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RESEARCH PAPER

Characterization of kinin receptors in human cultured detrusor smooth muscle cells

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Background and purpose: Kinins have an important role in inflammatory cystitis and in animal pathophysiological models, by acting on epithelium, fibroblasts, sensory innervation and smooth muscle. The aim of this study was to characterize the receptors responsible for direct motor responses induced by kinins on human detrusor.
Experimental approach: Human detrusor cells from biopsies were isolated and mantained in culture. B_1 and B_2 kinin receptors were characterized by means of radioligand and functional experiments (PI accumulation and PGE_2 release).
Key results: [^{3}H]-[desArg^9]-Lys-BK and [^{3}H]-BK saturation studies indicated receptor density (B_{max}) and K_d values of 19 or 113 fmol mg^{-1}, and 0.16 or 0.11 nM for the B_1 or B_2 receptors, respectively. Inhibition binding studies indicated the selectivity of the B_1 receptor antagonist [desArg^{9}Leu^8]-Lys-BK and of the B_2 receptor antagonists Icatibant and MEN16132. [DesArg^9]-Lys-BK and BK induced PI accumulation with an EC_{50} of 1.6 and 1.4 nM and different maximal responses (E_{max} of [desArg^9]-Lys-BK was 10% of BK). BK also induced prostaglandin E_2 release (EC_{50} 2.3 nM), whereas no response was detected with the B_1 receptor agonist. The incubation of detrusor smooth muscle cells with interleukin 1β (IL-1β) or tumour necrosis factor-α (TNF-α) (10 ng ml^{-1}) induced a time-dependent increase in radioligand-specific binding, which was greater for the B_1 than for the B_2 receptor.
Conclusions and Implications: Human detrusor smooth muscle cells in culture retain kinin receptors, and represent a suitable model to investigate the mechanisms and changes that occur under chronic inflammatory conditions.
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Keywords: human urinary bladder; PLC activation; upregulation; interleukin-1β; tumour necrosis factor-α; icatibant; MEN16132

Abbreviations: BK, bradykinin; B_1R, B_1 receptor; B_2R, B_2 receptor; PI, inositolphosphate; PGE_2, prostaglandin E_2; IL-1β, interleukin-1β; TNF-α, tumour necrosis factor-α

Introduction

Kinins are potent mediators of inflammation, causing pain, vasodilatation, increased vascular permeability, fluid secretion from epithelia and smooth muscle contraction. These actions are mediated by two receptor subtypes B_1 and B_2 (Regoli and Barabè, 1980): bradykinin (BK, H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH) is a selective agonist for the B_2 receptor, whereas the B_1 receptor exhibits greater selectivity for BK metabolites lacking the C-terminal Arg. The B_1 receptors are generally poorly expressed in normal physiological conditions, but rapidly induced *in vivo* under stress conditions (Marceau *et al*., 1998) and *in vitro* in some cell types, including fibroblasts and smooth muscle cells, after exposure to noxious stimuli such as interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) (Galizzi *et al*., 1994; Phagoo *et al*., 2000). The B_2 receptors are constitutively expressed in relatively high numbers in many tissues and cultured cells, and this expression may be upregulated by cytokines, as IL-1β and TNF-α (Schmidlin *et al*., 1998; Haddad *et al*., 2000; Newton *et al*., 2002).

In anaesthetized rats, BK evokes the micturiction reflex by activating capsaicin-sensitive sensory neurons (Lecci *et al*., 1995), and participates in the genesis of detrusor overactivity in the cyclophosphamide-induced cystitis (Maggi *et al*., 1993). In the rat isolated urinary bladder, prostanoids seem to be largely responsible for BK-induced contractions (Marceau *et al*., 1980; Pinna *et al*., 1992; Meini *et al*., 1998). On the other hand, after induction of experimental cystitis, B_1 receptors have also been reported to be involved in the contraction of rat bladder both *in vivo* and *in vitro* (Marceau *et al*., 1980; Meini *et al*., 1998; Belichard *et al*., 1999; Lecci *et al*., 1999).

Both kinin receptors have been demonstrated to produce detrusor smooth muscle contraction in various species (Maggi, 1997; Andersson and Wein, 2004), and B_1 and B_2

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receptor-mediated inositolphosphate (PI) turnover and prostanoids generation have been shown in the rabbit and rat detrusor smooth muscle, respectively (Nakahata *et al*., 1987; Butt *et al*., 1995; Meini *et al*., 1998). In the human isolated urinary bladder smooth muscle preparations indomethacin-sensitive contractile responses produced by exogenously administered BK have been shown to undergo upregulation following incubation *in vitro* (Andersson *et al*., 1992; Sjuve *et al*., 2000).

Smooth muscle cells from human bladder have been previously used to verify the effect of different culture conditions on cell growth (Baskin *et al*., 1993; Lai *et al*., 2002) or to characterize muscarinic or histamine receptors (Boselli *et al*., 2002; Neuhaus *et al*., 2006). The aim of the present study was to evaluate the pharmacological and coupling characteristics of kinin receptors in human detrusor smooth muscle cells, and to study the effect of long-term culture conditions or of proinflammatory cytokines (IL-1β and TNF-α) on the expression of kinin receptors, revealed through radioligand binding of agonists. The characterization of the receptors was carried out on membranes by means of saturation and inhibition experiments with B_1 and B_2 tritiated agonist radioligands. The responses of B_1 and B_2 receptor agonists ([desArg^9]Lys-BK and BK, respectively) in inducingPI accumulation and prostaglandin E_2 (PGE_2) production were investigated. Selectivity was checked by means of the B_1 receptor selective antagonist, [des Arg^{9}Leu^8]Lys-BK (Couture *et al*., 1981), or the B_2 receptor selective antagonists, icatibant (H-DArg-Arg-Pro-Hyp-Gly-Thi-Ser-Dtic-Oic-Arg-OH; Hock *et al*., 1991) 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 hydrochloride; Cucchi *et al*., 2005).

Methods

Isolation and culture of human detrusor smooth muscle cells

All procedures and protocols were approved by the local ethical committee. Specimens from the dome of human urinary bladder were obtained from five patients (males, mean age 60 ± 7 years) by full thickness biopsy. All the patients underwent radical cystectomy for muscle invasive transitional cell carcinoma and the samples were obtained 5 cm from the tumour. Haematoxylin–eosin staining confirmed that all the specimens considered normal showed no dysplasia or tumour cells. The smooth muscle tissues, dissected from the mucosa, were maintained in gassed (95% O_2, 5% CO_2) Krebs solution (NaCl 119 mM; $NaHCO_3$ 25 mM; KH_2PO_4 1.2 mM; $MgSO_4$ 1.5 mM; $CaCl_2$ 2.5 mM; KCl 4.7 mM and glucose 11 mM). Twelve detrusor muscle strips (0.5–0.8 mm wide and 10–15 mm long) from each specimen were washed five times in wash medium (minimum essential Eagle's medium α-modification, α-MEM, Sigma cat. no M4526) containing penicillin (100 μg ml^{-1}) streptomycin (100 μg ml^{-1}), and fungizone (2.5 μg ml^{-1}). Then tissue pieces were digested by incubation at 37°C in 10 ml of wash medium added with collagenase type II (0.15% wt vol^{-1}), pronase (0.015% wt vol^{-1}), soybean trypsin inhibitor type II (0.01% wt vol^{-1}), bovine serum albumin (BSA, 0.1% wt vol^{-1}) and gentamycin (4 μg ml^{-1}). After 20 min the supernatant was transferred, through filtration on Nytex (BDH, nylon mesh 250 μm), in 5 ml of foetal bovine serum (FBS, Hyclone, Logan, UT, USA) and the remaining tissue was digested twice as above. The obtained cell suspensions were pooled and centrifuged (5 min, 300 *g*) at room temperature. The pellet was resuspended in culture medium (wash medium added with non-essential aminoacids (NEAA, 1%), endothelial cell growth supplement (ECGS, 30 μg ml^{-1}), glutamine 1% and FBS 20%), and plated onto four tissue culture flasks (75 cm^2). The culture medium was changed after 2 days and, in these conditions, cells grew elongated and spindle-shaped to confluence as a monolayer in 7 days. Cells were subcultured up to 10th passage by incubation with 0.05% trypsin– 0.5 mM ethylenediaminetetraacetic acid at a ratio of 1:3, twice weekly. All patients yielded specimens that resulted in a separate cell line. Indirect immunocytochemistry detection confirmed that all primary cultures were positive to α-actin (Gown *et al*., 1985). Briefly, the cells were seeded in 24-well plates where they were fixed with phosphate-buffered formalin 10%. The cells were then incubated for 1 h at 37°C with fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal anti-α smooth muscle actin. Excess antibody was removed by gently rinsing for 3-times with phosphate-buffered saline (PBS) and the fluorescence was read under an ultraviolet fluorescent microscope. The α-actin content was not passage-dependent but remained constant along subculture passages.

For treatments of the cells with IL-1β and TNF-α, cells at confluence in 6- or 12-well plates were made quiescent by incubation with culture medium containing FBS 1% (the minimum percentage of serum necessary to maintain cells attached and viable in our culture conditions) for 24 h and then incubated at 37°C for different periods of time (3, 6 or 24 h) in the absence (control) or presence of IL-1β (10 ng ml^{-1}) or TNF-α (10 ng ml^{-1}).

Radioligand binding experiments with whole cells

Binding assays were performed in 6- or 12-well plates in α-MEM medium containing BSA (0.1 wt vol^{-1}) and captopril (1 μM). Cells were incubated with [^{3}H][desArg^9]Lys-BK or [^{3}H]BK (1.5 nM concentration) in the presence (nonspecific binding) or absence (total binding) of 10 μM appropriate unlabelled ligand in a total volume of 0.5 ml. After 60 min at 4°C, the incubation was terminated by rapid aspiration of medium, then cells were washed with ice-cold *N*-tris [hydroxymethyl]methyl-2-amino ethanesulphonic acid (TES 10 mM, pH 7.4) containing BSA (0.1 wt vol^{-1}) (3×0.5 ml), and dissolved with 0.5 ml/well NaOH (0.3 M). The cell lysed was transferred in a scintillation vial, added with scintillation fluid (Cytoscint ES, MP Biomedicals, Solon, OH, USA) (10 ml vial^{-1}), and the radioactivity was determined by β-scintillation counter (2200 CA, Packard Instrument Company). Each experiment was performed in triplicate.

Radioligand-binding studies with membranes

Cell membranes were prepared as previously described (Cucchi *et al*., 2005) from cells at confluence. The buffer

used was TES (10 mM, pH 7.4) containing 1,10-phenanthroline (1 mM), bacitracin (140 μg ml^{-1}) and BSA (0.1 wt vol^{-1}). Experiments were performed at room temperature in a final volume of 0.5 ml, and an incubation time of 60 min was chosen from preliminary time course experiments. Preliminary experiments showed that the radioligand-specific binding was directly proportional to membrane protein concentration (data not shown), and the concentration of 150 or 250 μg ml^{-1} was chosen for B_1 or B_2 receptor-binding assay, respectively. In the inhibition studies, the chosen [^{3}H][desArg^9]Lys-BK or [^{3}H]BK concentration was comparable to the calculated K_d value (0.10–0.15 nM), and the bound was less than 10% of the total added radioligand concentration. Nonspecific binding was defined as the amount of radiolabelled ligand bound in the presence of 1 μM of the appropriate unlabelled ligand. Competing ligands were tested in a wide range of concentrations (1 pM–10 μM). All incubations were terminated by rapid filtration through UniFilter-96 plates (Perkin Elmer, Shelton, CT, USA), pre-soaked for at least 2 h in polyethylenimine 0.6%, and using a MicroMate 96 Cell Harvester (Perkin Elmer). The tubes and filters were then washed five times with 0.5 ml aliquots of tris(hydroxymethyl)aminomethane (TRIS buffer 50 mM, pH 7.4, 4°C). Filters were dried, soaked in Microscint 40 (50 μl/well, Perkin Elmer), and bound radioactivity was counted by a TopCount Microplate Scintillation Counter (Perkin Elmer). Each experiment was performed in duplicate.

Measurement of PI levels

Cells were grown in 24-well tissue culture plates and labelled for 24 h with *myo*-[1,2-^{3}H]inositol (0.5 ml, 1 μCi ml^{-1}) in α-MEM containing 1% characterized FBS and L-glutamine (2 mM). The cells were incubated at 37°C for 15 min in the stimulation buffer (PBS Ca^{2+} Mg^{2+} free 135 mM, HEPES 20 mM, $CaCl_2$ 2 mM, $MgSO_4$ 1.2 mM, ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid 1 mM, glucose 11.1 mM, captopril 10 μM, BSA 0.05%) added with LiCl (25 mM) to attenuate inositolphosphate metabolism. When used, antagonists were added at this stage. Agonists were added for 30 min at 37°C. This time was selected by appropriate time course experiments. The reaction was stopped by addition of 1 ml of an ice-cold mixture of methanol and HCl 0.1 N (2:1, v/v), and samples were applied to Bio-Rad Laboratories (Hercules, CA, USA), AG1X8 columns. Total PI levels were determined as previously described (Cucchi *et al*., 2005).

Measurement of prostaglandin E_2 (PGE_2) release

Cells were grown at confluence in 24-well tissue culture plates in α-MEM containing 1% FBS and L-glutamine (2 mM). Incubation with the agonists was performed with the above stimulation buffer at 37°C for 10 min. This time was selected by appropriate time course experiments. The reaction was stopped on ice, the medium was removed and stored at –20°C until assayed. The amount of PGE_2 present in the supernatants was determined by a commercially available enzyme immunoassay (EIA, Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions.

Materials

[^{3}H]BK (specific activity 90 Ci mmol^{-1}), [^{3}H][desArg^9]Lys-BK (specific activity 80 Ci mmol^{-1}), and *myo*-[1,2-^{3}H]inositol (specific activity 74.7 Ci mmol^{-1}) were provided by Perkin Elmer New England Nuclear (Boston, MA, USA). BK, [desArg^9]Lys-BK, [desArg^{9}Leu^8]Lys-BK were obtained from Neosystem (Strasbourg, France). All salts used were purchased from Merck. FITC-conjugated mouse monoclonal anti-α smooth muscle actin, IL-1β and TNF-α were obtained from Sigma (St Louis, MI, USA). Icatibant and MEN16132 were synthesized in Menarini Ricerche (Florence, Italy). MEN16132 was dissolved in dimethilsulphoxide up to 100 μM. All compounds were stored at −25°C.

Analysis of data

Independent experiments were performed with each cell line. After appropriate analysis between passages 2 and 10, to determine that binding and functional responses were constant, results were pooled. Each value in the text or figures is mean ± s.e.m. or the mean and 95% confidence limits (CI, in parentheses) of the given number of experiments (*n*). All data were analysed by means of Prism 4 (GraphPad, San Diego, CA, USA).

Radioligand binding data were analysed to determine the maximum binding site density (B_{max}), and the radioligand affinity constant (K_d) from saturation experiments, and the ligand concentration inhibiting the radioligand binding by the 50% half-maximal inhibitory concentration (IC_{50}) from competition experiments. The −log of K_i (pK_i) values were calculated from IC_{50} using the Cheng-Prusoff equation ($K_i = IC_{50}/(1 + [\text{radioligand}/K_d])$) according to the concentration and K_d of the used radioligand.

In functional experiments, the concentration producing the 50% (EC_{50}) of the agonist maximal response (E_{max}) was calculated by sigmoidal nonlinear regression of the concentration–response curves. The PI production and the PGE_2 release were expressed as percentage over the basal output.

The effect produced by the incubation of cells with cytokines was expressed as percentage of the time-matched controls in each experimental session.

Multiple comparisons were performed by one-way analysis of variance (ANOVA) and individual differences tested by Dunnett's test after the demonstration of significant intergroup differences by ANOVA.

Results

Pharmacological characterization of kinin B_1 and B_2 receptors through radioligand binding experiments

The pharmacological characterization of radioligand binding was performed with membrane preparations. Analysis of saturation data obtained with the selective B_1 and B_2 receptor agonist radioligands, [^{3}H][desArg^9]Lys-BK and [^{3}H]BK, respectively, indicated the presence of a single class

of high-affinity binding sites for both receptors (Figure 1a and b). The K_d and B_{max} values obtained were 0.16 nM (95% CI 0.10–0.21) and 19 fmol mg^{-1} of proteins (95% CI 17–21) for [^{3}H][desArg^9]Lys-BK and 0.11 nM (95% CI 0.07–0.15) and 113 fmol mg^{-1} of proteins (95% CI 104–122) for [^{3}H]BK.

The [^{3}H][desArg^9]Lys-BK binding was inhibited with high affinity by the peptide B_1 receptor agonist and antagonist, [desArg^9]Lys-BK and [desArg^{9}Leu^8]Lys-BK, pK_i 10.2 (95% CI 10.1–10.3) and pK_i 10.0 (95% CI 9.9–10.1), respectively. The peptide B_2 receptor antagonist icatibant showed a lower affinity, pK_i 6.7 (95% CI 6.5–6.8) for the B_1 receptor agonist radioligand, whereas BK and the non-peptide B_2 receptor antagonist MEN16132 at 10 μM produced only a partial inhibition (Figure 2a).

The [^{3}H]BK binding was inhibited with high affinity by unlabelled BK, pK_i 9.8 (95% CI 9.7–9.8), and with even higher affinity by icatibant, pK_i 10.5 (95% CI 10.5–10.6), or MEN16132, pK_i 10.6 (95% CI 10.5–10.7), whereas both [desArg^9]Lys-BK and [desArg^{9}Leu^8]Lys-BK were ineffective up to 1 μM (Figure 2b).

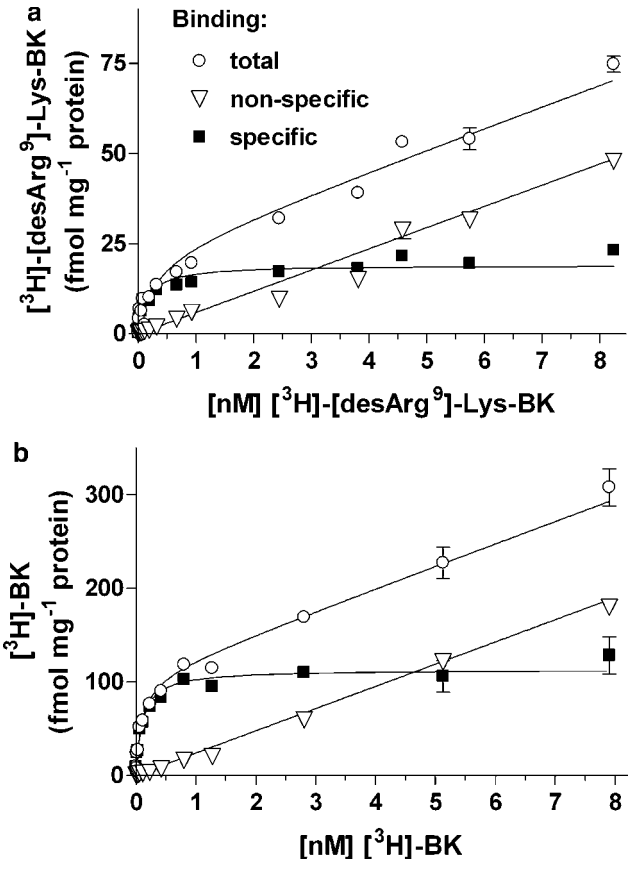

Figure 1 Saturation binding of [^{3}H][desArg^9]Lys-BK (**a**) and [^{3}H]BK (**b**) to human detrusor smooth muscle membranes. Total, non-specific and specific binding is represented. Increasing concentrations of [^{3}H][desArg^9]Lys-BK or [^{3}H]BK were incubated with membranes at room temperature for 60 min. Nonspecific binding was determined with the appropriate unlabelled ligand (1 μM). Data are expressed as mean ± s.e.m. (vertical lines), and are representative of five independent experiments from different cell preparations.

B_1 and B_2 receptor activated signalling

BK (0.1 nM–1 μM) induced concentration-dependent PI formation with an EC_{50} value of 1.4 nM (95% CI 0.7–2.9). The BK produced E_{max} was 20 ± 5-fold over the basal response (Figure 3). The B_1 receptor agonist [desArg^9]Lys-BK (0.1 nM–10 μM) produced concentration-dependent PI accumulation with an EC_{50} value of 1.6 nM (95% CI 0.4–6.6), and E_{max} of 2.0 ± 0.5-fold over the basal levels (Figure 3, inset panel). The E_{max} (1 μM) obtained with B_1 and B_2 receptor agonists was similar for the different subcultures (Figure 4). The effect of a submaximal concentration (10 nM) of both agonists in the absence and in the presence of selective antagonists was also tested as shown in Figure 5. None of the antagonists studied modified the basal response at the indicated concentrations. Icatibant prevented the BK (94 ± 0.3% of inhibition) but not [desArg^9]Lys-BK induced PI accumulation at 100 nM or 1 μM, respectively. In contrast, the B_1 receptor antagonist [des Arg^{9}Leu^8]Lys-BK was ineffective in blocking the response

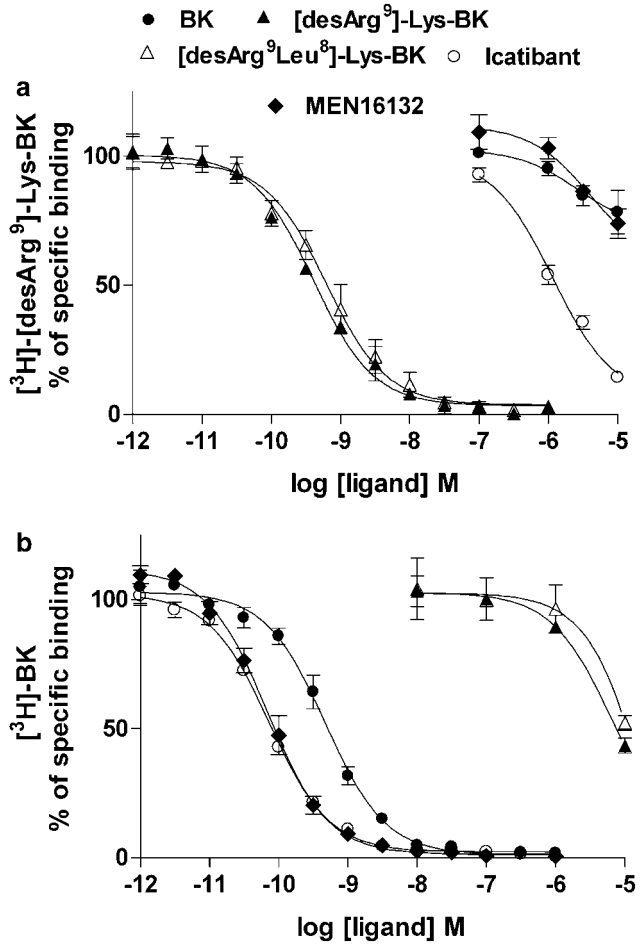

Figure 2 Inhibition curves for a number of ligands for [^{3}H][des Arg^9]Lys-BK (**a**) and [^{3}H]BK (**b**) specific binding to human detrusor smooth muscle membranes. Membranes were incubated at room temperature for 60 min with [^{3}H][desArg^9]Lys-BK or [^{3}H]BK, at a concentration comparable with their K_d value, and varying concentrations of competing ligands as described in Methods. Data points represent the mean ± s.e.m. (vertical lines) of five independent experiments from different patients, each one performed in duplicate.

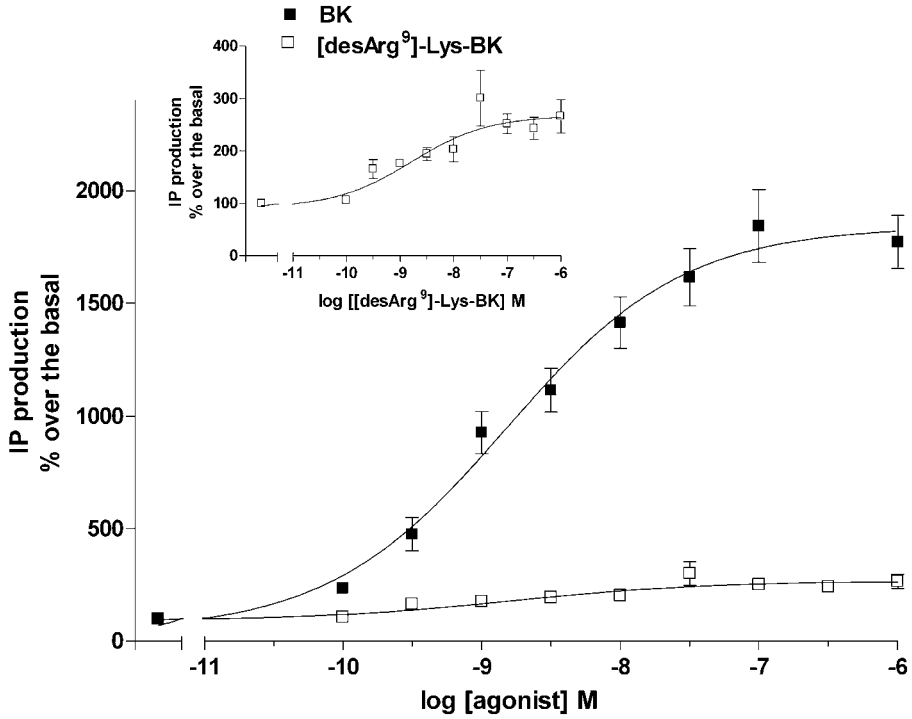

Figure 3 Concentration–response curve to [desArg^9]Lys-BK and BK in the PI production assay performed in human cultured smooth muscle cells. The inset shows the effects of B_1 stimulation on an expanded scale. Cells were incubated for 30 min at 37°C with the indicated agonist concentrations. Results are expressed as percentage of the basal output measured in the absence of agonist. Values are the means ± s.e.m. (vertical lines) of at least five independent experiments from different patients, each one performed in duplicate.

evoked by BK, whereas it inhibited the [desArg^9]Lys-BK-induced PI production by 85 ± 1%. Results obtained with the non-peptide antagonist MEN16132 were comparable to those obtained with icatibant (Figure 5): at 10 nM MEN16132 inhibited the BK response by 96 ± 2%, whereas at 1 μM it was devoid of any antagonist effect towards the [desArg^9]Lys-BK-induced response.

BK (0.1 nM–1 μM) produced a concentration-dependent PGE_2 release with an EC_{50} value of 2.3 nM (95% CI 1.1–4.8), and the E_{max} was increased 20 ± 1-fold over the basal (Figure 6). In contrast, [desArg^9]Lys-BK was not able to induce a detectable PGE_2 release up to 1 μM (data not shown).

Modulation of B_1 and B_2 receptor radioligand binding by IL-1β and TNF-α

The specific binding of the two B_1 and B_2 receptor agonist radioligands on human whole detrusor smooth muscle cells was measured in each culture from passage 2 up to passage 10. At equal cell densities, the specific binding of [^{3}H][des Arg^9]Lys-BK and [^{3}H]BK did not significantly change during subculturing (Figure 7), being 375 ± 31 and 6820 ± 169 sites/cell, respectively ($n = 12$ each).

The effect of the treatment with IL-1β and TNF-α was examined. Exposure of the cells to IL-1β (10 ng ml^{-1}) or TNF-α (10 ng ml^{-1}) time-dependently (3, 6, 24 h, Figure 8a) augmented the specific binding of [^{3}H][desArg^9]Lys-BK, the maximum increase being produced after 6 h of incubation (4.5 ± 0.3 and 3.4 ± 0.3-fold higher than the control level, respectively). Both cytokines promoted an increase even of [^{3}H]BK specific binding which, after a 6-h period of exposure, was 1.6 ± 0.1 and 1.4 ± 0.1-fold over the time-matched control, respectively (Figure 8b).

Discussion

The aim of the present study was to characterize the pharmacological and coupling characteristics of kinin receptors in human detrusor smooth muscle cells in culture, thus ruling out interference from stimulation of kinin receptors present on sensory nerves, fibroblasts or epithelium (Maggi *et al*., 1989; Chopra *et al*., 2005). Contrary to what has been observed for muscarinic (Boselli *et al*., 2002) or tachykinin receptors (unpublished observations), the present data indicate that isolated smooth muscle cells from human detrusor specimens express both B_1 and B_2 kinin receptors which are retained despite subculture conditions. Radioligand binding experiments (saturation curves obtained with tritiated agonists) with membranes prepared from cultured cells, indicate a receptor density comparable to that measured in other human cell types, such as fibroblasts (WI38, Meini *et al*., 1999; HLF-1, Cucchi *et al*., 2005), or smooth muscle cells (Schmidlin *et al*., 1998). The pharmacological profile obtained with the binding inhibition experiments overlaps that previously obtained for both native and recombinant human kinin receptors, thus supporting the selectivity of the antagonists used: that of [desArg^{9}Leu^8]Lys-BK for the B_1 receptor (Couture *et al*., 1981), and that of the peptide icatibant and the non-peptide

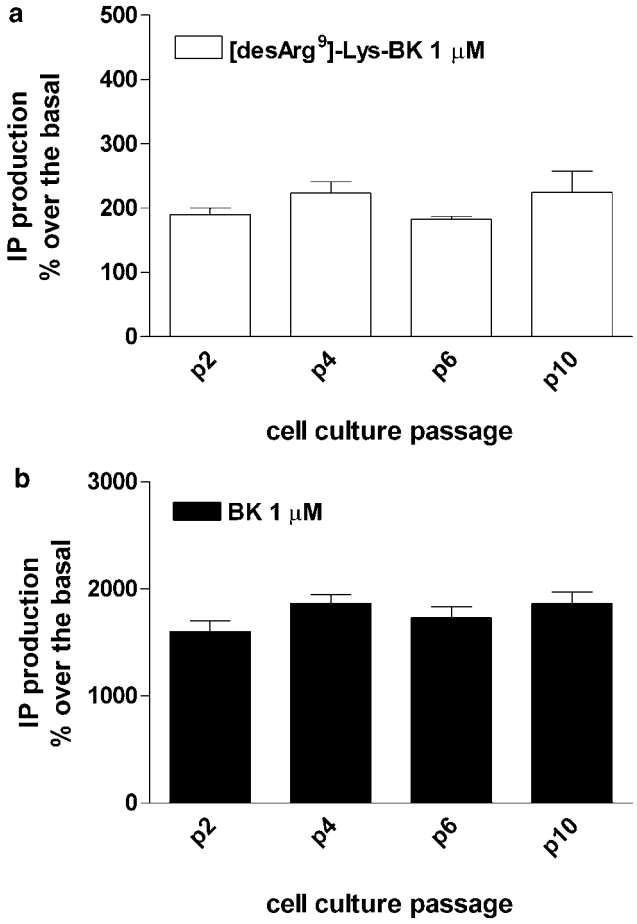

Figure 4 Effect of increased culture passage number of human detrusor smooth muscle cells on [desArg^9]Lys-BK (**a**) and BK (**b**) induced PI production. Cells were stimulated with a maximal concentration (1 μM) of B_1 or B_2 receptor agonist for 30 min at 37°C. Results are expressed as a percentage of the basal output measured in the absence of agonist. Values are the means ± s.e.m. (vertical lines) of at least five independent experiments from different patients, each one performed in duplicate.

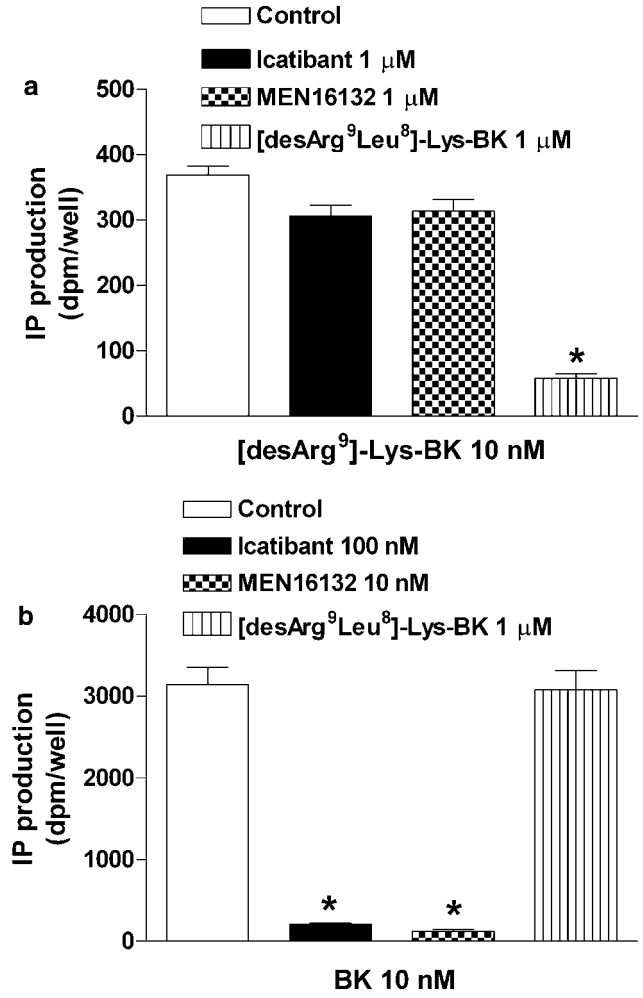

Figure 5 Icatibant, MEN16132 and [desArg^{9}Leu^8]Lys-BK selectively inhibited the production of PI stimulated by [desArg^9]Lys-BK (**a**) or BK (**b**), respectively. [desArg^9]Lys-BK or BK were added at a final concentration of 10 nM. Antagonists were added 15 min before agonist incubation. Data points are the mean ± s.e.m. (vertical lines) of at least four experiments from different patients.*$P<0.05$ versus the control.

MEN16132 for the B_2 receptor (Bastian *et al*., 2000; Cucchi *et al*., 2005). Conversely, the selectivity of the agonists in the functional experiments was proved by selective block of their responses with the antagonists (Figure 5). We had previously defined the potency of icatibant and MEN16132 in blocking the responses produced by BK in contractility studies of the isolated human detrusor smooth muscle strips (icatibant pK_B 8.4, Meini *et al*., 2000; MEN16132 pK_B 9.9, Cucchi *et al*., 2005), and the values obtained were in agreement with those found at the recombinant human B_2 receptor in antagonizing the BK-induced PI accumulation (icatibant pA_2 8.5, Bellucci *et al*., 2004; MEN16132, pK_B 10.3, Cucchi *et al*., 2005).

As stated in the Introduction, in the isolated smooth muscle preparation only indirect evidence (i.e. inhibition by indomethacin) has indicated the participation of prostanoids as a mechanism responsible for BK-induced contractions (Andersson *et al*., 1992; Sjuve *et al*., 2000). The data presented confirm that BK induces release of PGE_2, which can in turn induce contraction as we previously showed in the rat isolated urinary bladder (Meini *et al*., 1998), but also phospholipase C (PLC) activation. Whether or not the activation of PLC by BK in the present cell model, and the consequent intracellular rise in Ca^{2+}, is responsible for phospholipase A_2 (PLA_2) phosphorylation, and thus prostanoid generation, has not been determined at present (Leeb-Lundberg *et al*., 2005; Andersson and Wein, 2004).

An enhancement of the contractile response produced by BK occurs in isolated human detrusor smooth muscle strips has also been shown to occur following *in vitro* incubation (Sjuve *et al*., 2000). By measuring the binding sites with selective agonist radioligands and functional responses in terms of PLC activation, in our experiments with isolated smooth muscle cells, we found that the expression and consequently the coupling of both B_1 and B_2 receptors do not change over the subculture passages. Taken together these results (Sjuve *et al*., 2000; present study) suggest that other factors produced by connective tissue, that is, fibroblasts present along the smooth muscle tissue, can

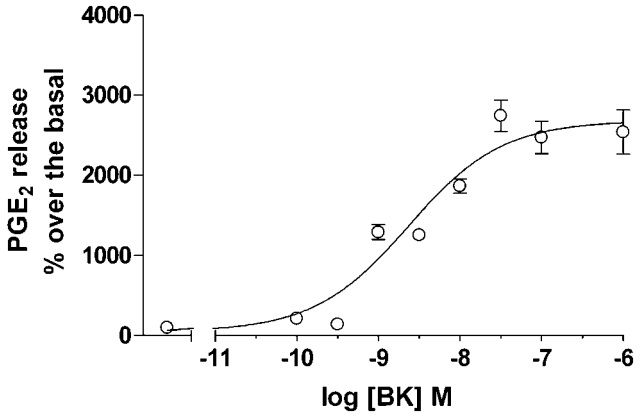

Figure 6 BK induces a concentration-dependent increase in PGE_2 production in detrusor smooth muscle cells. Cell were incubated for 10 min at 37°C with the indicated agonist concentrations. The results are expressed as percentage of the basal output measured in the absence of agonist. Data points represent the mean ± s.e.m. (vertical lines) of three independent experiments from different patients, each one performed in duplicate.

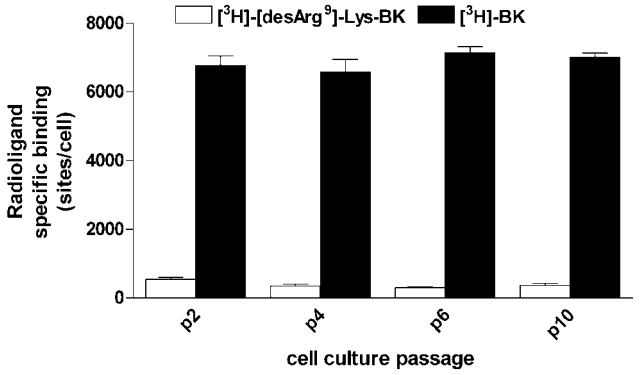

Figure 7 [^{3}H][desArg^9]Lys-BK and [^{3}H]BK specific binding in human detrusor smooth muscle cells during the different subculture periods. Cells were incubated with [^{3}H][desArg^9]Lys-BK or [^{3}H]BK (1.5 nM concentration, respectively) for 60 min at 4°C. Nonspecific binding was determined with the appropriate unlabelled ligand (10 μM). Data represent the mean ± s.e.m. (vertical lines) of five independent experiments performed on different cell preparations from different patients.

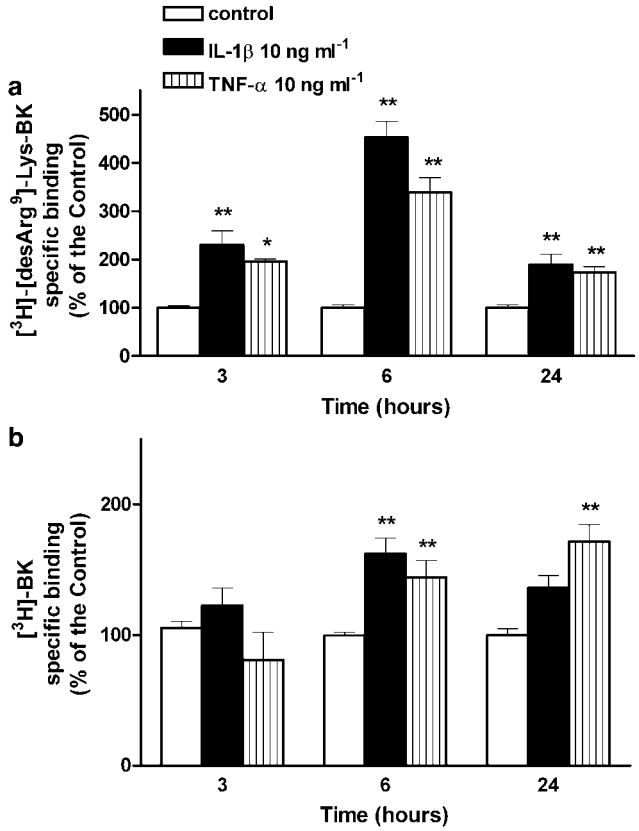

Figure 8 Effect of time-dependent stimulation of IL-1β or TNF-α on B_1 or B_2 receptor binding in detrusor smooth muscle cells. Cell were treated with IL-1β (10 ng ml^{-1}) or TNF-α (10 ng ml^{-1}) for various times as indicated and then assayed for [^{3}H][desArg^9]Lys-BK (**a**) or [^{3}H]BK (**b**) binding. The results are expressed as percentage of control where 100% refers to the response to α-MEM treatment alone. Data points represent the mean ± s.e.m. (vertical lines) of three independent experiments from different patients, each one performed in duplicate. *$P<0.05$; **$P<0.01$ versus the time-matched control.

release factors that, in turn, are responsible for the increased expression of kinin receptors (Phagoo *et al*., 1999; Sabourin *et al*., 2001). In fact, the incubation of smooth muscle cells with the proinflammatory cytokines IL-1β or TNF-α, exogenouosly administered, induces a time-dependent upregulation of B_1 receptor expression and, to a lesser extent, of B_2 receptors. The time course of this upregulation is consistent with that previously reported in other human cell types, such as fibroblasts or bronchial smooth muscle cells (Schmidlin *et al*., 1998; Haddad *et al*., 2000). On the other hand, in the present cell model we failed (data not shown) to produce any autologous upregulation with BK, as it has been previously documented for IMR-90 fibroblasts (Phagoo *et al*., 1999), thus reflecting the inability of smooth muscle cells to release cytokines following BK stimulation, in agreement with previous suggestions in other smooth muscle cell systems (Sabourin *et al*., 2001).

In summary, the present results are the first to provide evidence indicating that human detrusor smooth muscle cells in culture express and retain kinin B_1 and B_2 receptors. Throughout the subculture, the expression and consequently the coupling (PLC activation and prostanoids generation) of the B_2 receptor largely predominates over the B_1 receptor. On the other hand, the heterologous receptor upregulation induced by long-term incubation with IL-1β and TNF-α affects the B_2 much more the B_1 receptor.

Overall, the present findings indicate that the human detrusor smooth muscle cells are a suitable model for determining the interactions that occur between kinin receptors and the mechanisms activated when they are stimulated, and, also, the changes that are likely to occur following subchronic inflammation.

Conflict of interest

No financial links including consultancies with manufacturers of material or devices described in the paper as well as

links to the pharmaceutical industry or regulatory agencies or any other potential conflicts of interest.

References

Andersson KE, Hedlund H, Stahl M (1992). Contractions induced by angiotensin I, angiotensin II and bradykinin in isolated smooth muscle from human detrusor. *Acta Physiol Scand* **145**: 253–259.

Andersson KE, Wein AJ (2004). Pharmacology of the lower urinary tract: basis for current and future treatments of urinary incontinence. *Pharmacol Rev* **56**: 581–631.

Baskin LS, Howard PS, Duckett JW, Snyder HM, Macarak EJ (1993). Bladder smooth muscle cells in culture: I. identification and characterization. *J Urol* **149**: 190–197.

Bastian S, Pruneau D, Loillier B, Robert C, Bonnafous JC, Paquet JL (2000). Identification of a key region of kinin B(1) receptor for high affinity binding of peptide antagonists. *J Biol Chem* **275**: 6107–6113.

Belichard P, Luccarini JM, Defrene E, Faye P, Franck RM, Duclos H *et al*. (1999). Pharmacological and molecular evidence for kinin B1 receptor expression in urinary bladder of cyclophosphamide-treated rats. *Br J Pharmacol* **128**: 213–219.

Bellucci F, Meini S, Cucchi P, Catalani C, Giuliani S, Zappitelli S *et al*. (2004). The N-terminal of Icatibant and bradykinin interact with the same Asp residues in the human B_2 receptor. *Eur J Pharmacol* **491**: 121–125.

Boselli C, Govoni S, Vicini D, Lanni C, Racchi M, D'Agostino G (2002). Presence and passage dependent loss of biochemical M3 muscarinic receptor function in human detrusor cultured smooth muscle cells. *J Urol* **168**: 2672–2676.

Butt SK, Dawson LG, Hall JM (1995). Bradykinin B_1 receptors in the rabbit urinary bladder: induction of responses, smooth muscle contraction, and phosphatidylinositol hydrolysis. *Br J Pharmacol* **114**: 612–617.

Chopra B, Barrick SR, Meyers S, Beckel JM, Zeidel ML, Ford AP *et al*. (2005). Expression and function of bradykinin B_1 and B_2 receptors in normal and inflamed rat urinary bladder urothelium. *J Physiol* **562**: 859–871.

Couture R, Mizrahi J, Regoli D, Devroede G (1981). Peptides and the human colon: an *in vitro* pharmacological study. *Can J Physiol Pharmacol* **59**: 957–964.

Cucchi P, Meini S, Bressan A, Catalani C, Bellucci F, Santicioli P *et al* (2005). MEN16132 a novel potent and selective non-peptide antagonist for the human bradykinin B_2 receptor. *In vitro* pharmacology and molecular characterization. *Eur J Pharmacol* **528**: 7–16.

Galizzi JP, Bodinier MC, Chapelain B, Ly SM, Coussy L, Giraud S *et al*. (1994). Up-regulation of [^{3}H]-desArg^{10}-kallidin binding to the bradykinin B_1 receptor by interleukin-1 beta in isolated smooth muscle cells: correlation with B_1 agonist-induced PGI_2 production. *Br J Pharmacol* **113**: 389–394.

Gown AM, Vogel AM, Gordon D, Lu PL (1985). A smooth muscle-specific monoclonal antibody recognizes smooth muscle actin isozymes. *J Cell Biol* **100**: 807–813.

Haddad E-B, Fox AJ, Rousell J, Burgess G, Mcintyre P, Barnes PJ *et al* (2000). Post-transcriptional regulation of Bradykinin B_1 and B_2 receptor gene expression in human lung fibroblasts by tumor necrosis factor-α: modulation by dexamethasone. *Mol Pharmacol* **57**: 1123–1131.

Hock FJ, Wirth K, Albus U, Linz W, Gerhards HJ, Wiemer G *et al* (1991). Hoe140 a new potent and long acting bradykinin antagonist: *in vitro* studies. *Br J Pharmacol* **102**: 769–773.

Lai JY, Yoon CY, Yoo JJ, Wulf T, Atala A (2002). Phenotypic and functional characterization of *in vivo* tissue engineered smooth muscle from normal and pathological bladders. *J Urol* **168**: 1853–1858.

Lecci A, Giuliani S, Meini S, Maggi CA (1995). Pharmacological analysis of the local and reflex responses to bradykinin on rat urinary bladder motility *in vivo*. *Br J Pharmacol* **114**: 708–714.

Lecci A, Meini S, Patacchini R, Tramontana M, Giuliani S, Criscuoli M *et al* (1999). Effect of dexamethasone on cyclophosphamide-induced cystitis in rats: lack of relation with bradykinin B_1 receptor-mediated motor responses. *Eur J Pharmacol* **369**: 99–106.

Leeb-Lundberg LMF, Marceau F, Muller-Esterl W, Pettibone DJ, Zuraw BL (2005). International Union of Pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. *Pharmacol Rev* **57**: 27–77.

Maggi CA (1997). Bradykinin as an inflammatory mediator in the urinary tract.. In: Farmer S (ed). *The Kinin System*. Academic Press: London, pp 235–247.

Maggi CA, Patacchini R, Santicioli P, Geppetti P, Cecconi R, Giuliani S *et al*. (1989). Multiple mechanismis in the motor responses of the guinea-pig isolated urinary bladder to bradykinin. *Br J Pharmacol* **98**: 619–629.

Maggi CA, Santicioli P, Del Bianco E, Lecci A, Guliani S (1993). Evidence for the involvement of bradykinin in chemically-evoked cystitis in anaesthetized rats. *Naunyn Schmiedebergs Arch Pharmacol* **347**: 432–437.

Marceau F, Barabe J, St-Pierre S, Regoli D (1980). Kinin receptors in experimental inflammation. *Can J Physiol Pharmacol* **58**: 536–542.

Marceau F, Hess JF, Bachvarov DR (1998). The B_1 receptors for kinins. *Pharmacol Rev* **50**: 357–386.

Meini S, Lecci A, Cucchi P, Catalioto RM, Criscuoli M, Maggi CA (1998). Inflammation modifies the role of cyclooxygenases in the contractile responses of the rat detrusor smooth muscle to kinin agonists. *J Pharmacol Exp Ther* **287**: 137–143.

Meini S, Patacchini R, Giuliani S, Lazzeri M, Turini D, Maggi CA *et al*. (2000). Characterization of bradykinin B_2 receptor antagonists in human and rat urinary bladder. *Eur J Pharmacol* **388**: 177–182.

Meini S, Quartara L, Rizzi A, Patacchini R, Cucchi P, Giolitti A *et al*. (1999). MEN11270, a novel selective constrained peptide antagonist with high affinity at the human B_2 kinin receptor. *J Pharmacol Exp Ther* **289**: 1250–1256.

Nakahata N, Ono T, Nakanishi H (1987). Contribution of prostaglandin E_2 to bradykinin-induced contraction in rabbit urinary detrusor. *Jpn J Pharmacol* **43**: 351–359.

Neuhaus J, Weimann A, Stolzenburg JU, Dawood W, Schwalenberg T, Dorschner W (2006). Histamine receptors in human detrusor smooth muscle cells: physiological properties and immunohistochemical representation of subtypes. *World J Urol* **24**: 202–209.

Newton R, Eddleston J, Haddad E-B, Hawisa S, Mak J, Lim S *et al* (2002). Regulation of kinin receptors in airway epithelial cells by inflammatory cytokines and dexamethasone. *Eur J Pharmacol* **441**: 193–202.

Phagoo SB, Poole S, Leeb-Lundberg LMF (1999). Autoregulation of bradykinin reecptors: agonists in the presence of interleukin 1β shift the repertoire of receptor subtypes from B_2 to B_1 in human lung fibroblasts. *Mol Pharmacol* **56**: 325–333.

Phagoo SB, Yaqoob M, Herrera-Martinez E, Mcintyre P, Jones C, Burgess GM (2000). Regulation of bradykinin receptor gene expression in human lung fibroblasts. *Eur J Pharmacol* **397**: 237–246.

Pinna C, Caratozzolo O, Puglisi L (1992). A possible role for urinary bladder epithelium in bradykinin-induced contraction in diabetic rats. *Eur J Pharmacol* **214**: 143–148.

Regoli D, Barabè J (1980). Pharmacology of bradykinin and related kinins. *Pharmacol Rev* **32**: 1–46.

Sabourin T, Guay K, Houle S, Bouthillier J, Bachvarov DR, Adam A *et al*. (2001). Absence of ligand-induced regulation of kinin receptor expression in the rabbit. *Br J Pharmacol* **133**: 1154–1162.

Schmidlin F, Scherrer D, Daeffler L, Bertrand C, Landry Y, Gies J-P (1998). Interleukin 1β induces bradykinin B_2 receptor gene expression through a prostanoid cyclic AMP-dependent pathway in human bronchial smooth muscle cells. *Mol Pharmacol* **53**: 1009–1015.

Sjuve R, Boels PJ, Uvelius B, Arner A (2000). Up-regulation of bradykinin response in rat and human bladder smooth muscle. *J Urol* **164**: 1757–1763.