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Effects of bradykinin on signal transduction, cell proliferation, and cytokine, prostaglandin E_2 and collagenase-1 release from human corneal epithelial cells

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1 We recently demonstrated the presence of phospholipase C-coupled bradykinin (BK) B_2-receptors in human primary and SV40 virus-immortalized corneal epithelial (CEPI) cells.

2 The aims of the present studies were to demonstrate the specific binding of [^{3}H]-BK to CEPI cell membranes and to study its pharmacological characteristics. In addition, we wished to study the functional coupling of the BK receptors to various physiological and pathological mechanisms in the CEPI cells, including phosphoinositide (PI) turnover, intracellular Ca^{2+}-mobilization ($[Ca^{2+}]_i$), cell proliferation (via [^{3}H]-thymidine incorporation), and the release of various cytokines, collagenase-1 (matrix metalloproteinase-1) and prostaglandin E_2 (PGE_2).

3 Specific [^{3}H]-BK binding comprised $83 \pm 2\%$ of the total binding, and was of high affinity ($K_d = 1.66 \pm 0.52$ nM, $n = 5$), saturable ($B_{max} = 640 \pm 154$ fmol g^{-1} wet weight) and reversible. Competition studies yielded the following affinity values for BK and a number of BK-related peptides: Hoe-140 (D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]BK; icatibant): $K_i = 0.17 \pm 0.07$ nM; BK: $K_i = 1.0 \pm 0.11$ nM; [Tyr^8]-BK: $K_i = 12.9 \pm 2.3$ nM; [des-Arg^9]-BK: $K_i > 9{,}200$ nM (all $n = 3-5$)).

4 BK potently stimulated PI turnover ($EC_{50} = 2.3 \pm 0.3$ nM; $n = 7$) and $[Ca^{2+}]_i$ mobilization ($EC_{50} = 8-20$ nM) in CEPI cells and both responses were inhibited in a concentration-dependent manner by 100 nM – 10 μM Hoe-140, a selective B_2-receptor antagonist, and also inhibited by the selective phospholipase C (PLC) inhibitor, U73122 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione) ($IC_{50} = 3.0 \pm 1.6$ μM). BK-induced $[Ca^{2+}]_i$ mobilization was reduced by about 30% in the presence of 4 mM EGTA, but was not significantly affected by 100 nM nifedipine.

5 BK (0.1 nM – 10 μM) significantly ($P < 0.05-0.001$) stimulated [^{3}H]-thymidine incorporation into CEPI cellular DNA. However, while interleukin-1α (IL-1α; 10 ng ml^{-1}) potently stimulated the release of IL-6, IL-8 and granulocyte macrophage colony-stimulating factor from CEPI cells, BK (0.1 nM – 10 μM) was without effect.

6 Whilst phorbol-12-myristate-13-acetate (PMA; 3 μg ml^{-1}) and 10% foetal bovine serum (positive control agents) significantly stimulated the release of both MMP-1 and PGE_2 from CEPI cells, BK (0.1 nM – 10 μM) was without any significant effect under these conditions.

7 In conclusion, these data indicate that the CEPI cells express high-affinity [^{3}H]-BK binding sites representing B_2-subtype BK receptors coupled to PI turnover and $[Ca^{2+}]_i$ mobilization which appear to stimulate [^{3}H]-thymidine incorporation into cellular DNA. In contrast, BK failed to elicit the release of PGE_2, various cytokines and MMP-1 from CEPI cells. These results suggest that BK may have a potential role in corneal epithelium wound healing by stimulating cell proliferation.

Keywords: Bradykinin; bradykinin receptors; corneal epithelium; inflammation; wound healing; cytokine; collagenase-1; conjunctivitis; Hoe-140; PGE_2

Introduction

The corneal epithelium and the associated tear film are involved in light refraction and protection of the eye from undesirable allergens, airborne pathogens, u.v. radiation and chemicals (Beuerman *et al*., 1989; Rosenbaum *et al*., 1995). Provocation of the human conjunctiva leads to the release of relatively high concentrations of several mast cell mediators including histamine, platelet activating factor (PAF) and prostanoids into the tear-film (Allansmith & Ross, 1988; Proud *et al*., 1990; Abelson & Schaefer, 1993). The increased conjunctival vascular permeability induced by these mediators may then be responsible for causing the leakage of other chemicals and mediators like the peptides bradykinin (BK; Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9) and Lys-BK (kallidin) into the tear-film bathing the cornea and conjunctiva (Proud *et al*., 1990). Other sources of BK may be through local production on the ocular surface by the kallikriens secreted in the tears by the lacrimal gland (Proud *et al*., 1990). Regardless of the exact source(s) of these kinins, as much as 200 nM BK has been detected in the tear fluid of ocular allergic patients challenged with an allergen (Proud *et al*., 1990). Since topically applied BK also causes plasma extravasation in the guinea pig conjunctiva (Figini *et al*., 1995; Hall *et al*., 1995) and in other ocular tissues (Elliot *et al*., 1995), BK and related kinins may have important physiological/pathological roles in the ocular surface tissues. Chronic inflammation of the corneal surface can result in perforation of the anterior chamber, neovascularization (BenEzra *et al*., 1997), fibrosis and scarring (Abelson & Schaefer, 1993; Proia, 1994; Reim *et al*., 1997) which seriously reduces visual acuity and can lead to blindness in the affected eye. Therefore, a better understanding of the physiology and pathology of the

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corneal/conjunctival epithelial cells may lead to the development of suitable therapeutic agents to address these sight threatening problems (Reim *et al*., 1997).

We recently identified and characterized BK receptors coupled to phospholipase C (PLC) in human corneal epithelial (CEPI) cells, with the primary (P-CEPI) and SV40 virus-immortalized (CEPI-17-CL4) cells exhibiting near identical pharmacological responsiveness to BK agonists and antagonists (Wiernas *et al*., 1997). The aims of the present studies were to study the binding of [^{3}H]-BK to CEPI cells and to define its pharmacology, relating this to the signal transduction mechanism. In addition, we aimed to investigate further the functional coupling of the BK receptor in the these cells and to define some of the ultimate biological functions BK may have in the corneal epithelium. Hence, we investigated the effects of BK and related agonists and antagonists on intracellular calcium ($[Ca^{2+}]_i$) mobilization and [^{3}H]-thymidine incorporation, and the effects of BK on the release of cytokines, prostaglandin E_2 (PGE_2) and collagenase-1 (matrix metalloproteinase-1; MMP-1).

Methods

Human CEPI cell isolation and cultures

The procedures for isolating and culturing human normal, primary CEPI (P-CEPI) cells have been previously described (Wiernas *et al*., 1997; Sharif *et al*., 1997b). In brief, human corneas were aseptically dissected from cadaver eyes within 8–12 h of death and transported from eye banks in ice-cold Dexol or Optisol corneal preservation medium. Corneas from different donors were kept separate and rinsed in phosphate buffered saline (PBS) before being exposed to dispase at 10 u ml^{-1} in 50% Hank's buffered salts and keratinocyte growth medium (KGM) containing 0.05 mM calcium at 4°C for 24–48 h. The KGM was prepared by adding bovine pituitary extract (30 μg ml^{-1}), hydrocortisone (0.5 μg ml^{-1}), amphotericin B (0.05 μg ml^{-1})/gentamicin (50 μg ml^{-1}), insulin (5 μg ml^{-1}), transferrin (10 μg ml^{-1}), murine epidermal growth factor (10 μg ml^{-1}) and 0.05 mM $CaCl_2$ to keratinocyte basal medium (KBM). Following this incubation, the epithelium was gently removed with a scalpel blade. This tissue was then gently triturated to produce individual cells which were then washed in Dulbecco's modified Eagle medium (DMEM) containing 10% foetal bovine serum by a low speed centrifugation/re-suspension procedure. The CEPI cell pellet was resuspended in low calcium (0.05 mM) KGM medium, the cells plated into T25 flasks (previously coated with collagen type IV and fibronectin) and incubated at 37°C under a humidified atmosphere of 95% air/5% CO_2. The culture medium was changed 24 h later and then every 2 days thereafter. The cells became confluent in approximately 10 days at which point they were sub-cultured (passage 1; P1) by rinsing with PBS, incubating in dispase for up to 1 h until the cells detached, washing in DMEM by centrifugation and plated on collagen-coated 24-well plates. All experiments on P-CEPI cells were performed with the P1 cells.

Immortalized CEPI cells

The SV40 virus-induced immortalization and the initial genetic, morphological and preliminary pharmacological characterization of the primary (P-CEPI) and the latter immortalized (CEPI-17-CL4) cells have been recently described (Wiernas *et al*., 1997; Sharif *et al*., 1997b). These latter studies (Sharif *et al*., 1997b; Wiernas *et al*., 1997) have established that CEPI-17-CL4 cells represent the P-CEPI cells well in terms of the above-mentioned parameters, but especially in relation to the PLC-coupling and the pharmacological characteristics of the B_2-receptor present on both these cell-types (Wiernas *et al*., 1997). Accordingly, the term 'CEPI cells' will be used from here onward to denote generically the corneal epithelial cells.

[^{3}H]-BK binding studies

In view of the limited availability and slow growth characteristics of human P-CEPI cells (Kahn *et al*., 1993; Araki-Sasaki *et al*., 1995) and the small amount of CEPI-tissue that is potentially obtainable from cadaver eyes, all binding experiments were performed with CEPI-17-CL4 cells. Confluent CEPI-17-CL4 cells were gently scraped from the flasks with a rubber policeman and homogenized in 25 mM TES (N-tris[hydroxymethyl]methyl-α-aminoethansulphonic acid) buffer (pH 6.8), containing 1 mM 1,10 phenanthroline and a mixture of various peptide inhibitors (140 μg ml^{-1} bacitracin, 1 μM captopril, 1 mM dithiothreitol and 0.1% bovine serum albumin), by use of a Polytron tissue disruptor (setting 3 for 5 s). The homogenates were centrifuged at 30,000 *g* (20 min at 4°C) and the cell pellets gently dispersed in the above-mentioned buffer (at 30 mg wet weight tissue ml^{-1}) for the binding assays. Receptor binding of [^{3}H]-BK was determined by previously published procedures (Sharif & Whiting, 1991; 1993). Briefly, cell membranes (400 μl aliquots) were incubated with 50 μl of various concentrations of the test unlabelled compound (1 pM – 10 μM final) or buffer and 50 μl of [^{3}H]-BK (0.5 nM final) in polypropylene tubes at 23°C for 90 min in order to reach equilibrium. Non-specific binding was determined with 10 μM unlabelled BK or Hoe-140. Assays were terminated by rapid vacuum filtration on a Tomtec cell harvester through Wallac 'B' glass fibre filters (pre-soaked in 0.3% polyethyleneimine) by washing three times with 3 ml ice-cold 50 mM TrisHCl buffer (pH 7.4) washes. Bound radioactivity was measured at 50% efficiency by liquid scintillation spectrometry on a Wallac Beta-plate scintillation counter. Data analysis was performed as described below.

PI turnover studies

PI turnover studies were performed as previously described (Sharif & Xu, 1996; Wiernas *et al*., 1997; Sharif *et al*., 1997a,b). In brief, CEPI cells (approx. 2.5×10^5 cells/well) cultured in 24-well plates were incubated with [^{3}H]-*myo*-inositol (2 μCi ml^{-1}; 15–17 Ci mmol^{-1}) in DMEM for 24 h at 37°C in order to label the cell membrane lipids. The medium was then aspirated and the cells exposed to BK in DMEM/F-12 medium (+15 mM HEPES buffer) containing 10 mM LiCl for 60 min at 37°C in order to facilitate the accumulation of [^{3}H]-inositol phosphates ([^{3}H]-IPs) (Berridge *et al*., 1982). To determine the effects of antagonists, the latter drugs were added to the cells 30 min before the addition of BK. The medium was aspirated at the end of the incubation, and the assay terminated by the addition of 1 ml of ice-cold 0.1 M formic acid and the [^{3}H]-IPs quantified by standard ion exchange chromatography (Berridge *et al*., 1982) and liquid scintillation spectrometry.

Intracellular Ca^{2+}-mobilization studies

BK-stimulated mobilization of intracellular Ca^{2+} ($[Ca^{2+}]_i$) in CEPI-17-CL4 cells was studied with the Fura-2 fluorescent Ca^{2+} chelator method (Grynkiewicz *et al*., 1985) on stirred suspensions of cells in cuvettes (Griffin *et al*., 1997). To avoid

trypsin degradation of the membrane receptors, cells were detached with 0.05% EDTA in PBS (without Mg^{2+} and Ca^{2+}), containing 0.1% glucose, for 30 min at 23°C. The cell suspension was centrifuged at 1,000 *g* for 10 min at 23°C, washed twice by gently re-suspending in Hank's BSS containing 10 mM HEPES buffer pH 7.4 and 0.1% bovine serum albumin (BSA) (designated as BSS-BSA buffer) followed by centrifugation as above. The cells were then suspended in 2 μM Fura-2 AM (acetoxymethyl) ester in the BSS-BSA buffer and maintained at 23°C in the dark for 60 min. Free extracellular Fura-2 AM was removed by washing the cells 3 times with the BSS-BSA buffer as described above. Cell counts and cell viability were determined by the trypan blue exclusion method, and a concentrated stock suspension of the Fura-2 loaded cells in the BSS-BSA buffer was stored on ice in the dark to minimize leakage and photobleaching of Fura-2 inside the cells.

For the agonist stimulation experiments with BK, cells were diluted to a concentration of 1.0×10^6 ml^{-1} in a total volume of the BSS-BSA buffer sufficient for several experiments. Following gentle, thorough dispersion of this cell suspension, 1.5 ml aliquots were transferred to matched quartz cuvettes. This cell suspension was then equilibrated at 23°C for 60–90 min and gently dispersed before the agonist (15 μl) was added. Fluorescence changes were measured with a Perkin-Elmer MPF-66 fluorescence spectrophotometer in stirred cell suspensions maintained at 23°C and stirred with an overhead paddle-type stirrer. Excitation wavelength was 340 nm (with 1 nm excitation slit width) and emission wavelength was 510 nm, with the emission slit width adjusted to optimize the net fluorescence change determined by the calibration procedure (Grynkiewicz *et al*., 1985). After the agonist-stimulated response had decayed, the fluorescence signal was calibrated by first adding 50 μg ml^{-1} digitonin and then 6.6 mM EGTA plus 5 mM Tris base to achieve the maximal and minimal responses, respectively (Grynkiewicz *et al*., 1985). Based upon the maximal and minimal responses determined by this calibration procedure, the agonist-dependent maximal increase in intracellular fluorescence was converted to $[Ca^{2+}]_i$ by standard equations; a K_d value of 224 nM was assumed (Grynkiewicz *et al*., 1985). For the determination of potential antagonist effects, each test compound (6–15 μl) was added to the cell suspension (1.5 ml) for 20 min before the addition of BK. EGTA (1 mM stock) was utilized to chelate extracellular calcium and, therefore, define the importance of extracellular Ca^{2+} influx for the effect of BK on $[Ca^{2+}]_i$. A selective phospholipase C inhibitor (U73122; (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione)) was utilized to block the formation of IPs and, thus, help establish the importance of this pathway for the effect of BK on $[Ca^{2+}]_i$. In addition, a calcium channel blocker (nifedipine 100 nM final) was used as a further control to prevent influx of available extracellular Ca^{2+} via dihydropyridine-sensitive Ca^{2+}-channels. The B_2-receptor selective antagonist, Hoe-140 (100 nM final), was utilized to confirm that BK-induced $[Ca^{2+}]_i$ release can be blocked with an appropriate B_2-receptor antagonist. Since desensitization of the response to BK occurred (as determined by the response elicited by repeated stimulation), only one BK stimulation experiment was performed on each cuvette of cells.

[^{3}H]-thymidine incorporation studies

The effects of BK on mitogenesis of CEPI-17-CL4 cells grown in culture were determined by measuring [^{3}H]-thymidine incorporation into cellular DNA by use of methods modified from those previously published (Tones *et al*., 1988; Sharif & Stevenson, 1993). The normal culture medium containing growth factors and bovine pituitary extract (BPE) was aspirated and the CEPI-17-CL4 cells rinsed (1 ml/well) and subsequently grown in 0.5 ml KGM lacking the growth factors and BPE but containing BK (100 pM–10 μM final) or vehicle as control for 17 h at 37°C. Following this incubation period, the cells were exposed to 20 μl of 0.25 μCi ml^{-1} [^{3}H]-thymidine for 5 h at 37°C to allow incorporation of the [^{3}H]-thymidine into cellular DNA. The incubation solutions were aspirated and the cells washed with 0.5 ml ice-cold 0.15 M sodium chloride to remove the non-incorporated [^{3}H]-thymidine. A 10% solution of trichloroacetic acid (0.5 ml) was then added to the cells and they were left undisturbed at 23°C for 15 min to terminate the uptake of [^{3}H]-thymidine (Tones *et al*., 1988). After the cells had been washed with a total of 2 ml (4×0.5 ml) distilled water, they were exposed to 0.5 ml 1% sodium dodecyl sulphate at 37°C for 15 min to disrupt the cell membranes. Cell lysates were then transferred to separate scintillation vials and each pipette tip was rinsed with 0.15 ml distilled water. Another 0.5 ml 1% sodium dodecyl sulphate was added to each well and transferred to the appropriate vial. The contents of each vial were then thoroughly mixed with 5 ml of a water-accepting scintillation fluid (Opti-Fluor) and the amount of [^{3}H]-thymidine incorporated into cellular DNA was then quantified by liquid scintillation spectrometry on a beta-counter.

Cytokine release studies

Confluent P-CEPI cells were incubated for 24 h at 37°C with 5 μl of various concentrations of BK (100 pM–10 μM final) in 0.5 ml of low calcium (serum-free) DMEM medium per well. Interleukin-1α (IL-1α; 10 ng ml^{-1} final) was used as a positive control. Following this incubation period, commercially available ELISA kits were used according to the manufacturer's directions to quantify the levels of various cytokines (interleukin-6, (IL-6), interleukin-8 (IL-8) and granulocyte macrophage colony stimulating factor (GM-CSF)) released into the incubation medium via spectrophotometry at 450 nm, by use of a series of reference standards of known concentration, with a microplate reader. All the latter kits utilized employ the quantitative sandwich enzyme immunoassay procedure.

Briefly, for IL-6, the standards were prepared with the calibrator diluent RD5A (supplied in the kit) as stipulated by the manufacturer. The assay itself was conducted by adding 100 μl of the supplied assay diluent to each well of the supplied 96-well plate, followed by 100 μl of the standard or test sample (from above) and then incubating for 2 h at 23°C. Following this incubation period, contents of each well were aspirated and the wells washed 4 times with the supplied wash buffer. Then, 200 μl of the supplied conjugate was added to each well and incubated for 2 h at 23°C. Once again, contents of each well were aspirated and the wells washed 4 times with the supplied wash buffer. At this time, 200 μl of the supplied substrate solution was added to each well and incubated for 20 min at 23°C. The stop solution (50 μl) supplied was added to each well and the plate was read at 450 nm within 30 min. For IL-8, the procedures were identical to those described above, except that the aspiration and wash steps were performed in triplicate rather than in quadruplicate.

For GM-CSF, the standards were prepared in the calibrator diluent RD5 (supplied in the kit) as stipulated by the manufacturer. The assay itself was conducted by adding 200 μl of the standard or test sample (see above) to each well and then

incubating for 2 h at 23°C. Following this incubation period, contents of each well were aspirated and washed 4 times with the supplied wash buffer. Then, 200 μl of the supplied conjugate was added to each well and incubated for 2 h at 23°C. Contents of each well were then aspirated and washed 4 times with the supplied wash buffer. At this time, 200 μl of the supplied substrate solution was added to each well and incubated for 20 min at 23°C. The stop solution (50 μl) supplied was added to each well and the plate was read at 450 nm within 30 min. The limits of detection (sensitivity) for each cytokine were as follows: IL-6 0.7 pg ml^{-1}, IL-8 6 pg ml^{-1}, GM-CSF $<$ 2.8 pg ml^{-1}.

PGE_2 release studies

PGE_2 release from P-CEPI and CEPI-17-CL4 cells following stimulation by various agonists was determined by use of a commercially available radioimmunoassay (RIA) kit according to the manufacturer's instructions. The cell stimulation was similar to that described above for cytokine release, except that the incubation period was 60 min and 10% foetal bovine serum was used as a positive control. All standards and reagents supplied in the kit were prepared as stipulated in the instructions. Assay buffer, diluted standard and sample extracts (culture medium from the cell stimulation experiments) (diluted 1 : 100) were then pipetted into the appropriate tubes as specified in the kit instructions. Tracer solution (100 μl) was then added to each tube and mixed, followed by 100 μl antiserum to the appropriate tubes and thorough vortexing for 2 – 5 s. All tubes were then incubated overnight (24 h) at 4°C. Following this incubation, all tubes were placed in an ice bath and 1 ml cold precipating reagent (supplied in the kit) was pipetted into all appropriate tubes and vortexed thoroughly for 2 – 5 s followed by incubation in an ice bath at 4°C for 25 min. All tubes were then centrifuged in a refrigerated centrifuge at 2,000 $\times g$ for 30 min. The supernatants of all appropriate tubes were decanted as specified in the kit instructions and the RIA samples were analysed on a gamma-counter. The levels of PGE_2 released into the culture medium were then quantified in comparison with the PGE_2-standard curve. The limit of detection (sensitivity) of the PGE_2 RIA was 0.44 pg/well.

Collagenase-1 release studies

The procedures used for these studies with P-CEPI and CEPI-17-CL4 cells were basically identical to those utilized for the cytokine release studies. The only differences were the use of KGM medium, 10 min preincubation of the BK-treated cells with the angiotensin converting enzyme inhibitor (SQ 20881, pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro; 10 μM final), PMA (3 μg ml^{-1}) as the positive control and a commercially available collagenase-1 (Matrix Metalloproteinase-1; MMP-1) ELISA kit. This kit also utilizes the quantitative sandwich enzyme immunoassay procedure mentioned above with the MMP-1 levels being determined via spectrophotometry, by use of a series of reference standards of known concentration. The kits were utilized according to the manufacturer's instructions. All reagents and working standards were prepared as stipulated in the kit instructions. Following this step, 100 μl supplied assay buffer 2, standard or test sample were pipetted into the appropriate wells of the supplied 96-well plate, the plates were covered and incubated at 23°C for exactly 2 h. Following this incubation period, the contents of all wells were aspirated and the wells washed 4 times with the supplied wash buffer and blotted to remove any residual fluid. Antiserum (100 μl) was then added to each well and the plates were covered again and incubated at 23°C for 2 h. Following this incubation period, the contents of the wells were aspirated and each well washed 4 times with the supplied wash buffer and blotted to remove any residual fluid. Peroxidase conjugate (100 μl) was then added to each well and the plates were covered again and incubated at 23°C for 1 h. Following this incubation period, the contents of the wells were aspirated and the latter washed 4 times with the supplied wash buffer and blotted to remove any residual fluid. Immediately following this step, 100 μl room temperature equilibrated TMB substrate supplied in the kit was dispensed into all wells, the plates were covered and incubated for exactly 30 min at 23°C while mixing on a microplate shaker. The reaction was then stopped by adding 100 μl 1 M sulphuric acid to all wells and the plates were read at 450 nm within 30 min. The limits of detection (sensitivity) of the MMP-1 ELISA kit was 1.7 ng ml^{-1}.

Materials

The reagents, chemicals, drugs and materials used in the present studies were purchased or were gifts from the following sources: Optisol or Dexol from Chiron Ophthalmics, Irvine, CA (U.S.A.); dispase from Collaborative Biomedical, Bedford, MA (U.S.A.); KGM from Clonetics Corp., San Diego, CA (U.S.A.); DMEM and DMEM/F-12 media, BSS, trypsin/EDTA, phosphate-buffered saline without Ca^{2+} or Mg^{2+} and Hank's BSS from Life Technologies, Grand Island, NY (U.S.A.); culture plates from Corning/Costar, Cambridge, MA (U.S.A.); Becton Dickenson, Oxnard (U.S.A.), CAFNC-matrix from Biological Research Faculty and Facility, Inc., Ijamsville, MD (U.S.A.); all peptides and SQ20881 were from Peninsula Labs., Belmont, CA (U.S.A.), except Hoe-140 D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8] BK; (icatibant) which was a generous gift from Hoechst AG (Frankfurt, Germany); MMP-1 ELISA kits and [^{3}H]-*myo*-inositol from Amersham Corp., Arlington Heights, IL (U.S.A.); cytokine ELISA kits from R&D Systems, Minneapolis, MN (U.S.A.); [^{3}H]-BK (78.7 Ci mmol^{-1}) and [^{3}H]-thymidine (102.7 Ci mmol^{-1}) from New England Nuclear (NEN), Boston, MA (U.S.A.); Fura-2 AM from Molecular Probes, Eugene, OR, (U.S.A.); AG1x8 resin, Econo-columns and sodium dodecyl sulphate from Biorad, Richmond, CA (U.S.A.); BSA, digitonin, formic acid, ammonium formate, LiCl, polyethylenimine, nifedipine and trichloroacetic acid from Sigma, St. Louis, MO (U.S.A.); PMA from L.C. Laboratories, San Diego, CA (U.S.A.); gentamicin, penicillin/streptomycin from Gibco/BRL, Grand Island, NY (U.S.A.); U73122 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione) and U73343 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl) amino)hexyl)–2,5-pyrrolidine-dione) from Biomol Research Laboratories, Plymouth Meeting, PA (U.S.A.); Ecolume scintillation cocktail from ICN, Costa Mesa, CA (U.S.A.); Opti-Fluor scintillation cocktail from Packard Instrument Co., Meriden, CT (U.S.A.); Origin Scientific Graphics software package from Microcal, Northampton, MA (U.S.A.).

Data analysis

The original data (d.p.m. bound; d.p.m./well; nM $[Ca^{2+}]_i$ mobilized) were analysed using a non-linear, iterative curve-fitting computer program (Michel & Whiting, 1984; Sharif & Whiting, 1991) and Origin software (Sharif *et al*., 1996; 1997a) incorporating a logistic function. The competition data for BK vs [^{3}H]-BK were processed by use of the 'EBDA' suite of computer programs (McPherson, 1983a,b) to perform Scatch-

ard analysis and thus derive the apparent receptor affinity (K_d) and apparent density (B_{max}) parameters. The initial inhibition constants (IC_{50}s) for various peptides competing for [^{3}H]-BK binding were converted to equilibrium dissociation constants (K_is) by use of the standard Cheng-Prusoff equation (Cheng & Prusoff, 1973).

Agonist potency (EC_{50}) was defined as the concentration of the compound required to stimulate 50% of the maximal PI turnover response. Individual concentration-response curves for each agonist were analysed as above to obtain the potency values. However, in order to present composite data for each peptide from several experiments (e.g. Table 1), the data were normalized and presented as a percentage of the maximal specific binding, or for functional assays, basal (unstimulated) levels of [^{3}H]-IPs or $[Ca^{2+}]_i$ were set to zero and the maximally-stimulated levels of [^{3}H]-IPs or $[Ca^{2+}]_i$ at the top of each of the concentration-response curves were set to 100%. All intermediate levels of [^{3}H]-IPs accumulation or $[Ca^{2+}]_i$ were then calculated as a percentage of the maximal stimulation. Some data were presented as histograms to depict the concentration-response relationship for BK or the relative activity of reference compounds. Statistical analysis utilized Student's *t* test for determining the level of significance between the basal responses and the magnitude of the effects of the test compounds. A P value of <0.05 indicated the minimal level of statistical significance between the afore-mentioned parameters. The Origin Scientific Graphics software package was utilized to construct the figures shown.

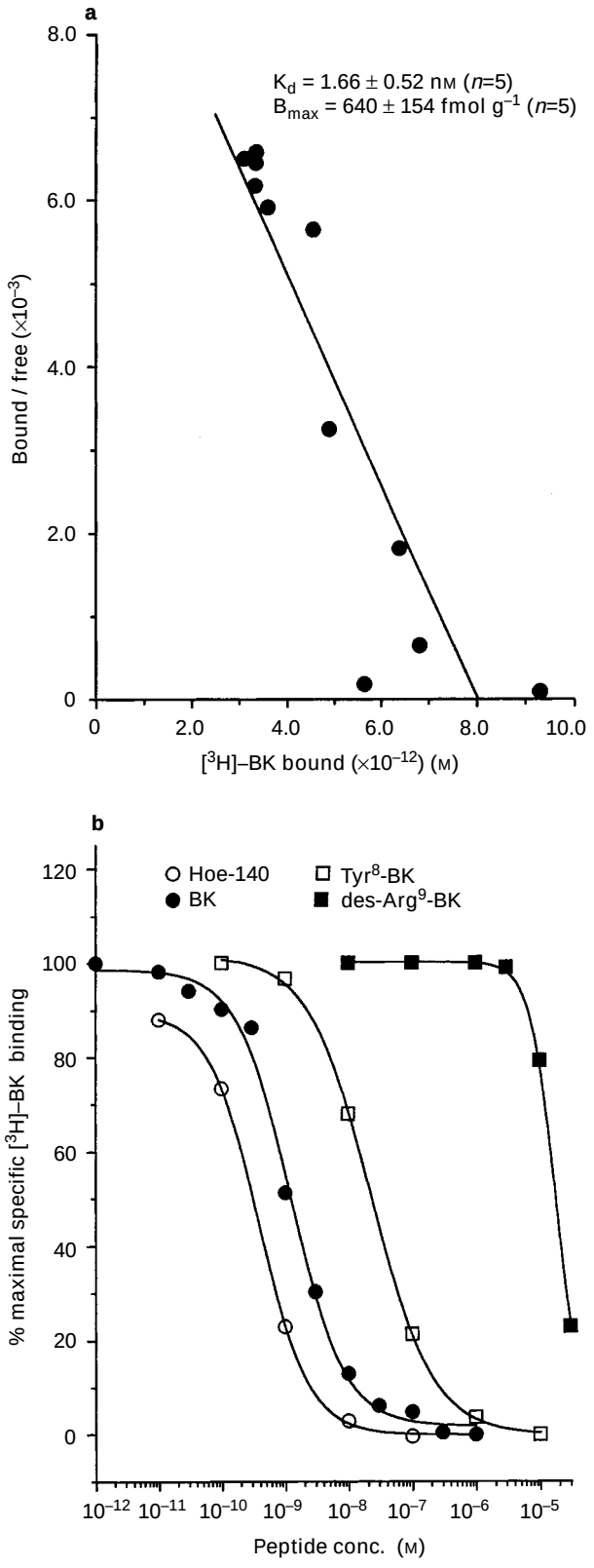

Figure 1 Scatchard analysis of specific [^{3}H]-bradykinin ([^{3}H]-BK) binding (a) and competition curves (b) for various unlabelled peptides competing for specific [^{3}H]-BK binding to CEPI-17-CL4 cell membranes. Data shown are from a single representative experiment. Composite data (mean ± s.e.mean) from several such experiments are shown in Results and in Table 1.

Results

[^{3}H]-BK binding

Specific [^{3}H]-bradykinin ([^{3}H]-BK) binding comprised 83 ± 2% of the total binding, was of high affinity (K_d = 1.66 ± 0.52 nM, n = 5) and reflected interaction with a finite number of saturable binding sites (B_{max} = 640 ± 154 fmol g^{-1} wet weight) (Figure 1a). The relative affinities of BK analogues, as well as the specific antagonist Hoe-140, at inhibiting specific [^{3}H]-BK binding were as follows (all n = 3–5): Hoe-140: IC_{50} = 0.22 ± 0.09 nM, K_i = 0.17 ± 0.07 nM; BK: IC_{50} = 1.32 ± 0.14 nM, K_i = 1.0 ± 0.11 nM; [Tyr^8]BK: IC_{50} = 16.8 ± 2.9 nM, K_i = 12.9 ± 2.3 nM; [des-Arg^9]BK: IC_{50} > 12,000 nM, K_i > 9,200 nM. None of the Hill coefficients was statistically different from unity (P > 0.05) (Table 1).

PI turnover studies

As previously shown (Wiernas *et al*., 1997), bradykinin (BK) induced the production of [^{3}H]-IPs in P-CEPI and CEPI-17-CL4 cells in a concentration-dependent manner (EC_{50} = 2.3 ± 0.3 nM, n = 7) (Figure 2a). Furthermore, the BK-induced

Table 1 Affinities of bradykinin-related agonists and antagonist competing for [^{3}H]-bradykinin binding to CEPI-17-CL4 cell membranes

Compound	*Affinity* (IC_{50}, nM)	*Affinity* (K_i, nM)	*Hill coefficient* (n_H)
Hoe-140	0.22 ± 0.09	0.17 ± 0.07	1.5 ± 0.48
BK	1.32 ± 0.14	1.0 ± 0.11	0.89 ± 0.05
[Tyr^8]-BK	16.8 ± 2.9	12.9 ± 2.3	1.06 ± 0.02
[des-Arg^9]-BK	> 12,000	> 9,200	–

Data are mean ± s.e.mean from 3–5 independent experiments with cells from several different passages. The Hill coefficients were not significantly different from unity (P > 0.05). BK = bradykinin.

PI turnover was very similar in both cell-types and was substantially ($\geqslant$80%) blocked by nanomolar concentrations of Hoe-140, a B_2-receptor selective antagonist (Figure 2b). The specific phospholipase C (PLC) inhibitor, U73122, inhibited BK-induced PI turnover ($IC_{50} = 3.0 \pm 1.6$ μM, $n = 3$), while its inactive analogue, U73343 (10 nM – 10 μM), was without any effect (data not shown).

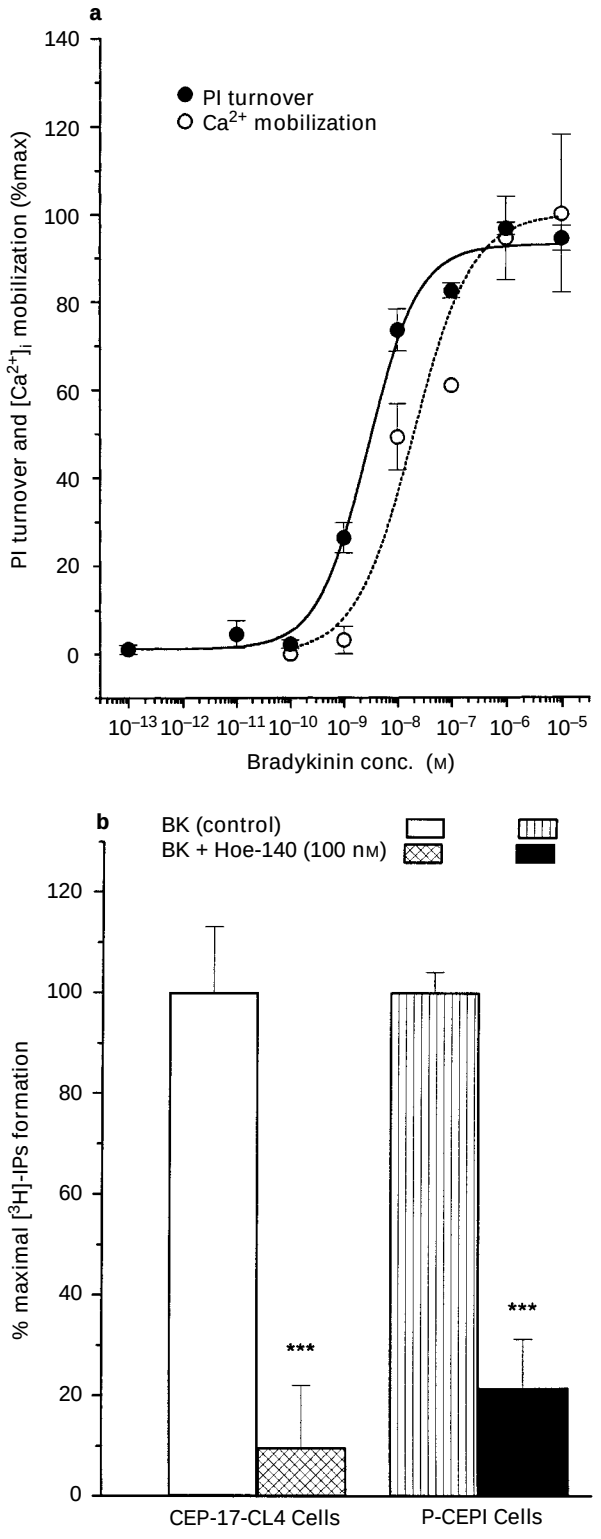

Figure 2 Bradykinin (BK)-induced phosphoinositide (PI) turnover and intracellular Ca^{2+} ($[Ca^{2+}]_i$) mobilization in CEPI cells. (a) The concentration-response curves for BK in stimulating phosphoinositide (PI) turnover and $[Ca^{2+}]_i$ mobilization and (b) The antagonistic effects of Hoe-140 (D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]BK; 100 nM) on the BK-induced PI turnover responses in CEPI-17-CL4 and P-CEPI cells. Data are mean from 7 experiments for PI turnover and from up to 5 experiments for the $[Ca^{2+}]_i$ mobilization studies; vertical lines show s.e.mean. ***$P < 0.001$ for antagonism by Hoe-140 relative to the BK-induced PI turnover by Student's t test.

$[Ca^{2+}]_i$ mobilization studies

Bradykinin (BK) potently stimulated mobilization of $[Ca^{2+}]_i$ in CEPI-17-CL4 cells ($EC_{50} = 8 - 20$ nM) which correlated reasonably well with its potency in stimulating PI turnover (Figure 2a; also see Wiernas *et al*., 1997). Representative tracings of $[Ca^{2+}]_i$ mobilized in response to control and test agents are shown in Figure 3. The response to a maximally effective concentration of BK (1 μM) was less than that to a maximally effective concentration of histamine (1 mM) (Figure 3a). However, Hoe-140 (10 μM) blocked the BK-induced $[Ca^{2+}]_i$ mobilization and did not influence the histamine-induced response (Figure 3b; Figure 4; $n = 3 - 5$). The specific phospholipase C (PLC) inhibitor, U73122 (4 μM) effectively prevented BK- and histamine-induced $[Ca^{2+}]_i$ mobilization (Figure 3c; Figure 4; $n = 3 - 5$). However, U73343 (a known inactive analogue of U73122) neither inhibited BK- nor histamine-induced $[Ca^{2+}]_i$ mobilization (data not shown).

In order to determine the contribution of extracellular Ca^{2+} to the BK-induced $[Ca^{2+}]_i$ mobilization, the effects of the calcium chelator EGTA (4 mM final) and the calcium channel

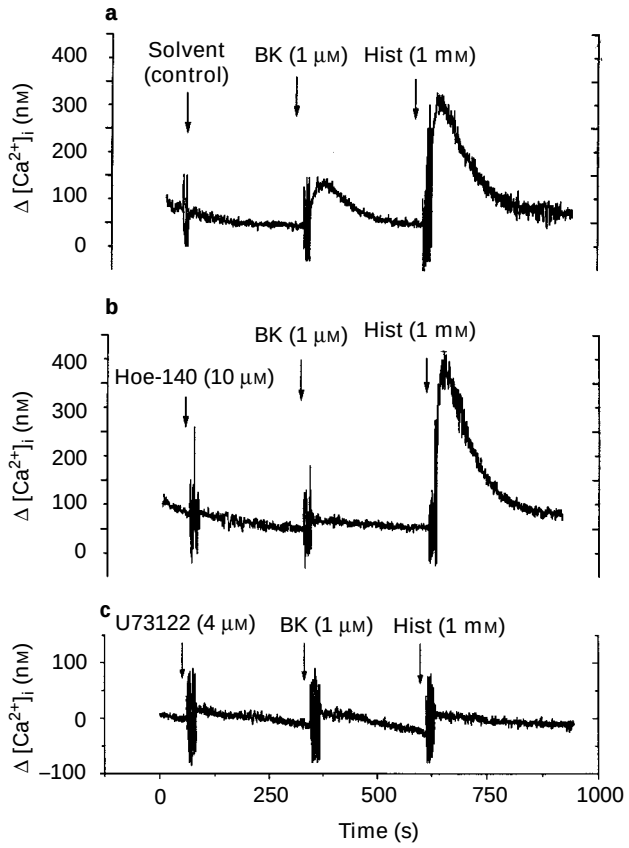

Figure 3 Effects of various agents on intracellular Ca^{2+} ($[Ca^{2+}]_i$) mobilization in CEPI-17-CL4 cells. Representative traces of the $[Ca^{2+}]_i$ mobilization signals induced by bradykinin (BK) and histamine (a) are shown. Effects of the B_2-receptor antagonist, Hoe-140 (D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]BK), on BK- and histamine-induced Ca^{2+}-responses are shown in (b). Effects of the phospholipase C inhibitor, U73122, on BK- and histamine-induced Ca^{2+}-responses are shown in (c). Representative traces from up to five experiments are shown.

blocker nifedipine (100 nM final) were investigated. EGTA appeared to reduce the BK-induced $[Ca^{2+}]_i$ mobilization by about 30%, but nifedipine did not produce any significant effects (Figure 4; $n=3-5$).

[^{3}H]-thymidine incorporation/cell proliferation studies

BK (0.1 nM – 10 μM final) stimulated the incorporation of [^{3}H]-thymidine into CEPI-17-CL4 cellular DNA in a concentration-dependent manner and this effect plateaued at a maximal stimulation of twice the basal level ($P<0.005$; $n=3$) (Figure 5).

Cytokine release studies

Bradykinin (BK ; 0.1 nM – 10 μM) had no significant effect on the release of interleukin-6 (IL-6), interleukin-8 (IL-8) or granulocyte macrophage colony stimulating factor (GM-CSF) by P-CEPI cells ($n=3$) (Figure 6). However, the positive control agent, interleukin-1α (IL-1α; 10 ng ml^{-1} final), potently stimulated IL-6, IL-8 and GM-CSF secretion from these cells when tested in parallel with BK (Figure 6).

PGE_2 and collagenase-1 release studies

Bradykinin (BK; 0.1 nM – 10 μM final) had no significant effect on the release of either PGE_2 (Figure 7) or collagenase-1 (MMP-1) from P-CEPI/CEPI-17-Cl4 cells ($n=3-4$) (Figure 8). However, 10% foetal bovine serum stimulated PGE_2 release (Figure 7) and the phorbol ester, phorbol-12-myristate-13-acetate (PMA; 3 μg ml^{-1} final), stimulated

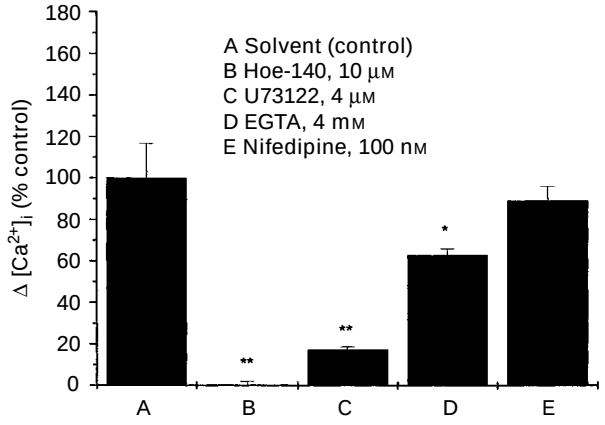

Figure 4 Effects of various agents on bradykinin (BK)-induced intracellular Ca^{2+} ($[Ca^{2+}]_i$) mobilization in CEPI-17-CL4 cells. Pre-treatment of the cells with Hoe-140, U73122, EGTA and nifedipine was followed by exposure to 1 μM BK and the $[Ca^{2+}]_i$ mobilization signals represented as a percentage of the control. Data are mean from 3–5 experiments; vertical lines show s.e.mean. $^*P<0.05$ and $^{**}P<0.01$ as compared to controls by Student's t test.

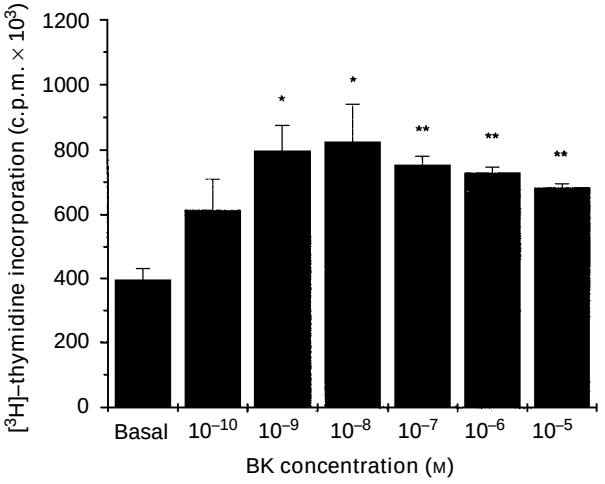

Figure 5 [^{3}H]thymidine incorporation into CEPI-17-CL4 cellular DNA in response to bradykinin (BK). Data are mean from a representative experiment performed in triplicate using BK (10^{-10} – 10^{-5} M); vertical lines show s.e.mean. Similar data were obtained in two additional determinations. $^*P<0.05$, $^{**}P<0.005$ as compared to the basal level by Student's t test.

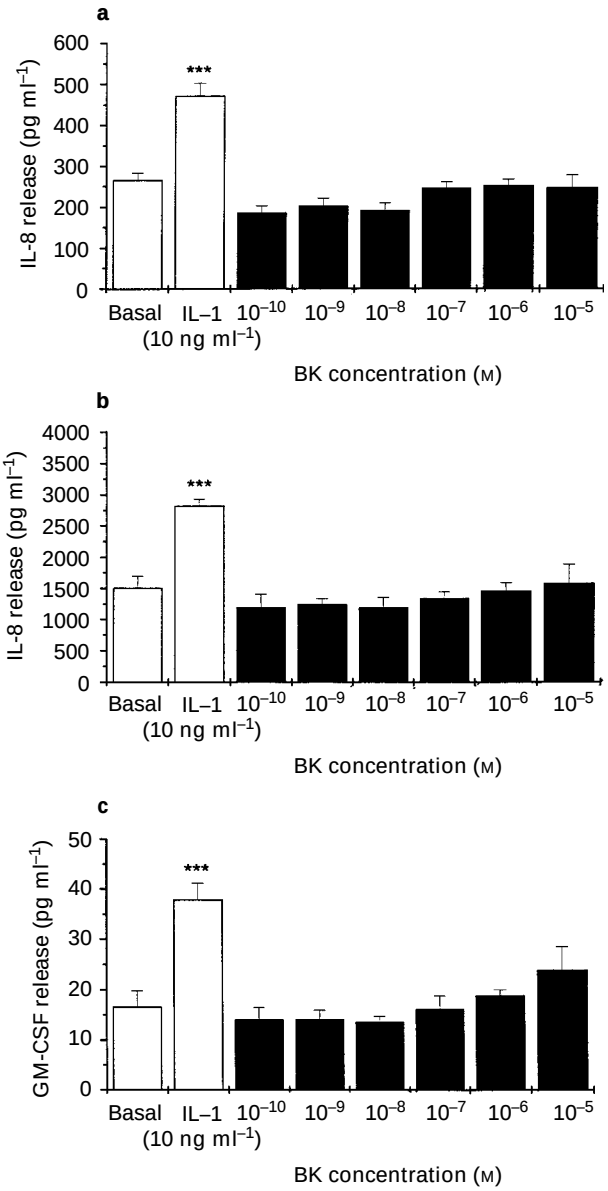

Figure 6 Effects of bradykinin (BK) and interleukin-1α (IL-1α) on cytokine release from P-CEPI cells. (a) The effects of various concentrations of bradykinin (BK; $10^{-10}-10^{-5}$ M) and 10 ng ml^{-1} interleukin-1α (IL-1α) on interleukin-6 (IL-6) release; (b) shows interleukin-8 (IL-8) release and (c) granulocyte macrophage colony stimulating factor (GM-CSF) release. Data are mean from 4 donors; vertical lines show s.e.mean. Similar results were obtained with the CEPI-17-CL4 cells. $^{***}P<0.001$ as compared to the basal level by Student's t test.

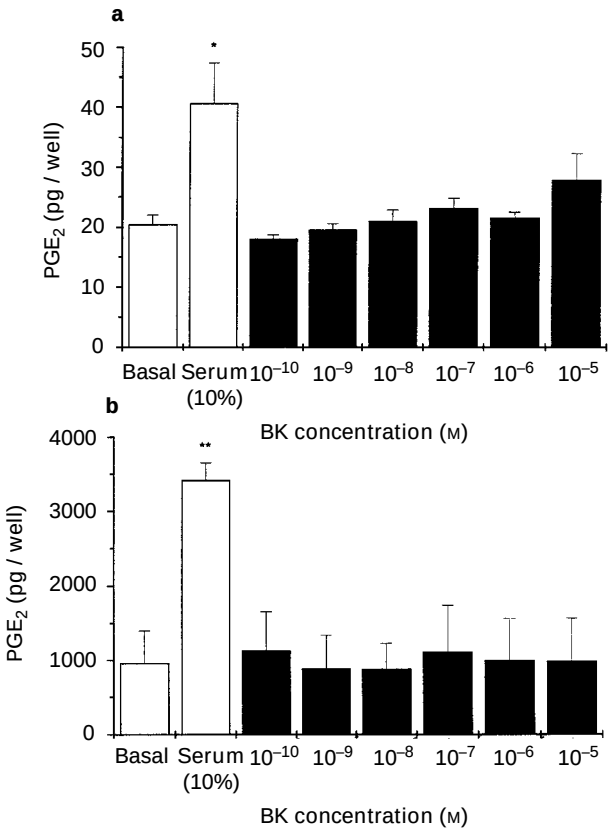

Figure 7 Effects of bradykinin (BK) and foetal bovine serum on prostaglandin E_2 (PGE_2) release from CEPI-17-CL4 (a) and P-CEPI (b) cells. Data are from 3–4 experiments/donors with BK (10^{-10}–10^{-5} M) or serum (10%) to stimulate the cells; vertical lines show s.e.mean. $^*P<0.05$; $^{**}P<0.01$ as compared to the basal level by Student's t test. Note the difference in the basal and stimulated levels of PGE_2 in the two cell types; note also that the positive control agent (10% serum) markedly stimulated the release of PGE_2 in both cell types.

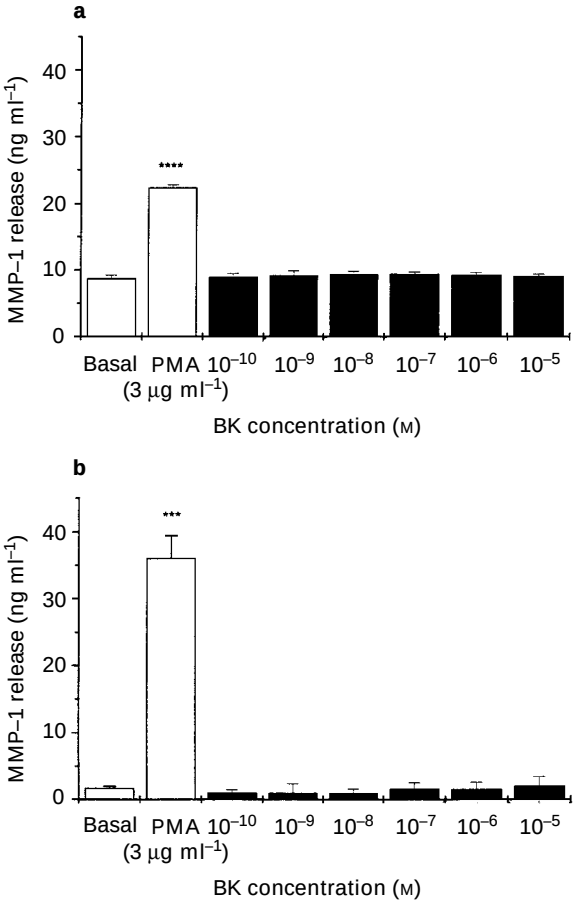

Figure 8 Effects of bradykinin (BK) and phorbol-12-myristate-13-acetate (PMA) on matrix metalloproteinase-1 (MMP-1) release from CEPI-17-CL4 (a) and P-CEPI (b) cells. Data are from 3–4 experiments/donors using BK (10^{-10}–10^{-5} M) or PMA (3 μg ml^{-1}) to stimulate the cells; vertical lines show s.e.mean. $^{***}P<0.001$; $^{****}P<0.0001$ as compared to the basal level by Student's t test.

MMP-1 release when tested in parallel with BK (Figure 8). Interestingly, the amount of PGE_2 released from P-CEPI cells exceeded that released from CEPI-17-CL4 cells ($n=3$–4) (Figure 7).

Discussion

We have previously demonstrated the presence of bradykinin (BK) B_2-receptors coupled to phospholipase C (PLC) in human CEPI cells and shown numerous BK-related agonists and antagonists to exhibit very similar pharmacological effects in P-CEPI and CEPI-17-CL4 cells (Wiernas *et al*., 1997). The present studies have confirmed some of those previous observations and have considerably extended the information on the B_2-receptors in these cell-types. Thus, the present [^{3}H]-BK binding studies indicated the presence of a single high-affinity ($K_d=1.66$ nM) binding site on the B_2-receptor with a rank order of pharmacological specificity/affinity (Hoe-140 > BK > [Tyr^8]-BK > > > [des-Arg^9]-BK) identical to that previously described for PI turnover in these cells (Wiernas *et al*., 1997). These data indicate that the high-affinity binding site detected by radioligand binding experiments represents the receptor site mediating the functional coupling to the PLC and the phosphoinositide (PI) turnover/$[Ca^{2+}]_i$ mobilization signal transduction pathway. The affinities of BK and related peptides and apparent B_2-receptor density found on the CEPI cells compare well with values obtained for human fibroblasts (Roscher *et al*., 1990), human pituitary cells (Sharif *et al*., 1988), murine fibrosarcoma cells (Sharif & Whiting, 1993) and various tissues such as guinea-pig ileum, lung, heart, brain and spinal cord (Sharif & Whiting, 1991; also see Hall, 1992 for review).

The stimulant effects and the potency of BK on PI turnover and $[Ca^{2+}]_i$ mobilization in CEPI cells and the blockade of these responses by the selective B_2-receptor antagonist, Hoe-140 (icatibant), are fully consistent with similar findings in several other cells (Sharif *et al*., 1988; Ransom *et al*., 1991; Sharif & Whiting, 1993; Smith *et al*., 1995; Sharif & Xu, 1996) and tissues (Butt *et al*., 1995; see Hall, 1992 for review) implicating a B_2-receptor involvement. The selectivity of Hoe-140 for the BK-induced responses was further evident from its inactivity against the histamine-induced Ca^{2+}-response in CEPI cells. The fact that the selective PLC inhibitor, U73122, effectively blocked both the PI turnover and $[Ca^{2+}]_i$ mobilization activated by BK in the CEPI cells strongly suggested that the product(s) of PI hydrolysis are directly involved in mobilizing $[Ca^{2+}]_i$ in CEPI cells. Again, these results are very similar to findings on the potency and effects of U73122 on various other cell-based systems, including platelets and neutrophils (Bleasdale *et al*., 1990), Swiss 3T3 cells (Griffin *et al*., 1997) and several other cell types (Berridge & Irvine,

1984). The BK-induced $[Ca^{2+}]_i$ mobilization in CEPI cells appeared to be primarily derived from intracellular stores since pre-treatment of the cells with the Ca^{2+}-chelator, EGTA, partially reduced the Ca^{2+}-signal and the Ca^{2+}-channel blocker, nifedipine, was without any significant effect. These results suggest that the BK-induced functional responses in the CEPI cells depend largely on the PLC-mediated generation of IP_3 to release $[Ca^{2+}]_i$ from the cellular endoplasmic reticulum, and also perhaps on the diacylglycerol (DAG) which is concomitantly generated from PI hydrolysis (Berridge & Irvine, 1984). The potential role(s) of DAG in CEPI cell function(s) remains to be defined.

Even though corneal epithelial cells express mRNAs for pro-inflammatory cytokines (Sharif *et al*., 1997a) and they are capable of releasing cytokines in response to various stimuli (Wakefield & Lloyd, 1992; Cubitt *et al*., 1993; Faquet *et al* ., 1997) and also during the development of the pathology of pseudophakic bullous keratopathy (Rosenbaum *et al*., 1995), BK did not stimulate the secretion of IL-6, IL-8 or GM-CSF from the human CEPI cells. However, it should be noted that the positive control agent (IL-1α) tested alongside with BK was a potent cytokine secretagogue in the CEPI cells. These results suggest that BK apparently does not have a pro-inflammatory function in the corneal epithelium as far as the release of cytokines is concerned. Similarly, while BK and related peptides have previously been shown to stimulate the production of PGE_2 in murine fibrosarcoma cells (Becherer *et al*., 1982) and in monocytes (Lerner *et al*., 1989), our studies showed that BK had no significant effect on PGE_2 release from CEPI cells. Once again, the cells in our experiments were responsive to serum, which was used as a positive control to induce PGE_2 release from the CEPI cells. It was interesting that the PGE_2 levels (basal and stimulated) in the P-CEPI cells were substantially higher than those observed in the CEPI-17-CL4 cells. One possible explanation for this may be that the trauma associated with enucleation and subsequent transport of the human cadaver eyes, and further the dispase treatment of the corneal epithelium, may have caused the up-regulation of the cyclo-oxygenases in the primary CEPI cells. In contrast, the immortalized cells had already been in culture over several passages and perhaps had a lower level of trauma associated with them relative to the primary cells. Other factors may also have been responsible for the observed difference in PGE_2 levels in the two cell types but this requires further study.

BK and prostaglandins are well established mediators of exudative and inflammatory phases of wound healing, mediating their proliferative effects via stimulation of $[Ca^{2+}]_i$ and DAG production leading to elevation of DNA synthesis (Ziche *et al*., 1992; Berridge, 1993; Lowe *et al*., 1993; Weber *et al*., 1996; Vernhet *et al*., 1996). Accordingly, in our experiments, nanomolar concentrations of BK potently stimulated the PI turnover and $[Ca^{2+}]_i$ mobilization (see above) and also stimulated incorporation of [^{3}H]-thymidine into the DNA of CEPI cells indicative of a mitogenic effect of BK in these cells. Such a cell proliferative effect of BK on CEPI cells appears to be in line with a possible role of BK (released into the tear-film during corneal injury/trauma) in corneal wound healing. This possible mitogenic function of corneal BK receptors is supported by similar findings of BK-induced cell proliferation for tissue repair, a protective mechanism, in cardiac tissue (Weber *et al*., 1996), liver cells (Vernhet *et al*., 1996), human lung fibroblasts (Dalemar *et al*., 1996), smooth muscle cells like DDT1MF-2 (Graber *et al*., 1996), and in various other cells/tissues (see Marceau *et al*., 1983; Hall, 1992 for reviews). Since P-CEPI and CEPI-17-CL4 cells express high levels of mRNAs for epidermal growth factor (EGF), platelet derived growth factor, transforming growth factor-alpha (TGF-α), TGF-β and also mRNA for EGF-receptors (Sharif *et al*., 1997a), it is also possible that BK could stimulate the release of these growth factors from the CEPI cells to enhance further CEPI cell proliferation, such as during repair of corneal wounds. However, these aspects require careful study, especially since BK can also inhibit DNA synthesis as shown for human mammary cells (Patel & Schrey, 1992) or inhibit DNA synthesis induced by EGF (McAllister *et al*., 1993).

In conclusion, our studies have shown the presence of high-affinity [^{3}H]-BK binding sites on human CEPI cells which represent the pharmacologically responsive B_2-receptors mediating PI hydrolysis, leading to the generation of IPs, which in turn stimulate $[Ca^{2+}]_i$ mobilization predominantly from intracellular stores. The consequence of BK receptor activation in the human CEPI cells appears to be the stimulation of [^{3}H]-thymidine incorporation into cellular DNA, thus reflecting a potential role of BK in cell proliferation as may be necessary during corneal wound healing. The present studies, under the defined experimental conditions, did not show the release of MMP-1 or various cytokines or PGE_2 from these cells in response to BK. Taken together, it seems that BK acting via B_2-receptors primarily stimulates CEPI cell proliferation by inducing [^{3}H]-thymidine incorporation in CEPI cells and thus BK appears to have a potential role in corneal wound healing. *In vivo* studies to address the latter issue appear warranted and should be performed in the future.

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