, 2000; Bianchi *et al.*, 2007; Požgan *et al.*, 2010), and are released by chondrocytes under inflammatory stimuli (Guerne *et al.*, 1990; Henrotin *et al.*, 1999; Sanchez *et al.*, 2002; Legendre *et al.*, 2005).

Several earlier studies (reviewed in Meini and Maggi, 2008) have shown that BK activates chondrocytes from different species, but the presence of BK B_2 receptors in chondrocytes from any species has not been pharmacologically quantified, nor have the corresponding affinity and potency of selective B_2 receptor antagonists been evaluated. The present study aimed to quantify the pharmacological profile of BK, to unravel new mechanisms related to OA pathology, and to characterize the effects of the nonpeptide B_2 receptor antagonist MEN16132. We have previously shown that MEN16132 is a selective kinin B_2 receptor antagonist, with *in vitro* bioassays in human and animal tissues (Cucchi *et al.*, 2005; Meini *et al.*, 2007; 2009; 2010). This compound has exhibited long-lasting antagonist properties in different *in vivo* preclinical models (Valenti *et al.*, 2005; 2008; Cialdai *et al.*, 2009).

To pharmacologically characterize the BK B_2 receptors present on human and rat chondrocytes, we performed radioligand binding experiments using tritiated BK. MEN16132 potency was compared with that of the well-known reference antagonist icatibant (Hock *et al.*, 1991) using the concentration-dependent accumulation of inositol phosphates (IP), induced by BK, as index of PLC activation. Moreover, the ability of BK to evoke the release of IL-6 and IL-8 after long-term incubation and the antagonist potency of MEN16132 was investigated in human chondrocytes. The B_2 receptor selectivity of BK actions is checked by using a kinin B_1 receptor antagonist in the functional assays. The involvement of the enzymic products of cyclooxygenase (COX) and lipoxygenase (LOX), as well as that of prostanoid receptors, was evaluated by using appropriate inhibitors and antagonists in the BK-induced release of IL-6 and IL-8. Lastly, the effect of the glucocorticoid dexamethasone and the downstream signalling pathways, activated by BK, were investigated exploiting a panel of inhibitors for mitogen-activated protein kinases (MAPK) and the nuclear factor-κB (NF-κB).

Our results indicate that BK B_2 receptors were expressed both in rat and human chondrocytes, and that their agonist and antagonist pharmacology was consistent with that previously observed in other cell or tissue models. Moreover, we present evidence that in human chondrocytes, BK can increase the production of pro-inflammatory cytokines such as IL-6 and IL-8, and thus might potentially contribute to stages in the degradation of cartilage occurring in OA pathology.

Methods

Cell culture

Chondrocytes originating from knee cartilage of Sprague-Dawley rats were purchased as frozen vials (DPK-CACC-R, Lots No. RA04505U2C, RA05406U2C, RA03506U2F) from Dominion Pharmakine (Bilbao, Spain). Cells were handled as recommended by the producer: the seeding density for attachment was 7000 cells cm^{-2} on plastic wells, and the medium was DMEM : F12 (1:1) (Sigma) added with 50 $U \cdot mL^{-1}$ penicillin plus 50 $\mu g \cdot mL^{-1}$ streptomycin (Sigma), and 10% foetal bovine serum (FBS) (Hyclone). When cells reached 90% of confluency, they were split by using trypsin/EDTA solution (Gibco) and seeded in 12-well plates (125 000 cells per well).

Normal human knee chondrocytes were purchased as frozen vials (NHAC-Kn, lot no. 5F1452) from Lonza (Walkersville, MD). Cells were handled as recommended by the producer: the seeding density for attachment was 10 000 cells cm^{-2} on plastic wells, and the culture medium was CGM BulletKit (Lonza). When cells reached 90% of confluency, they were split by using a subculture reagent kit according to the manufacturer's instructions (Lonza) and seeded in 75 cm^2 flasks (10 000–15 000 cells cm^{-2}) for cell culture maintenance, and in 12-well plates or 24-well plates (25 000–35 000 cells cm^{-2}) for binding or functional experiments, respectively.

Both rat and human cells were used between two and five passages, during which their phenotype was maintained. The chondrocyte phenotype was assessed by the round to polygonal cell morphology (Schnabel *et al.*, 2002).

Radioligand binding

Binding assay was performed on adherent cells in the presence of Nutrient mixture F-12 Ham (F12, Sigma-Aldrich) medium containing BSA (0.1% wt·vol^{-1}), NaN_3 (0.1%) to prevent receptor internalization, and captopril (1 μM), bacitracin (140 μg·mL^{-1}) and 1,10-phenanthroline (1 mM) (pH 7.4 at 4°C) to inhibit enzyme activities. In saturation experiments, cells were incubated with different [^{3}H]-BK concentrations (100 pM–30 nM) in the absence (total binding) or presence of unlabelled BK (1 μM) to determine nonspecific binding. Preliminary experiments indicated that no differences in terms of specific binding were obtained by using a higher unlabelled BK concentration (10 μM). Competition experiments were performed with 1 nM of [^{3}H]-BK and varying concentrations of ligands. At this radioligand concentration, the specific binding was 80 ± 6% and 65 ± 4% of the total [^{3}H]-BK binding in human and rat chondrocytes, respectively. Subsequent experimental steps were as follows: the culture medium was aspirated and the binding medium was added to wells (800 μl), then the unlabelled ligand (10x) and the radioligand (10x) were added in sequence. BK, MEN16132 and icatibant were tested in a range of concentrations between 1 or 10 pM and 1 μM. After an incubation of 2 h on ice (4°C), the reaction was terminated by rapid aspiration of the medium and three consecutive washings (3 × 1 mL per well) with ice-cold binding buffer. Cells were lysed with 0.5 mL·well NaOH (0.3 M) for 30 min. The content of each well was transferred in ponyvials and 10 mL of Cytoscint (MP Biomedicals, code 0188245301) was added each vial. Radioactivity was determined by a liquid β-scintillation counter (2200 CA, Packard). In each experimental section, the number of cells was determined in order to normalize the maximum binding site density calculation (B_{max}).

Inositol phosphate (IP) accumulation

Cells at confluence were preincubated with myo-[1,2-^{3}H(N)]inositol (1 μCi·mL^{-1}) for 48 h in Dulbecco's modified Eagle's medium (Sigma-Aldrich) and F12 medium (1:1) supplemented with foetal bovine serum (FBS, 1%), penicillin (50 μg·mL^{-1}), streptomycin (50 μg·mL^{-1}), amphotericin B (15 μg·mL^{-1}) and glutamine (2 mM) (0.5 mL final volume). On the day of the experiment, the medium was removed and the antagonist was added at the indicated concentrations in IP buffer (final volume 0.5 mL; IP buffer composition (mM): PBS Ca^{2+} and Mg^{2+} free (135), Na HEPES (20), $CaCl_22H_2O$ (2), $MgSO_47H_2O$ (1.2), EGTA (1), glucose (11.1), LiCl (25), with BSA (0.05%), captopril (1 μM), thiorphan (10 μM), bacitracin (2 μg·mL^{-1}). Peptidase inhibitors were included in order to prevent BK degradation. Preliminary experiments performed with both MEN16132 and icatibant indicated overlapping results, using an antagonist equilibration time of 15 or 60 min, therefore a 15 min antagonist pre-incubation time was chosen. After the pre-incubation with antagonist, the appropriate concentration of agonist was added and the cells incubated for an additional 60 min at 37°C. Reaction was stopped on ice, IP buffer was removed and cells were lysed with MeOH and HCl (1:1; 1 mL per well, 4°C). Total IP levels were determined as previously described (Bellucci *et al.*, 2004).

Determination of IL-6 and IL-8 contents

Human chondrocytes were incubated for 24 h at 37°C with increasing concentrations of BK (1 nM–10 μM) in F12 medium supplemented with FBS (1%), penicillin (50 μg·mL^{-1}), streptomycin (50 μg·mL^{-1}), amphotericin B (0.75 μg·mL^{-1}), glutamine (2 mM) and captopril (1 μM) (1 mL final volume). Antagonists and inhibitors were preincubated for 30 min, before the addition of BK (100 nM), except for indomethacin, NDGA and dexamethasone which were preincubated for 60 min. Concentrations were selected from our previous experience or from published papers (Clarke *et al.*, 2004; Jeffrey and Aspden, 2007; Molloy *et al.*, 2008; Bellucci *et al.*, 2009; Li *et al.*, 2009). None of the used drugs produced cell morphological changes indicative of cytotoxicity. At the end of agonist incubation, the medium of stimulation was removed and stored at –80°C until assay.

IL-6 and IL-8 contents in the supernatant were assayed by commercially available enzyme immunoassay kits, according to the procedure described by the manufacturer (in parentheses the sensitivity of each test): IL-6 (PK-EL-61606; 1.56–100 pg·mL^{-1}) and IL-8 (PK-EL-61806; 15.6–1000 pg·mL^{-1}) from Promokine (Heidelberg, Germany).

Analysis of data

Each value in the text represents the mean ± SEM or the mean and 95% confidence limits (c.l.), in parentheses, of the reported number of independent experiments (n) that each one performed in triplicate.

Binding data were fitted by the appropriate nonlinear regression using GraphPad Prism 4.0 (San Diego, CA, USA), in order to determine (i) the maximum binding site density (B_{max}) and the equilibrium dissociation constant (K_d) from saturation experiments or homologous inhibition curves; and (ii) the ligand concentration inhibiting the radioligand binding of 50% (IC_{50}) from heterologous inhibition experiments. Inhibitory affinity constant values (K_i) were obtained by normalizing the obtained IC_{50} by the radioligand dissociation constant (K_d) and its concentration ([L*]) according to the Cheng-Prusoff relationship $K_i = IC_{50}/(1 + [L^*]/K_d)$ (Cheng and Prusoff, 1973).

Functional data were fitted by sigmoidal nonlinear regression (GraphPad Prism 4.0) to determine the agonist concentration producing 50% (EC_{50}) of the maximal response (E_{max}) from the concentration–response curves, and the antagonist concentration producing 50% inhibition (IC_{50}) of the control agonist response.

In IP accumulation experiments, the responses to BK either in the absence (control) or presence of antagonist were normalized towards the E_{max} of control BK.

Antagonist potency was calculated as follows. When the antagonist produced parallel shifts of concentration-response curves of the agonist and did not depress the agonist control E_{max} by more than 50%, the concentration-ratio (CR) was calculated from equi-effective concentrations of agonist (EC_{50}) obtained in the presence and in the absence of

antagonist. In this case the estimate of the antagonist potency was calculated from the equation $pA_2 = \log(CR - 1) - \log[B]$ where B is the used antagonist concentration (Kenakin, 2006). Schild regression was constructed by plotting the estimates of log[CR – 1] against log[B] (Arunlakshana and Schild, 1959), to determine the slope of linear regression. When the antagonist depressed the agonist control E_{max} by more or equal to 50%, then the dissociation constant of the antagonist was calculated with the Gaddum double-reciprocal method for the measurement of noncompetitive antagonist affinity (Gaddum *et al.*, 1955). Equiactive agonist concentrations in the absence [A] and in the presence [A′] of antagonist [B] were determined, and their reciprocal values (*x*: 1/A′; *y*: 1/A) were plotted in order to obtain a linear regression. The estimate of the equilibrium dissociation constant of the antagonist-receptor complex (K_B) is given by $K_B = [B]/(\text{slope} - 1)$ (Kenakin, 2006).

Antagonist potency values are given in the text as the mean ± SEM of the data obtained in the single experimental sessions.

Data obtained from IL-6 and IL-8 release experiments are shown as $pg \cdot mL^{-1}$, or normalized towards the BK control response, obtained in untreated cells (in the absence of antagonist or inhibitor) in each experimental session.

Statistical analysis was performed using GraphPad Prism 4.0 and comparisons were made by combining two-way analysis of variance (ANOVA) and Bonferroni's *post hoc* test, as indicated in the text.

Materials

[^{3}H]-BK (specific activity 80 $Ci \cdot mmol^{-1}$) and myo-[1,2-^{3}H(N)]inositol (specific activity 60 $Ci \cdot mmol^{-1}$) were from PerkinElmer (Boston, MA, USA). The kinin B_2 receptor agonist BK, the kinin B_1 receptor agonist Lys-[desArg^9]BK, and the kinin B_1 receptor antagonist Lys-[Leu^8][desArg^9]BK were obtained from PolyPeptide (Strasbourg, France), the neutral endopeptidase inhibitor thiorphan was from Bachem (Essex, UK). The cytokine tumor necrosis factor α (TNFα), the angiotensin converting enzyme inhibitor captopril, the protease inhibitor 1,10-phenanthroline, the aminopeptidase inhibitor bestatin, the nonselective COX inhibitor indomethacin, the synthetic glucocorticoid dexamethasone, and the NF-κB inhibitor BAY-117085 were from Sigma-Aldrich (St. Louis, MO, USA). The nonselective LOX inhibitor nordihydroguaiaretic acid (NDGA) was from Cayman (Ann Arbor, MI, USA). The p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580, the c-Jun N (JNK) terminal MAPK inhibitor SP600125, the ERK 1/2 MAPK inhibitor PD98059, the prostanoid EP_1 and EP_2 receptor antagonist AH6809, the prostanoid EP_3 antagonist L-798,106, and the prostanoid EP_4 receptor antagonist L-161,982 were from Tocris Bioscience (Bristol, UK). All salts used were purchased from Merck (Darmstadt, Germany). Kinin B_2 receptor antagonists were synthesized at Menarini Ricerche (Chemistry Departments of Florence and Pomezia, Italy). Icatibant (Hock *et al.*, 1991; DArg[Hyp^3,Thi^5,DTic^7,Oic^8]BK) (batches 254-21, 30061-3 and 30058-2) and MEN16132 [4-(S)-Amino- 5-(4-{4- [2,4-dichloro- 3-(2,4-dimethyl-8-quinolyloxymethyl) phenylsulphonamido]-tetrahydro- 2*H*-4- pyranylcarbonyl}piperazino)-5-oxopentyl](trimethyl)ammonium chloride hydro chloride) (batches 3, 6 and 7) were dissolved in distilled water at 10 mM and 1 mM concentrations (stock solutions) and stored at –25°C. All other compounds were dissolved at 10 mM concentration (stock solutions) in water or DMSO as indicated by data sheets and stored at –25°C. Further working dilutions were made in the appropriate buffer.

Results

Affinity of bradykinin, MEN16132 and icatibant in radioligand binding experiments in rat or human chondrocytes

Homologous displacement curves performed on adherent rat chondrocytes revealed a low level of [^{3}H]-BK binding sites, with a calculated maximum receptor density (B_{max}) of 2800 ± 1100 sites per cell (n = 3). The affinity constant (K_d) of BK deriving from these experiments was 1.13 nM (0.21–5.96, 95% c.l., n = 4). Both MEN16132 and icatibant fully inhibited the [^{3}H]-BK specific binding in a concentration-dependent manner. The inhibitory affinity constant (K_i) values were 0.65 nM (0.47–0.90, 95% c.l., n = 3) for MEN16132 and 4.60 nM (2.81–7.52, 95% c.l., n = 3) for icatibant (Figure 1A).

Saturation experiments were carried out in human chondrocytes with [^{3}H]-BK (100 pM–30 nM): the calculated K_d value was 3.10 nM (1.68–7.51, 95% c.l., n = 3), and the B_{max}

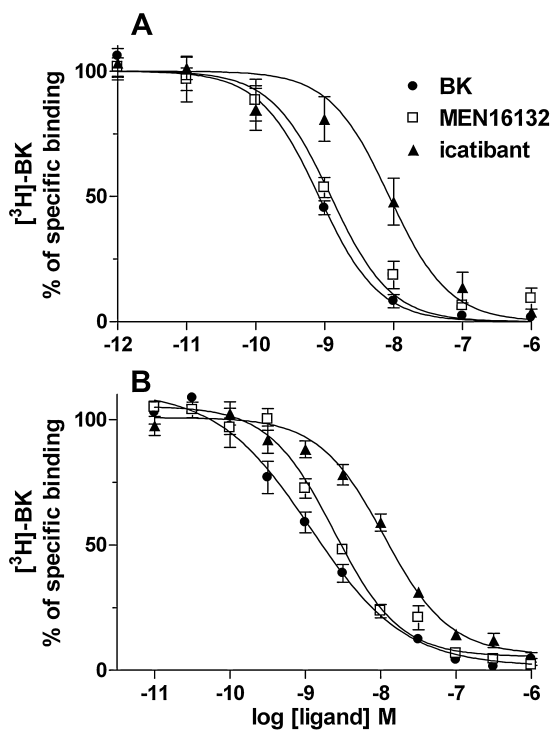

Figure 1

BK, MEN16132 and icatibant inhibition curves of [^{3}H]-BK specific binding to rat (A) and human (B) chondrocytes. Cells were incubated for 2 h at 4°C with [^{3}H]-BK (1 nM) and varying concentrations of competing ligands as described in Methods. Data are expressed as mean ± SEM of three independent experiments, each one performed in triplicate.

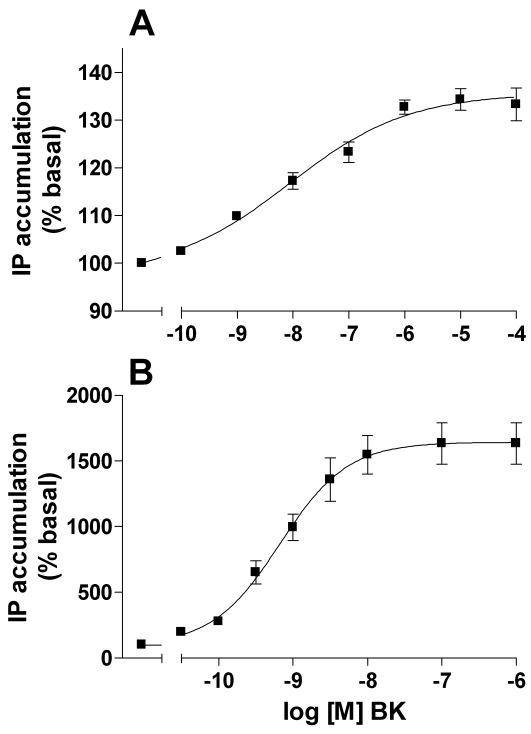

Figure 2

Concentration-dependent increase in IP accumulation by BK in rat (A) and human (B) chondrocytes. BK was incubated for 60 min at the indicated concentrations. Data are expressed as percentage of basal IP accumulation. Data points are the mean ± SEM from 4–6 independent experiments, each one performed in triplicate.

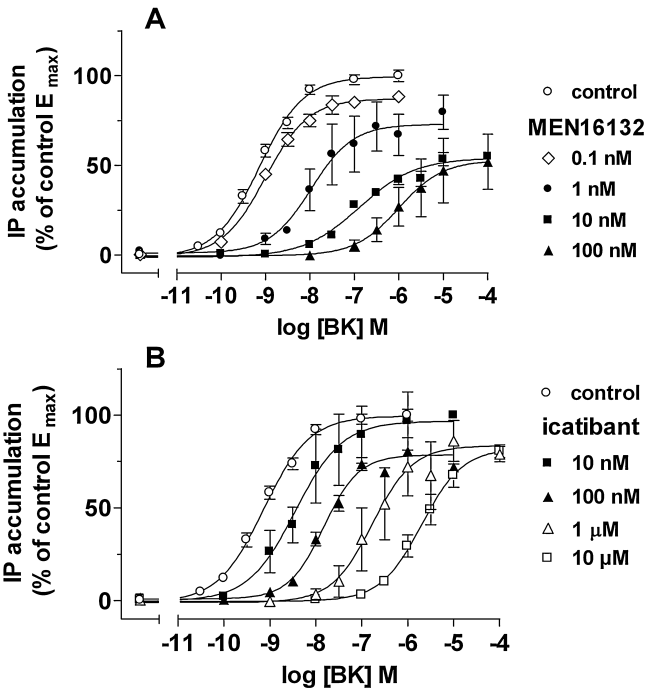

Figure 3

Antagonism by MEN16132 (A) or icatibant (B) of BK-induced activation of IP production in human chondrocytes. Antagonists were added at the indicated concentrations 15 min before the incubation with agonist (60 min). Data are expressed as percentage of BK-induced maximal response (E_{max}) obtained in the absence of antagonists. Data points are the mean ± SEM from 4–5 independent experiments, each one performed in triplicate.

was significantly greater than that measured in the rat chondrocytes being 84 880 ± 5380 sites per cell (n = 3, Figure S1). To have a direct comparison with data obtained with rat chondrocytes, homologous inhibition curves of BK were analyzed also in human chondrocytes, and indicated K_d and B_{max} values of 0.34 nM (0.10–1.11, 95% c.l.) and 36 704 ± 3573 sites per cell, respectively.

In parallel experiments, the affinity of MEN16132 and icatibant were evaluated through inhibition curves at the [^{3}H]-BK binding sites (Figure 1B). The two antagonists concentration-dependently (10 pM–1 µM) inhibited all the radioligand specific binding and K_i values deriving from one-site competition model were of 1.62 nM (1.27–2.08, 95% c.l.) for MEN16132 and 7.48 nM (5.54–10.09, 95% c.l.) for icatibant.

BK activation of phospholipase C (IP accumulation assay) in rat and human chondrocytes

Cell activation by BK in rat and human chondrocytes was evaluated by the IP accumulation assay and results were consistent with the significantly different number of BK binding sites.

In rat chondrocytes, BK at 10 µM concentration induced a 0.35-fold increase of basal IP accumulation (Figure 2A). This effect was concentration-dependent, and the response curve was quite shallow (Hill slope 0.46, 0.18–0.74, 95% c.l., n = 4). The EC_{50} value deriving from the fit of data was 11.3 nM (2.7–46.8, 95% c.l., n = 4). On the contrary, the BK-induced maximal response in human chondrocytes was much more consistent: the E_{max} was 17-fold over the basal at 100 nM BK concentration (Figure 2B). The EC_{50} value obtained from concentration-response curves to BK was 0.73 nM (0.57–0.93, 95% c.l., n = 5), and the slope of the nonlinear regression close to unity (Hill slope 0.87, 0.71–1.03, 95% c.l., n = 6).

Comparison of antagonist potency of MEN16132 and icatibant in the BK-induced IP accumulation assay in human chondrocytes

The antagonist potency of the two kinin B_2 receptor antagonist MEN16132 and icatibant was measured towards the BK-induced concentration-response curve in the IP accumulation assay performed with human chondrocytes (Figure 3). Both antagonists, incubated 15 min before the agonist, did not modify the basal IP accumulation at any of the tested concentrations (data not shown).

Icatibant (10 nM to 10 µM) induced a rightward shift of the BK curve without depressing the obtainable E_{max} as compared with the controls. Data were analyzed with the Schild regression that indicated a competitive antagonism (slope value not statistically different from unity: 1.091,

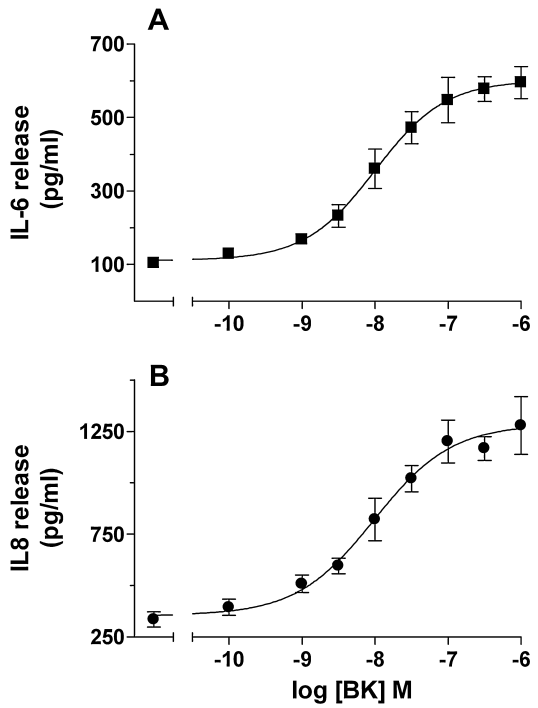

Figure 4

BK-induced release of IL-6 (A) and IL-8 (B) in human chondrocytes. Cells were incubated for 24 h with BK (0.1 nM–1 μM). Data are expressed as the mean ± SEM of four independent experiments, each one performed in triplicate.

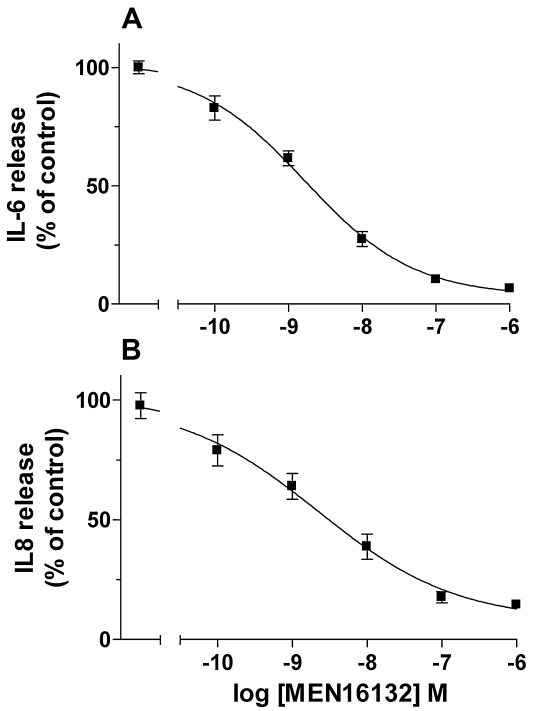

Figure 5

Inhibitory effect of MEN16132 on BK-induced IL-6 (A) or IL-8 (B) release. Human chondrocytes were preincubated for 30 min with the indicated concentrations of MEN16132 before BK (100 nM, 24 h) stimulation. Data are expressed as percentages of control response to BK (in the absence of antagonist), and represent the mean ± SEM of five independent experiments, each one performed in triplicate.

0.845–1.336, 95% c.l.). The apparent antagonist potency of icatibant calculated as pK_B from single experiments was 8.55 ± 0.13 (n = 16), overlapping to that deriving from the Schild regression (8.39, x intercept when y = 0).

A different pattern was observed for MEN16132 (1 nM–1 μM) which displayed insurmountable antagonist behaviour, as it depressed the BK E_{max}, concentration-dependently. The apparent antagonist potency was measured from single experiments, using different calculation methods dependently on the depression exerted (see Analysis of data in Methods). In 5 out of 15 experiments, MEN16132 inhibited the BK E_{max} by 34–50%. The mean of antagonist potency values (as pK_B) from single experiments was 10.16 ± 0.13 (n = 16).

Long-term incubation of human chondrocytes with BK increases the release of IL-6 and IL-8 which is blocked by the nonpeptide B_2 receptor antagonist MEN16132.

Preliminary time course experiments with human chondrocytes were conducted to confirm the reproducibility of IL-6 and IL-8 release produced by BK (1 μM), and the time of 24 h was selected for the subsequent studies. Cells were incubated with different concentrations of BK (0.1 nM–1 μM) that stimulated the release of IL-6 in a concentration-dependent manner (Figure 4A). The EC_{50} value calculated from the obtained curves was 10.0 nM (5.4–18.6, 95% c.l., n = 4). In the same supernatants, IL-8 was also measured and the corresponding EC_{50} value was 9.7 nM (3.7–25.2, 95% c.l., n = 4) (Figure 4B).

Both the IL-6 and IL-8 release stimulated by BK were inhibited by the nonpeptide B_2 receptor antagonist. As shown in Figure 5, MEN16132 (0.1 nM–1 μM) prevented, in a concentration-dependent manner, the release of cytokines induced by BK (100 nM). The calculated IC_{50} values were 1.68 nM (1.00–2.82, 95% c.l., n = 5, Figure 5A) for inhibition of the BK-induced release of IL-6 and 2.21 nM (0.68–7.13, 95% c.l., n = 5, Figure 5B) for the inhibition of IL-8 release.

Similar cytokine release was induced by 0.1 ng·mL^{-1} of the cytokine TNFα under the same experimental conditions: the stimulated IL-6 release was 814 ± 350 pg·mL^{-1}, and that of IL-8 was 1778 ± 198 pg·mL^{-1} (n = 3). In a different set of experiments, MEN16132 (1 μM) did not block cytokine release induced by TNFα. For IL-6, the release of IL-6 was 445 ± 51 and 549 ± 104% of basal with or without MEN 161632. The corresponding values for IL-8 were 1160 ± 153 and 1305 ± 207% of basal release, respectively (n = 3).

The kinin B_1 receptor antagonist Lys-[Leu^8][desArg^9]BK does not affect BK mediated effects

In order to evaluate the contribution of the kinin B_1 receptor in the effects produced by BK, the effects of a kinin B_1 receptor antagonist were assessed in the IP accumulation assay.

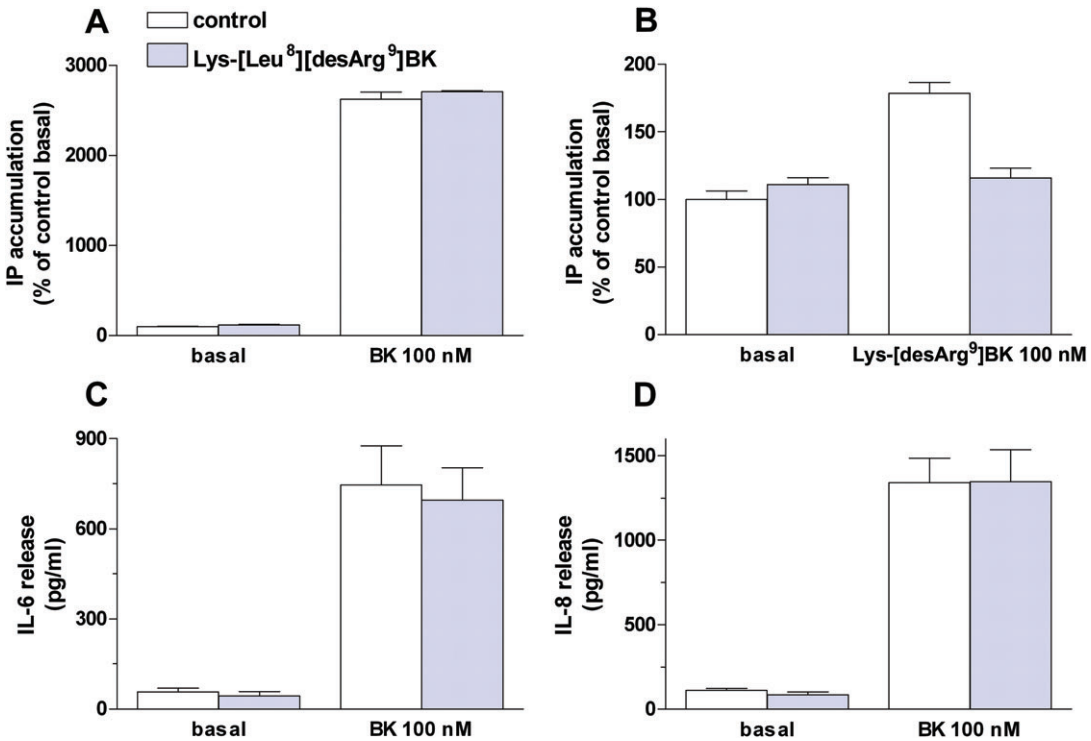

Figure 6

Lack of effect of the kinin B_1 receptor antagonist Lys-[Leu^8][desArg^9]BK on BK-induced effects. Human chondrocytes were preincubated for 15 min with Lys-[Leu^8][desArg^9]BK (10 μM) and then cells were exposed for 60 min to BK (A) or to the kinin B_1 agonist Lys-[desArg^9]BK (B) in the IP accumulation assay, or for 24 h for IL-6 (C) and IL-8 (D) determination. Data are expressed as percentage of control basal (A and B) or in pg·mL^{-1} of released IL-6 (C) or IL-8 (D), and represent the mean ± SEM of three independent experiments, each one performed in triplicate.

Lys-[Leu^8][desArg^9]BK at a concentration of 10 μM did not alter the IP accumulation induced by BK (100 nM) (Figure 6A) whereas it blocked the effect produced by the kinin B_1 receptor agonist Lys-[desArg^9]BK used as positive control (Figure 6B).

At the same concentration, Lys-[Leu^8][desArg^9]BK did not affect the production of IL-6 or IL-8 induced by 24 h incubation of human chondrocytes with BK (Figure 6C and D).

Different effects exerted by a series of inhibitors to explore the mechanisms involved in the IL-6 and IL-8 release induced by BK in human chondrocytes

Human chondrocytes were exposed to pretreatment with the nonselective COX inhibitor indomethacin (10 μM), the nonselective LOX inhibitor NDGA (10 μM), or the glucocorticoid dexamethasone (100 nM) before BK stimulation (100 nM, 24 h). Data were analyzed to evaluate the statistical significance of treatments on both stimulated (BK) and unstimulated (basal) chondrocytes, and data were also compared with the release from cells washout any inhibitor treatments (control). All the treatments significantly reduced the BK-induced release of IL-6 (Figure 7A), whereas no significant differences could be detected through *post hoc* comparisons among the basal production of IL-6 after the different treatments. On the other hand, both the COX and LOX inhibitors and dexamethasone treatments did not completely prevent the stimulatory effect of BK, although the BK-induced increase of IL-6 after treatment with indomethacin and dexamethasone, but not after NDGA, was not statistically significant (Figure 7A). Despite these results, the two-way ANOVA statistical analysis also indicated that indeed indomethacin and dexamethasone treatments reduced both the basal and BK-stimulated release (F interaction = 6.960, d.f. 1.35, $P < 0.05$ for indomethacin group; F interaction = 12.30, 1.38 d.f., $P < 0.01$ for dexamethasone group); whereas after NDGA treatment, there was no significant interaction (F interaction = 3.919, d.f. 1.37, $P = 0.0552$). When measuring the IL-8 content in the same samples, no significant effect was produced by indomethacin or NDGA treatment (Figure 7B); whereas a slight decrease was observed in chondrocytes pretreated with dexamethasone, as the output produced by the BK stimulation did not reach statistical significance.

The possibility that prostanoids could contribute to the BK-induced release was explored in a further set of experiments in which the effect of antagonists with selectivity towards the different subtypes of EP receptors was evaluated. Neither the prostanoid EP_1 and EP_2 receptor antagonist AH6809 (10 μM), the prostanoid EP_3 antagonist L798,106 (200 nM), nor the prostanoid EP_4 receptor antagonist L-161,982 (1 μM) modified the output of IL-6 or IL-8 induced by BK (Table 1).

Last, a panel of inhibitors towards different intracellular signalling mechanisms were used to identify the pathways involved in BK-induced IL-6 and IL-8 release by human

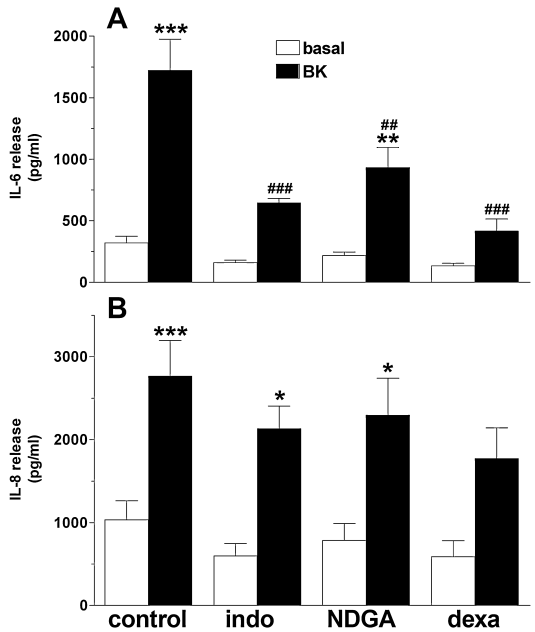

Figure 7

Effect of indomethacin, NDGA, and dexamethasone on BK-induced IL-6 (A) or IL-8 (B) release. Human chondrocytes were preincubated for 60 min with indomethacin (indo, 10 μM), NDGA (10 μM), or dexamethasone (dexa, 100 nM) before BK (100 nM, 24 h) stimulation. Data are expressed in pg·mL^{-1}, and represent the mean ± SEM of three to five independent experiments, each one performed in triplicate. *P < 0.05; **P < 0.01, ***P < 0.001 versus the respective basal condition; ##P < 0.01; ###P < 0.001 versus the corresponding control response; two-way ANOVA followed by Bonferroni's *post hoc* test.

chondrocytes. As shown in Table 1, the inhibitor for p38 MAPK SB203580 (10 μM) significantly inhibited BK-induced IL-6 release by 58% and the IL-8 release by 48%. The ERK 1/2 inhibitor PD98059 (30 μM) significantly inhibited the IL-8 release by 31%, whereas the JNK inhibitor SP600125 (30 μM) did not exert any inhibition. On the contrary, the NF-κB inhibitor BAY-117085 (5 μM) completely prevented the BK-induced release of IL-6 and IL-8.

Discussion and conclusions

The present study describes the pharmacology of BK and B_2 receptors in rat and human chondrocytes. It also highlights the potential role of this inflammatory peptide in the processes of cartilage degradation, which characterize the pathology of OA. Moreover, the nonpeptide B_2 receptor antagonist MEN16132 was shown to be fairly potent in inhibiting BK-induced effects after short- or long-term stimulation of human chondrocytes. The ability of this selective B_2 receptor antagonist to block BK effects, together with the lack of antagonism shown by Lys-[Leu^8][desArg^9]BK, which is selective for the human kinin B_1 receptor (Leeb-Lundberg *et al.*, 2005), confirmed the sole participation of B_2 receptors.

As mentioned in the Introduction, earlier studies have shown that BK activated chondrocytes from different species,

Table 1

Effect of prostanoid EP antagonists and different inhibitors on the IL-6 and IL-8 release induced by BK in human chondrocytes

	Production induced by BK 100 nM (% of control)	
	IL-6 release	IL-8 release
Control	100 ± 6	100 ± 4
AH6809 10 μM	99 ± 11	75 ± 13
L-798,106 200 nM	101 ± 16	71 ± 11
L-161,982 1 μM	102 ± 7	88 ± 14
SB203580 10 μM	42 ± 8*	52 ± 6*
PD98059 30 μM	76 ± 7	69 ± 8*
SP600125 30 μM	135 ± 29	102 ± 10
BAY-117085 5 μM	2 ± 1*	0.6 ± 0.3*

Cells were preincubated with each inhibitor or antagonist at the indicated concentration for 30 min and then exposed to BK (100 nM, 24 h). The contents of IL-6 and IL-8 were measured in the supernatant as described in Methods.
Data are expressed as the mean ± SEM of three independent experiments, each one performed in duplicate. *P < 0.01: statistical analysis (two-way ANOVA followed by Bonferroni *post hoc* test) was performed on the raw data and compared with control conditions. Since no significant differences were observed among basal values in each treatment, data were normalized according to the mean output of IL-6 or IL-8 produced by BK in control conditions in each experimental section.

but none had pharmacologically quantified the presence of BK B_2 receptors. From radioligand binding data, we showed that the numbers of [^{3}H]BK binding sites differed markedly between rat (B_{max} 2800 sites per cell) and human chondrocytes, even though, in human cells, the number depended on the experimental protocol used (saturation or homologous displacement, B_{max} 84 880 and 36 704 sites per cell respectively). Saturation studies could not be performed in rat chondrocytes because of the higher nonspecific binding with increasing concentrations of radioligand. However, the affinity of the B_2 receptor antagonists MEN16132 and icatibant derived from the present experiments was comparable to that found in other rat and human radioligand binding assays (Meini *et al.*, 2009). The difference in B_2 receptor density between rat and human chondrocytes correlated with the efficacy of BK in activating B_2 receptor-mediated PLC signalling, as indicated by the difference in E_{max} obtained in the IP accumulation assay carried out in rat (0.35-fold of basal) and human (17-fold of basal) cells. In spite of this good correlation, it is worth pointing out that data obtained with rat chondrocytes are more representative of the species as they originate from pooled cartilage tissues of several animals whereas the human chondrocytes are from a single donor. It is also relevant to point out that, although BK and B_2 receptors have been shown to contribute to the nociceptive effect which follows the intra-articular injection of monosodium iodoacetate into the rat knee (Cialdai *et al.*, 2009), the relatively low levels of B_2 receptors on rat chondrocytes would make it less possible to demonstrate the participation of BK in

the process of cartilage degradation, which characterizes this preclinical OA model.

The potency values of BK measured in this study (IP assay, EC_{50} values 0.73 and 11.3 nM in human and rat chondrocytes, respectively) are roughly in the same range of those previously obtained in guinea pig (EC_{50} 4.2 nM, Baragi *et al.*, 1989) and pig (EC_{50} 40 nM, Benton *et al.*, 1989) chondrocytes maintained in culture. In addition, using the IP accumulation assay, we characterized the two B_2 receptor antagonists by evaluating the concentration-dependency of their antagonism and the nature of their antagonism towards BK. Characterization experiments were conducted in the human chondrocytes only because of the poor and erratic response obtained in the rat cells. The profile of B_2 receptor antagonists in chondrocytes has not been reported thus far. Our data indicate the sub-nanomolar antagonist potency of the non-peptide antagonist MEN16132 (pK_B 10.16), although some 40-fold higher than that of the peptide icatibant (pK_B 8.55), was very comparable to that obtained from other cell models expressing the native (human fibroblast-like synoviocytes, MEN16132 pK_B 10.0, icatibant pK_B 8.1, Bellucci *et al.*, 2009) or the recombinant human B_2 receptor (CHO cells, MEN16132 pK_B 10.3, Cucchi *et al.*, 2005; icatibant pK_B 8.4, Meini *et al.*, 2004). Contrary to earlier results, in which both icatibant and MEN16132 showed a competitive antagonistic profile (Meini *et al.*, 2004; Cucchi *et al.*, 2005; Bellucci *et al.*, 2009), in the present cell model, the profiles differed. Icatibant was a competitive antagonist (slope of Schild analysis not different from unity) whereas MEN16132 was an insurmountable antagonist. Indeed, the concomitant displacement of the agonist concentration-response curve and depression of the agonist E_{max} by MEN16132, and the concentration-dependency of this effect, suggest an insurmountable orthosteric antagonism, which has been attributed to equilibrium conditions not being achieved in the assay (Kenakin, 2006). This last hypothesis can be ruled out by the fact that a prolonged time of preincubation with MEN16132 yielded similar results, whichever antagonist concentration was used. We do not have at present a reasonable explanation for the diverging behaviour of MEN16132 in this cell assay, in spite of the affinity and potency values consistent with those previously measured in different models.

As recently reported, BK is known to induce both IL-6 and IL-8 release in different human cell types, and also in fibroblast-like synoviocytes (Lee *et al.*, 2008; Bellucci *et al.*, 2009). In the present study, we show for the first time that, after a long-term incubation with human chondrocytes, BK increased, in a concentration-dependent manner and with comparable potency values, the release of IL-6 and IL-8. The amount of released cytokines by BK seems to be comparable to that produced by the pleiotropic cytokine TNFα at a concentration (0.1 ng·mL^{-1}) previously detected in the OA synovial fluid (0.08–0.17 ng·mL^{-1}, Kahle *et al.*, 1992), and occurring in a BK concentration range similar to that detected in the synovial fluid of OA patients (i.e. 98–427 ng·mL^{-1}, which corresponds to 1–4 × 10^{-7} M concentration) (Bond *et al.*, 1997). The negatively charged nature of the glycosaminoglycan components of the extracellular matrix and the increased tissue permeability in degenerating cartilagineous tissue (Jackson and Gu, 2009) can facilitate the diffusion of a small positively charged peptide like BK, and hint at the possibility that it could readily access the milieu of the chondrocytes, which is relatively avascular. Thus, the B_2 receptor-dependent augmented production of IL-6 and IL-8 from articular chondrocytes opens a novel scenario for BK and its participation in OA pathology. IL-6 has been correlated with the severity of knee damage, radiographically measured (Livshits *et al.*, 2009) and, apart from participating in the sensitization of afferent fibers (Brenn *et al.*, 2007), this cytokine can increase the expression of metalloproteinases and aggrecanases involved in the processes of cartilage degradation (Legendre *et al.*, 2005). On the other side, IL-8 has been shown to have a modulatory action on chondrocytes and to favour the production of IL-6 (Henrotin *et al.*, 1996), as well as being involved in bone resorption and osteoclastogenesis (Bendre *et al.*, 2003). Furthermore, in the present study, we show also that the treatment of articular chondrocytes with the B_2 receptor antagonist MEN16132 completely prevents the BK-induced release of both IL-6 and IL-8, with a potency similar to that evaluated in other assays (Bellucci *et al.*, 2009).

In the last part of this study, the mechanism(s) involved in the BK-induced release of IL-6 and IL-8 were investigated. By using inhibitors of COX (indomethacin) or LOX (NDGA), the BK-induced release of IL-6, but not that of IL-8, was partly reduced, suggesting that products of these enzymic pathways were involved in the production of IL-6 but not of IL-8. These data are in line with previously reported evidence both *in vitro* (human chondrocytes) and *in vivo* (cytokines content in synovial fluid from OA patients) reporting that COX inhibitors (nonsteroidal anti-inflammatory drugs, NSAIDs) differently regulate the release of these two inflammatory mediators, and indicating a contribution of prostanoids and/or eicosanoids, only in the production of IL-6 (Clausen *et al.*, 1996; Henrotin *et al.*, 1999; Bianchi *et al.*, 2007). On the other hand, as different prostanoid EP receptors have been described as being involved in the catabolic functions of chondrocytes (Alvarez-Soria *et al.*, 2007; Li *et al.*, 2009; Wang *et al.*, 2010), we tested a range of EP receptor antagonists (AH6809, L-798,106 and L-161,982). Here, our data suggested that the release of IL-6 and IL-8 induced by BK did not involve activation of EP_1, EP_2, EP_3, or EP_4 receptors. One possible explanation of our results is that other prostanoids, such as thromboxanes or prostacyclins, that do not act through EP receptors, are the COX products involved in IL-6 release by BK.

The downstream signalling activated by BK and responsible for the augmented production of IL-6 and IL-8 was explored, and data indicated that BK stimulated p38 MAPK and NF-κB activation. NF-κB is a transcription factor controlling the expression of several genes associated with inflammation and previous reports have shown its activation by BK (Pan *et al.*, 1996; Xie *et al.*, 2000). In the present context, the inhibitory activity of dexamethasone on the BK-induced release of IL-6 and, to a lesser extent that of IL-8, could be attributed to its inhibition of *de novo* protein synthesis (Miyazawa *et al.*, 1998).

Oral NSAIDs and intra-articular corticosteroids are currently used for the therapeutic and symptomatic treatment of OA, but these agents are associated with well documented undesirable side-effects, and the possible combination of these drugs with other treatments possessing differing side-effect profiles and mechanisms of action is considered to

likely reduce safety and tolerability risks in patients (Altman, 2009). In this context and based on the emerging role of BK and B_2 receptors in OA mechanisms, low MW molecules endowed with high antagonist potency and long-lasting activity towards such effects of BK may represent potential candidates for this type of combined, alternative OA treatments.

Overall, the present study represents the first quantitative characterization of BK and B_2 receptor antagonism in rat and human articular chondrocytes. It has also elucidated novel long-term pro-inflammatory effects produced by BK, which are likely to participate in joint inflammatory and degenerative diseases such as OA, and has shown MEN16132 to be a high affinity antagonist capable of preventing such effects.

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Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Saturation binding of [^{3}H]BK to human chondrocytes maintained in culture. In the *y*-axis, the specific binding is represented as calculated sites/cells, and in the *x*-axis, there are the used concentrations of [^{3}H]BK which were incubated with cells at 4°C temperature for 120 min. Nonspecific binding was determined with unlabelled BK(1 μM). Points are the mean ± SEM of one representative experiment performed in triplicate. The calculated K_d value was 3.10 nM (1.68–7.51, 95% c.l., $n = 3$), and the B_{max} was 84 880 ± 5380 sites per cell ($n = 3$).

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