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Pharmacological and functional characterization of bradykinin B_2 receptor in human prostate

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Abstract

The objective of this study was to pharmacologically characterize bradykinin receptors, a component of the kallikrein–kinin system, in normal human prostate cells. In primary cultured human prostate stromal cells, bradykinin, but not [des-Arg^9]bradykinin or [des-Arg^{10}]kallidin, produced calcium mobilization or inositol phosphates accumulation with potencies (pEC_{50}) of 8.8±0.2 and 8.2±0.2, respectively. This was consistent with abundance of bradykinin B_2 mRNA over bradykinin B_1 mRNA in prostate stromal cells. Although the prostate epithelial cells (prostate epithelium, BPH-1, and PC-3) expressed mRNA for bradykinin B_2 receptors (albeit in lesser amounts than stromal cells), bradykinin was not functionally efficacious in the epithelial cells. Increasing concentrations of D-arginyl-L-arginyl-L-prolyl-*trans*-4-hydroxy-L-prolylglycyl-3-(2-thienyl)-L-alanyl-L-seryl-D-1,2,3,4-tetrahhydro-3-isoquinolinecarbonyl-L-(2α,3β,7αβ)-octahydro-1*H*-indole-2-carbonyl-L-arginine (HOE-140), a bradykinin B_2-selective peptide antagonist, attenuated bradykinin concentration–response curves in human prostate stromal cells with apparent estimate of affinity similar to that for the human bradykinin B_2 receptor. Bradykinin (10 nM) caused proliferation of prostate stromal cells and phosphorylated extracellular signal-regulated kinases (ERK-1 and ERK-2) that were blocked by HOE-140 (1 μM). This study demonstrated that, in primary cultures of normal human prostate stromal cells, bradykinin activates bradykinin B_2 receptors that may play a significant role in proliferation via activation of ERK-1/2 pathways.

Keywords: Bradykinin; Human, prostate; Prostate stromal cell; Prostate epithelial cell

1. Introduction

The endogenous nonapeptide bradykinin is a product of a functional kallikrein–kinin system (Blaukat, 2003). Components of the kallikrein–kinin system are as follows: (a) kininogens, both high and low molecular weight, (b) kallikreins, enzymes that generate bradykinin, and (c) kininases, that metabolize bradykinin to [des-Arg^9]bradykinin (Campbell, 2003). Bradykinin and [des-Arg^9]bradykinin activate type I G-protein-coupled receptors, namely bradykinin B_2 and bradykinin B_1 receptors, respectively. Both of the human bradykinin receptor subtypes have been cloned and characterized (Hess et al., 1992; Menke et al., 1994). These receptor subtypes couple preferentially with $G_{\alpha q}$ subtype of G-proteins activating the inositol phosphates pathway (Blaukat, 2003). Bradykinin is a selective agonist for the bradykinin B_2 receptor, while the bradykinin B_1 receptor exhibits greater selectivity for agonism by the cleaved product of bradykinin, [des-Arg^9]bradykinin or [des-Arg^{10}]kallidin (Simpson et al., 2000). Both peptide and nonpeptide antagonists, with high selectivity and affinity, for either bradykinin B_1 or bradykinin B_2 have been discovered (Altamura et al., 1999; Wood et al., 2003). D-arginyl-L-arginyl-L-prolyl-*trans*-4-hydroxy-L-prolylglycyl-3-(2-thienyl)-L-alanyl-L-seryl-D-1,2,3,4-tetrahhydro-3-isoquinolinecarbonyl-L-(2α,3β,7αβ)-octahydro-1*H*-indole-2-carbonyl-L-arginine (HOE-140) and D-Arginyl-L-arginlyl-L-prolyl-*trans*-4-

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hydroxy-L-prolylglycyl-3-(2-thienyl)-L-alanyl-L-seryl-D-1,2,3,4-tetrahydro-3-isoquinolinecarbonyl-L-(2α, 3β, 7αβ)-octahydro-1*H*-indole-2-carbonyl ([des-Arg^{10}]HOE-140) are potent and selective peptide antagonists for the bradykinin B_2 and bradykinin B_1 receptors, respectively (Wirth et al., 1991a,b), and have been used in this study to characterize the bradykinin receptors in human prostate cells in culture.

All or some components of the kallikrein–kinin system modulate sensory afferent signals, regulate efferent neuronal drive and interact with trophic factors across many species including humans (Howl and Payne, 2003). Bradykinin receptor antagonists have been described as having potential therapeutic utility for respiratory disorders, inflammation, pain, and stroke (Ding-Zhou et al., 2003; Hirayama et al., 2003; Howl and Payne, 2003). In genitourinary tissues, the kallikrein–kinin system is functional and regulates the tone of the urinary bladder and prostate of many species (Steidle et al., 1990; Butt et al., 1995; Lecci et al., 1995, 1999; Belichard et al., 1999; Meini et al., 2000; Davis and Burgess, 2002; Srinivasan et al., 2004). Increased levels of bradykinin were apparent in the bladder wall of interstitial cystitis patients (Rosamilla et al., 1999) and increased bradykinin immunoreactivity was found in patients diagnosed with benign prostatic hyperplasia (Walden et al., 1999). Bradykinin also induced proliferation of both benign and malignant human prostate cells (Walden et al., 1999; Stewart et al., 2002; Taub et al., 2003). Kallikreins are expressed in human prostate (Stephan et al., 2003; Yousef et al., 2003) and have been evaluated as biomarkers for benign prostate hyperplasia (Scorilas et al., 2003). In fact, bradykinin receptor antagonists are pursued as novel therapeutic agents for prostate cancer (Stewart et al., 2002). Furthermore, the kallikrein–kinin system is also believed to be functional in prostatic seminal fluid (Schill and Miska, 1992; Charlesworth et al., 1999). In light of these findings, it is important to characterize bradykinin receptor subtypes in normal human prostate, as the fully characterized and functional receptor may be a 'druggable target' for lower urinary tract dysfunction. Therefore, the major objectives of this study were (i) to characterize the bradykinin receptor subtype in healthy human prostates of both stromal and epithelial cell types and (ii) to elucidate a function of bradykinin receptors that may be related to uncontrolled cell growth such as in prostate cancer and benign prostate hyperplasia.

2. Methods

2.1. Materials

Human prostate stromal and prostate epithelial cells, media for culturing prostate cells and reagents to subculture (trypsin, trypsin-inhibitor, and Hank's balanced salt solution), were obtained from Cambrex Biosciences (Walkersville, MD). The androgen-insensitive PC-3 and androgen-sensitive LNCaP prostate cancer cell lines were obtained from the American type culture collection (ATCC, Manassas, VA). BPH-1 cell line was obtained under a license agreement from The University of California at San Francisco. BPH-1 cell line is an epithelial cell line generated from a patient clinically diagnosed with benign prostate hyperplasia (Hayward et al., 1995). All the media and reagents used for cell culture were purchased from Invitrogen (Carlsbad, CA) unless otherwise indicated. Cell culture flasks and pipettes were obtained from BD Biosciences (Bedford, MA). Antibodies against smooth muscle actin, vimentin, and high-molecular-weight cytokeratin were obtained from Dako (Carpinteria, CA). The goat–anti-mouse secondary antibody conjugated to Alexa-488 was obtained from Molecular Probes (Eugene, OR). Bradykinin, [des-Arg^9]bradykinin, [des-Arg^{10}]kallidin, HOE-140, [des-Arg^{10}]HOE-140, lysophosphatidic acid, probenecid, lithium chloride, calcium chloride, methanol, and acetone were obtained from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture

Human prostate stromal and human prostate epithelial cells used were derived from prostate tissue of a 28-year-old and a 17-year-old donor, respectively. Prostate cells were grown and subcultured (maximum of 7 passages) as per manufacturer's instructions. Briefly, cells were grown in T-75 cm^2 Primaria™ flasks (BD Biosciences). Complete media for stromal cells contained fetal bovine serum, human fibroblast growth factor, insulin, and gentamicin/amphotericin-B. Media for epithelial cells did not contain serum but had a cocktail of bovine pituitary extract, hydrocortisone, human epithelial cell growth factor, epinephrine, insulin, triiodothyronine, transferrin, gentamicin/amphotericin-B, and retinoic acid. Cells were sub-cultured at approximately 90% confluency. For subculture, cells were first washed with Hank's balanced salt solution containing 30 mM HEPES and trypsinized (0.025%) at room temperature. The trypsin was neutralized with a trypsin-inhibitor and cells were then centrifuged at 1600×*g* for 5 min at room temperature. Supernatant was then aspirated and cell pellet was resuspended in appropriate media and aliquoted into new cell culture flasks. Cells were split at a ratio between 1:5 and 1:8.

Androgen-sensitive LNCaP cells and androgen-independent PC-3 cells were cultured in complete growth media comprised of Roswell Park Memorial Institute (RPMI)-1640 containing 10% fetal bovine serum, 20 mM HEPES and antibiotic/antimycotic cocktail or nutrient mixture (Ham's F-12) containing 10% fetal bovine serum and antibiotic/antimycotic cocktail, respectively. BPH-1 cells were cultured in RPMI-1640 with 5% fetal bovine serum, 25 mM HEPES, geneticin (250 μg/ml), and antibiotic/antimycotic cocktail as described previously (Hayward et al., 1995). Recombinant human bradykinin

B_2 receptors were expressed in Chinese Hamster Ovary (CHO) cells as described previously (Jarnagin et al., 1996). These cells were cultured in Ham's F-12 media supplemented with 10% serum containing antibiotic/antimycotic cocktail. All cells were maintained in a humidified atmosphere (5% CO_2) at 37 °C.

2.3. Immunocytochemistry of human prostate stromal and human prostate epithelial cells

Human prostate stromal (passage 6 through 7) and human prostate epithelial (passage 4 through 6) cells were grown on 6-well dishes containing sterile cover slips in appropriate complete growth media. Cells were fixed at −20 °C for 10 min, in a 7:3 mixture of methanol:acetone. Nonspecific binding sites were blocked using 5% bovine serum albumin for 30 min at 37 °C. Cells were then incubated with antibodies (mouse-monoclonal) against smooth muscle actin, vimentin, or cytokeratin (1:500 dilution in 5% bovine serum albumin) for 1 h at room temperature. Cells were then washed six times with phosphate-buffered saline containing 0.05% Triton X-100. Cells were then incubated with a goat–anti-mouse secondary antibody conjugated to Alexa-488 (1:1000 dilution, 5% bovine serum albumin) for 1 h at room temperature, followed by six washes with phosphate-buffered saline containing 0.05% Triton X-100. The cover slips were then mounted onto slides using Vectashield™ mounting media with 4′, 6′-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) and sealed with Cytoseal™ 60 (Richard-Allen Scientific, Kalamazoo, MI). To evaluate the specificity of the staining, only secondary control conditions were performed for each experiment. The labeling was viewed with a Nikon Microphot 5A microscope and images acquired using an attached digital camera (Diagnostic Imaging, Sterling Heights, MI).

2.4. TaqMan analysis

Total RNA was isolated from human prostate stromal, human prostate epithelial, BPH-1, LNCaP, and PC-3 cells using the Trizol method (O'Reilly et al., 2002). Human bradykinin B_1 and bradykinin B_2 receptor primers and probes were used to amplify 50 ng total RNA isolated from human prostate samples using "One-Step RT-PCR Master Mix Reagents" (Applied Biosystems, Foster City, CA). Amplification and detection was performed with the ABI Prism 7700 sequence detection system with the following cycle profile: 1 cycle at 48 °C for 30 min, 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. 18*S* rRNA primers were used for normalization. The following primers and probes were used: bradykinin B_1 receptor forward primer: 5′-TTGAGCATCCCCACATTCCT-3′; bradykinin B_1 receptor reverse primer: 5′-CAGGCGGTGATGTTCAGATCT-3′; bradykinin B_1 receptor probe: 5′-FAM-CTGCGAT CCATCCAAGCCGTCC-TAMRA-3′; bradykinin B_1 receptor standard curve oligo: 5′-TTGAGCATCCCCACATTCCTGCTGCGATCCATCCAAGCCGTCCCAGATCTGAACATCACCGCCTG-3′; bradykinin B_2 receptor forward primer: 5′-CAGGTCAGAACCCATTCAGATG-3′; bradykinin B_2 receptor reverse primer: 5′-CTGGCGTTCCACGGAGAT-3′; bradykinin B_2 receptor probe: 5′-FAM-CCATGGGCACACTGCGGACCT-TAMRA-3-; bradykinin B_2 receptor standard curve oligo: 5′-CAGGTCAGAACCCATTCAGATGGAGAACTCCATGGGCACACTGCGGACCTCCATCTCCGTGGAAC GCCAG-3′.

2.5. Measurement of intracellular calcium using the Fluorometric Imaging Plate Reader (FLIPR)

Intracellular calcium flux was measured by FLIPR, as described previously (Sullivan et al., 1999; Srinivasan et al., 2004). Briefly, cells (human prostate stromal cells and CHO cells expressing the human bradykinin B_2 receptor as positive control) were seeded into black-walled clear-bottom 96-well plates (Corning, Acton, MA) at a density of 25,000 cells per well. Cells were grown in appropriate complete growth media and cultured overnight and washed with FLIPR-buffer (Hank's balanced salt solution with 10 mM HEPES, 2.5 mM probenecid, 2 mM calcium chloride, and 0.1% bovine serum albumin). FLIPR-buffer containing 4 μM of *N*-[4-[6-[(acetyloxy)methoxy]-2,7- dichloro-3-oxo-3*H*-xanthen-9-yl]-2-[2-[2- [bis[2-[(acetyloxy)methoxy]-2- oxyethyl]amino]-5- methylphenoxy]ethoxy]phenyl]-*N*-[2- [(acetyloxy)methoxy]-2-oxyethyl]-, (acetyloxy)methyl ester (Fluo-3 AM; Teflabs, Austin, TX) was then added to the cells. Cells were allowed to uptake the Fluo-3 AM dye for 1 h at 37 °C in 5% CO_2. Cells were then washed in FLIPR-buffer. For the curve shift experiments, plates were incubated for 30 min at room temperature with either buffer alone (vehicle) or buffer containing various concentrations of the antagonists. The plates were then placed in the FLIPR device (Molecular Devices, Sunnyvale, CA) and relative changes in fluorescence upon addition of various agonists was monitored (λ_{ex}=488 nM, λ_{em}=540 nM).

2.6. Scintillation Proximity Assay (SPA) of inositol phosphates accumulation

The assay was performed as described by Brandish et al. (2003). Briefly, human prostate stromal cells in T-162 cm^2 flasks were grown until they were about 80% confluent. Cells were then washed with Hank's balanced salt solution containing 30 mM HEPES and then trypsinized (0.025%). The trypsin was neutralized using a defined trypsin inhibitor (Cascade Biologics, Denver) followed by centrifugation at 1600×*g* and the supernatant was then aspirated. Cells were resuspended in inositol-free medium containing 5 μCi/ml [^{3}H]myoinositol (Amersham, Piscataway, NJ) and 0.1% bovine serum albumin. Cells were then seeded onto a collagen-coated 96-well plate at

25,000 cells per well and the plate was incubated overnight at 37 °C in an incubator (5% CO_2). Inositol-free media containing 20 mM lithium chloride and 0.1% bovine serum albumin was then added for 30 min. Vehicle (water) or agonists were then added to the appropriate wells and the plate was incubated for 1 h. The incubation mixtures were then aspirated and 100 μl of 20 mM formic acid was added to each well. About 20 μl of formic acid extract was then transferred into a solid white scintillation proximity assay plate (Dynex Technologies, Chantilly, VA) and 80 μl of yttrium-silicate beads (1 mg cells/well final concentration) was added and the plate was shaken for 30 min. Yttrium-silicate beads were allowed to settle for 30 min and the radioactivity was counted using Topcount (Perkin–Elmer, Boston, MA).

2.7. Colorimetric cellular proliferation assay

Cellular proliferation of human prostate stromal, human prostate epithelial, PC-3 and LNCaP cells was measured using the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay (Roche Diagnostics, Mannheim, Germany). MTT, a pale yellow substrate, is cleaved only by metabolically active cells to form a formazan dye which can be detected colorimetrically (Berridge and Tan, 1993). Briefly, cells were seeded onto 96-well plates at a density of 2000 cells per well in 100 μl of appropriate complete growth media and were allowed to attach to the plates for 16–20 h in a humidified incubator at 37 °C (5% CO_2). Next day, after visual inspection to confirm that all the cells were attached, the complete media was substituted with appropriate basal media. Cells were then allowed to equilibrate in the basal media for 1 h and treated with appropriate drugs or vehicle. Plates were then placed in the incubator and cells were allowed to grow for another 48 h. A total of 48 h after the first addition of drugs or vehicle, 10 μl of the MTT dye was added per well and allowed to incubate for 4 h. About 100 μl of the solubilization buffer was then added to each well and the plates were incubated for additional 16 h after which they were read using a Shimadzu plate reader (Shimadzu, Columbia, MD) at 550 nm. Readings were also taken at 690 nm to measure background that was then subtracted from the 550-nm reading.

2.8. Western blots for extracellular signal-regulated kinase (ERK) activation

Human prostate stromal and prostate epithelial cells were seeded at a density of 200,000 cells/well in a 6-well plate and were grown to about 80% confluency. Growth media was aspirated and cells were serum-starved overnight in appropriate basal media. Cells were then treated with vehicle, 10 nM bradykinin or 10 nM [des-Arg^9]bradykinin for 10 min at 37 °C. For testing the antagonist, human prostate stromal cells were pretreated with 1 μM HOE-140 for 30 min and then treated with bradykinin or vehicle for 10 min. The plates were then placed on ice and the cells were washed two times with cold Hank's balanced salt solution (and 30 mM HEPES). About 500 μl of mammalian protein extraction reagent containing protease inhibitors (Pierce, Rockford, IL) was added per well and incubated at room temperature for 15 min. Cellular protein was then collected and centrifuged (14,000×g, 5 min, 4 °C) to remove debris. Sample buffer and reducing agent was added to 1 μg of protein, placed in boiling water for 10 min and then loaded onto a 10% Bis–Tris gel. Gels were run using 3-(*N*-morpholino)propanesulfonic acid (MOPS) in sodium dodecyl sulfate (SDS) running buffer as per manufacturers instructions (200 V, 1 h) and were then transferred onto nitrocellulose membranes using transfer buffer with 10% methanol for 90 min at room temperature. Equal loading and efficient transfer was confirmed by Ponceau S staining. Nonspecific binding sites on the membranes were then blocked with 3% non-fat dry milk in Tris buffered saline containing 0.05% Triton X-100 and 0.05% Tween for 1 h at room temperature. Membranes were then incubated with 1:2000 mouse anti-phospho ERK-1/2 antibodies (Cell Signaling, SD, CA) overnight at 4 °C and washed three times at 15-min intervals. Membranes were then incubated with 1:10,000 goat–anti-mouse antibodies conjugated to horseradish peroxidase (Jackson Laboratory, Bay Harbor, Maine) for 2 h at room temperature. Following incubation, membranes were washed, treated with Western blotting luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 min, and exposed to film which was developed using a developer. Membranes were then stripped at room temperature using a Restore™ Western blot stripping solution (Pierce) for 15 min. After repeated washing, the membranes were blocked in 3% milk for 1 h at room temperature and were incubated overnight in 3% milk containing 1:1000 rabbit anti-Total ERK-1/2 polyclonal antibodies at 4 °C. The following day, membranes were washed, incubated with 1:10,000 goat–anti-rabbit horseradish peroxidase for 2 h. After a final wash step, the membranes were treated with luminol and exposed to film which was then developed.

2.9. Data analyses

Data obtained from the FLIPR assay was collected as maximum fluorescence intensity (spatial uniformity correction was activated to normalize for cell number). Changes in fluorescence intensity were then expressed as a percentage of appropriate concentration of lysophosphatidic acid or bradykinin. Nonlinear regression analysis was performed on concentration–response curves to determine potency (pEC_{50}) of the agonists (Graph Pad Software, San Diego, CA). The nature of the interaction of HOE-140 with the bradykinin B_2 receptors was estimated by Schild analysis (Arunalakshana and Schild, 1959). Apparent antagonist dissociation constants (K_B) were determined for HOE-140 according to the equation K_B=[HOE-140]/(CR-1), where

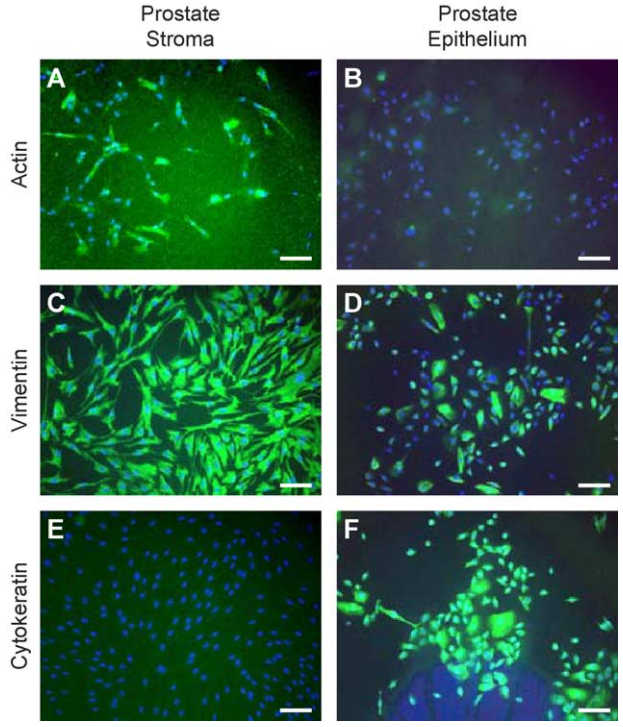


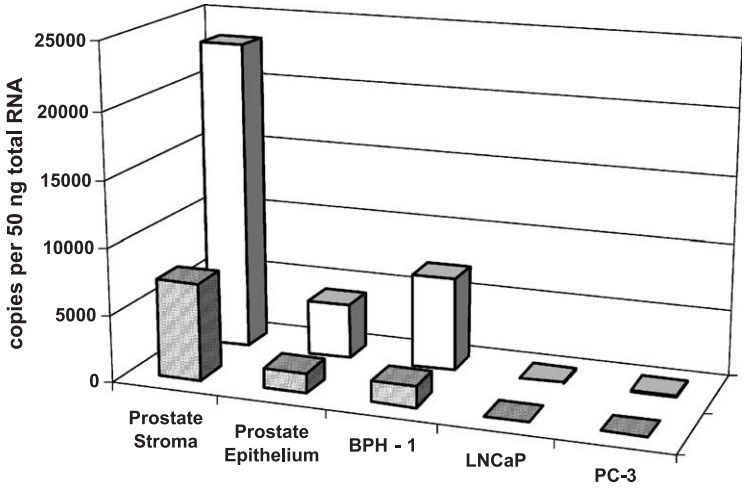
Fig. 1. Primary cultures of human prostate stromal and human prostate epithelial cells. Human prostate stromal (A, C, and E) and prostate epithelial (B, D, and F) cells were labeled with antibodies against smooth muscle actin (actin), vimentin, or high-molecular-weight cytokeratin (cytokeratin). Nuclear staining is indicated by the blue DAPI labeling. Data shown was taken at 20× magnification and is representative of three independent experiments. The horizontal bar represents 5 μm.

CR is the ratio of EC_{50} of bradykinin in the presence of HOE-140 divided by EC_{50} of bradykinin alone. Because HOE-140 antagonism in the human prostate stromal cells produced suppression of maximal response to bradykinin, K_B of HOE-140 was also calculated from a double-reciprocal plot (Kenakin, 1997). For the cellular proliferation assays, absorbance values at 550 (A_{550}) and 690 nm (A_{690}) were obtained and A_{550}–A_{690} values were calculated. Data was then expressed as a percent change from the vehicle group. Statistical significance, at 95% confidence interval, was calculated using a Student's *t*-test. Values reported in this study are mean±S.E.M for the number of experiments (n) stated.

3. Results

3.1. Immunohistochemical characterization of human prostate cells in culture

Human prostate stromal and epithelial cells were characterized using antibodies against smooth muscle actin, vimentin, or high-molecular-weight cytokeratin. Fig. 1 depicts a representative picture of cell-specific marker expression for all the passages of cells used in the study. Smooth muscle actin or vimentin are expressed in smooth muscle cells or fibroblasts respectively, while cytokeratins are specific to epithelial cells (Kooistra et al., 1995). Prostate stromal cells expressed smooth muscle



	Prostate Stroma	Prostate Epithelium	BPH-1	LNCaP	PC-3
Bradykinin B_1 receptor (hatched bars)	**7326**	**1545**	**1612**	**12**	**30**
Bradykinin B_2 receptor (white bars)	**23253**	**4098**	**7020**	**61**	**106**

Fig. 2. Bradykinin receptor transcript (mRNA) levels in human prostate cells in culture. The graph depicts relevant abundance of bradykinin B_1 (hatched bar) and bradykinin B_2 (white bar) receptor transcripts in human prostate stromal, prostate epithelial, BPH-1, LNCaP, and PC-3 cells. Absolute copy numbers of bradykinin B_2 or bradykinin B_1 receptor mRNA, per 50 ng total RNA, in various cell types, are presented in a tabular format at the bottom of the graph.

actin (Fig. 1A) and vimentin (Fig. 1C) but not high-molecular-weight cytokeratin (Fig. 1E), although there were cells in the field of view as indicated by nuclear staining. These results suggested that prostate stromal cells in culture were predominantly stromal. Human prostate epithelial cells on the other hand predominantly labeled with anti-cytokeratin (Fig. 1F) with no specific labeling for smooth muscle actin (Fig. 1B). Interestingly, a subpopulation of human prostate epithelial cells also expressed the fibroblastic marker vimentin (Fig. 1D). There were no significant changes in the expression pattern of these proteins with cell passages (maximum of 7) used in this study.

3.2. mRNA for bradykinin B_1 and bradykinin B_2 receptor

After characterizing the human prostate stromal and prostate epithelial cells, we first evaluated the presence of bradykinin receptor transcripts using TaqMan analysis. Transcripts for the bradykinin B_2 receptor were most abundant in human prostate stromal cells (Fig. 2). The human prostate epithelial and other prostate epithelial cells including BPH-1, LNCaP, and PC-3 cells had lower amounts of mRNA for bradykinin B_2. Bradykinin B_2 mRNA was greater than that for bradykinin B_1 mRNA in all cell types tested. There was significant message for the bradykinin B_1 receptor in human prostate stromal cells as well, although it was about three times less abundant than the bradykinin B_2 counterpart.

3.3. Bradykinin-induced calcium flux (FLIPR) in prostate cells

To study whether mRNA for bradykinin B_2 and bradykinin B_1 receptors correlated with functional protein expression, we studied changes in intracellular calcium using the FLIPR assay as described in the methods section. In the human prostate stromal cells, the bradykinin B_2 agonist bradykinin, but not the bradykinin B_1 agonist [des-Arg^9]bradykinin, produced a concentration-dependent mobilization of intracellular calcium (Fig. 3A) with a potency (pEC_{50}) of 8.8±0.2. In the human prostate epithelial cells, neither the bradykinin B_2 nor the bradykinin B_1 agonist produced any change in intracellular calcium (Fig. 3B). To rule out the possibility of an inefficient coupling machinery in cultured prostate epithelial cells that may account for the lack of response to bradykinin or [des-Arg^9]bradykinin, we stimulated lysophosphatidic acid receptors, that are expressed in these cells and are known to mediate signal transduction leading to mobilization of intracellular calcium (Fukushima and Chun, 2001; Daaka, 2002). As expected, lysophosphatidic acid increased intracellular calcium in human prostate epithelial cells with a pEC_{50} of 6.7±0.3 (Fig. 3B). Lysophosphatidic acid was equipotent (pEC_{50}: 7.1±0.1) in prostate stromal cells as well (Fig. 3A). These results suggested that, while the lysophosphatidic acid receptor coupling is functional in both

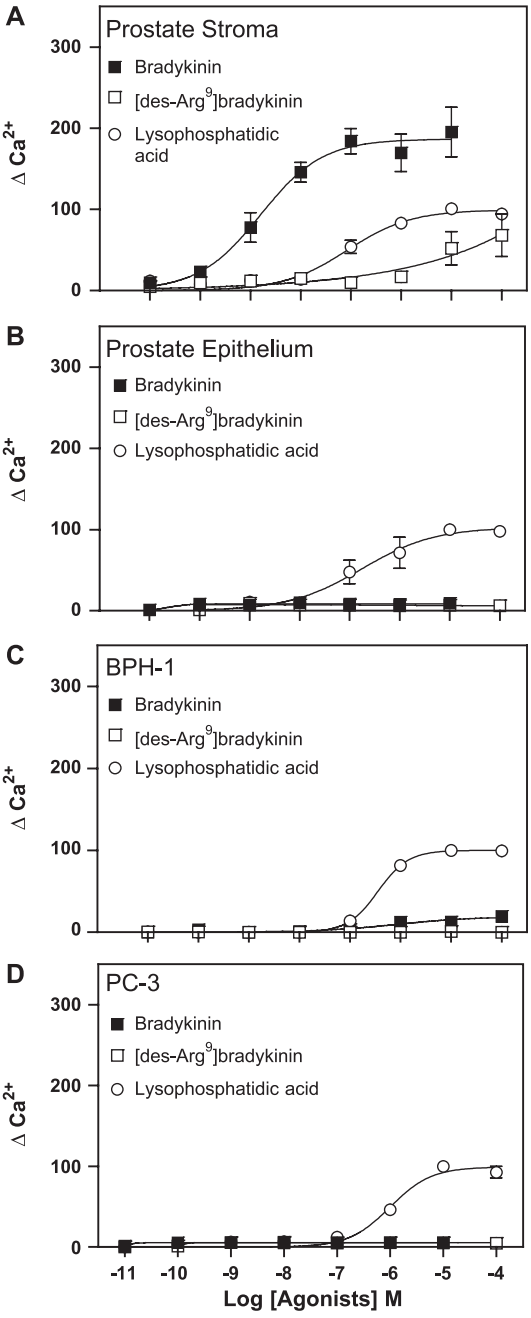


Fig. 3. Concentration-response curves for bradykinin (■), [des-Arg^9]bradykinin (□), and lysophosphatidic acid (○) in human prostate stromal (A), prostate epithelial (B), BPH-1 (C), and PC-3 (D) cells . Responses were measured as changes in fluorescence (ΔCa^{2+}) and expressed relative to 10 μM lysophosphatidic acid. Data points represent mean values and vertical lines indicate S.E.M. from three independent experiments.

stromal and epithelial cells, bradykinin B_2 receptors were only present in prostate stromal cells. Functional presence of bradykinin B_2 or bradykinin B_1 receptor was not evident in the normal prostate epithelial cells, at least with respect to changes in intracellular calcium.

The effect of bradykinin was also studied in both benign and malignant prostate cells, such as BPH-1 and PC-3 cells, respectively. Interestingly, in the BPH-1 cell line, bradyki-

nin, but not [des-Arg^9]bradykinin, induced mobilization of intracellular calcium, although the maximal response of bradykinin was significantly lower than that observed in the human prostate stromal cells (Fig. 3C). However, this observation may indicate increased coupling efficiency or expression of bradykinin B_2 receptors in prostatic epithelium during benign prostate hyperplasia. Contrary to this, in the PC-3 cells, neither bradykinin nor [des-Arg^9]bradykinin mobilized intracellular calcium (Fig. 3D). Again, lysophosphatidic acid was a potent agonist in both BPH-1 and PC-3 cells, with potency values of 6.4±0.1 and 6.0±0.1, respectively.

3.4. Antagonism of bradykinin B_2 receptors: dextral curve shift and Schild analysis

To confirm whether bradykinin-induced increases in intracellular calcium in the prostate stromal cells were indeed mediated by bradykinin B_2 receptors, we studied the ability of HOE-140 and [des-Arg^{10}]HOE-140, selective antagonists for bradykinin B_2 and bradykinin B_1 receptors, respectively, to attenuate bradykinin-induced FLIPR response in human prostate stromal cells (Fig. 4). Increasing concentrations of HOE-140 caused a rightward shift of the bradykinin-induced concentration–response curve in human prostate stromal cells while [des-Arg^{10}]HOE-140 failed to attenuate bradykinin-induced calcium fluxes in these cell types (Fig. 4A and C). Interestingly, in the prostate stromal cells, [des-Arg^{10}]HOE-140 consistently induced a potentiation of maximal response to bradykinin (Fig. 4C) but the potentiation was not concentration-dependent.

These results were consistent with the hypothesis that human prostate stromal cells expressed the bradykinin B_2 receptor subtype, based on agonist rank order described previously in Fig. 3. The recombinant human B_2 receptor (hB2-CHO) was also used in a parallel and similar assay as a positive control (Fig. 4B and D). As nicely depicted, HOE-140 caused a dextral shift as expected, while [des-Arg^{10}]HOE-140 did not shift bradykinin concentration–response curves in the recombinant system. Apparent affinity estimate for HOE-140 at the human prostate

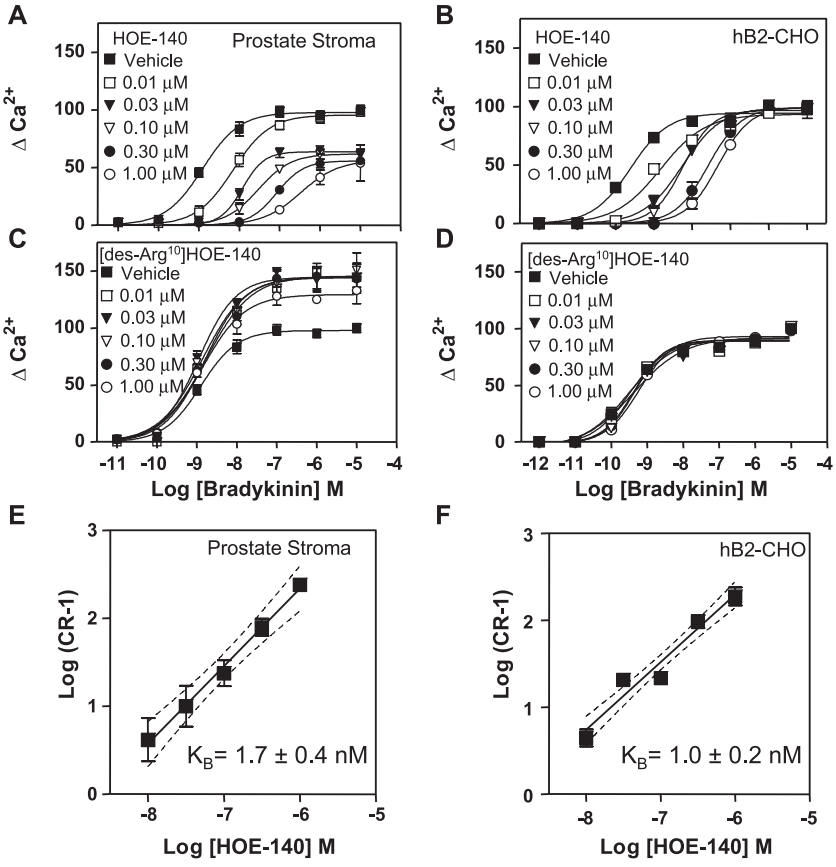


Fig. 4. Increasing concentrations of the bradykinin B_2-selective antagonist HOE-140 attenuated bradykinin-induced calcium mobilization in human prostate stromal (A) and in recombinant Chinese Hamster Ovary cells expressing the human bradykinin B_2 receptor (hB2-CHO; B). Contrary to this, the bradykinin B_1-selective antagonist [des-Arg^{10}]HOE-140 did not shift bradykinin concentration response curves in either human prostate stromal (C) or in hB2-CHO (D) cells. Responses were measured as changes in fluorescence (ΔCa^{2+}) and expressed relative to 10 μM bradykinin. Schild analysis of HOE-140 in prostate stromal and in hB2-CHO cells is depicted in panels E and F, respectively, with 95% confidence intervals (- - -). CR indicates concentration ratio. Log (CR-1) values were plotted against increasing concentrations of HOE-140. Calculated affinity estimate (K_B) values are listed (E and F; inset). Points represent mean values and vertical lines indicate S.E.M. from three independent experiments.

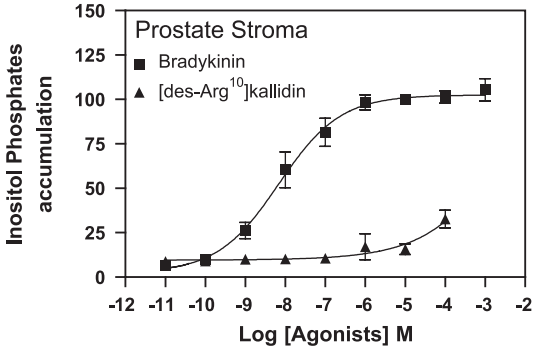


Fig. 5. Bradykinin-induced accumulation of total inositol phosphates in human prostate stromal cells. Increasing concentrations of bradykinin (■), but not [des-Arg^{10}]kallidin (▲), induced an accumulation of total inositol phosphates. Inositol phosphate accumulation was measured as radioactivity counts and normalized to 10 μM bradykinin. Points represent mean values and vertical lines indicate S.E.M. from three independent experiments.

stromal cells and at the cloned human bradykinin B_2 receptor were calculated by Schild analysis (Arunalakshana and Schild, 1959; Fig. 4E and F). Estimated affinity of HOE-140 at the recombinant human bradykinin B_2 receptor was 1.0±0.2 nM (slope: 0.85±0.05). Likewise, the Schild estimate of HOE-140 for the bradykinin receptor in the human stromal cells was 1.7±0.4 nM (slope: 0.88±0.1). Therefore, it appears that the bradykinin B_2 receptor is the predominant functional bradykinin receptor subtype in human prostate stromal cells.

3.5. Bradykinin B_2 receptor-induced inositol phosphates accumulation in human prostate stromal cells

Having determined functional presence of bradykinin B_2 receptors in human prostate stromal cells, we wanted to study inositol phosphates accumulation specifically, as calcium mobilization measured in a FLIPR assay is a global representation of intracellular calcium change. Because calcium is a ubiquitous intracellular messenger, calcium mobilization is a function of both inositol phosphates and noninositol phosphates pathway activation by cell surface receptors (Nowycky and Thomas, 2002). Bradykinin induced a concentration-dependent increase in total inositol phosphates accumulation with a pEC_{50} of 8.2±0.2 (Fig. 5). The bradykinin B_1-selective agonist, [des-Arg^{10}]kallidin, did not elicit accumulation of total inositol phosphates except at high concentrations (>1 μM), indicating that, in human prostate stromal cells, bradykinin B_2 receptors couple to the inositol phosphates pathway leading to increases in intracellular calcium.

3.6. Bradykinin-induced proliferation of human prostate cells

Bradykinin is known to induce cellular proliferation of benign human prostate cells (Walden et al., 1999; Barki-Harrington and Daaka, 2001). We evaluated whether bradykinin can stimulate proliferation of human prostate cells in culture (Fig. 6). Again, we used lysophosphatidic acid since it was established to have functional receptors in human prostate. In the human prostate stromal cells, bradykinin (white bars; 10 nM and 1 μM) significantly promoted cell growth, whereas lysophosphatidic acid (hatched bars) inhibited growth at the higher concentration (1 μM; Fig. 6A). Bradykinin did not induce cellular growth nor did it inhibit proliferation like lysophosphatidic acid did, in human prostate epithelial, PC-3 or LNCaP cells (Fig. 6B–D). Contrary to this,

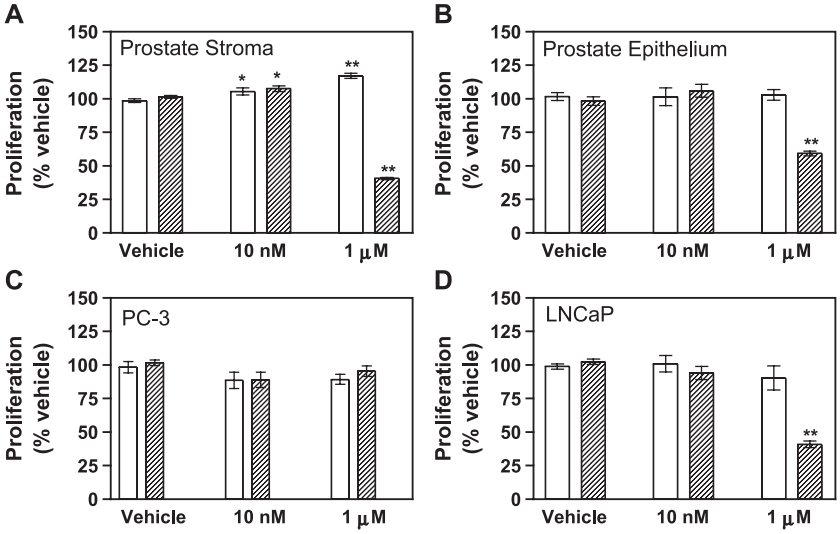


Fig. 6. Bradykinin induced cellular proliferation in serum starved human prostate cells. Bradykinin (white bars) caused proliferation of prostate stromal (A) but not prostate epithelial, PC-3, or LNCaP (B, C, and D, respectively) cells. 1 μM lysophosphatidic acid (hatched bars) significantly inhibited cellular growth of prostate stromal, prostate epithelial, and LNCaP (A, B, and D, respectively), but not of PC-3 (C), cells. Data was acquired as absorbance values (A_{550}–A_{690}) and normalized to vehicle. Data represents mean values and vertical lines indicate S.E.M. from three independent experiments. *P<0.05; **P<0.001.

lysophosphatidic acid (1 μM) inhibited growth of human prostate epithelial cells as well, although the effect of lysophosphatidic acid was more pronounced in the stromal cells over the epithelial cell types (Table 1). Interestingly, although bradykinin did not influence growth of PC-3 and LNCaP cells, lysophosphatidic acid led to a significant reduction in cell number of LNCaP cells at 1 μM, with no effect on the PC-3 cells, indicating a possible role of androgen receptors in lysophosphatidic acid-induced inhibition of growth in LNCaP cells (Fig. 6C and D).

3.7. Bradykinin-induced phosphorylation and activation of extracellular signal-regulated kinases (ERKs)

Cellular proliferation is often a consequence of activating components of the ERK cascade. Phosphorylation of ERK-1 and ERK-2 proteins are good indicators of ERK cascade activation. Having studied bradykinin-induced cellular proliferation in human prostate cells, we tested the hypothesis that bradykinin-induced proliferation of human prostate stromal cells could be mediated via activation of ERK-1/2 proteins. Bradykinin (10 nM) stimulated phosphorylation of ERK-1/2 proteins in human prostate stromal cells (Fig. 7A, top panel). As a negative control, we used the bradykinin B_1 agonist [des-Arg^9]-bradykinin, as we had established apparent absence of bradykinin B_1 receptors in human prostate stromal cells in the FLIPR assay. Unlike bradykinin, [des-Arg^9]bradykinin (10 nM) did not phosphorylate ERK-1/2 proteins. To ascertain that similar amounts of total ERKs were present on each lane of the gels, membranes were stripped and reprobed for total amounts of ERK-1/2 (Fig. 7A, bottom panel). As is clearly depicted, lack of [des-Arg^9]bradykinin-induced phosphorylation of ERK-1/2 was not due to absence of ERKs in our samples. In the human prostate epithelial cells, there was significant basal phosphorylation of ERK-1/2 as evident from higher than normal background staining in the vehicle-treated wells (Fig. 7A). Neither bradykinin nor [des-Arg^9]bradykinin was able to activate ERK-1/2 at significant levels over untreated prostate epithelial samples. Activation/phosphorylation of ERK-1/2 proteins by bradykinin (10 nM) in human prostate stromal cells was mediated by the bradykinin B_2 receptor as the effect was completely abolished in the presence of HOE-140 (1 μM; Fig. 7B).

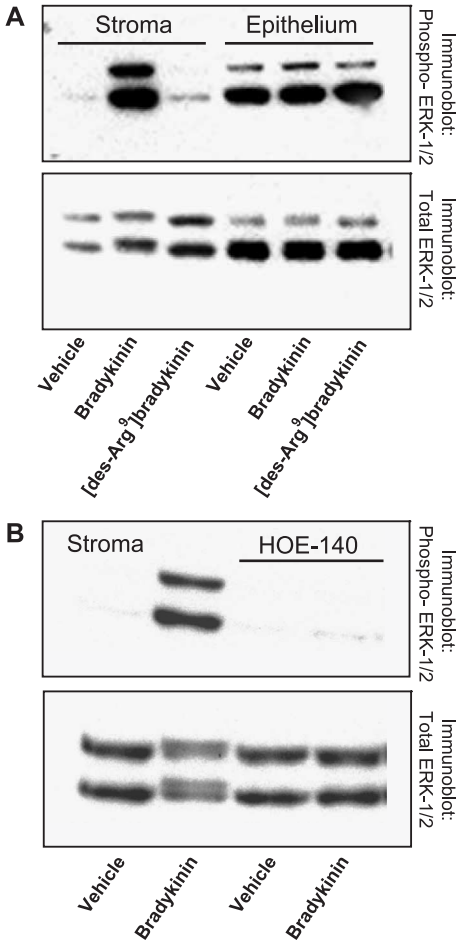


Fig. 7. Bradykinin-induced phosphorylation of extracellular signal regulated kinases (ERKs). Bradykinin (10 nM)-induced, but not [des-Arg^9]bradykinin (10 nM)-induced, phosphorylation of ERKs (phospho-ERK-1/2) in prostate stromal cell types (A). Prostate epithelial cell types exhibited higher basal phosphorylation of ERK-1/2 proteins (A). HOE-140 (1 μM) attenuated bradykinin (10 nM)-induced phosphorylation of ERK-1/2 in prostate stromal cells (B). Similar levels of total ERK-1/2 were present under all conditions tested (A and B lower panels). Western blot data shown is representative of three independent experiments.

Table 1
Bradykinin and lysophosphatidic acid-induced proliferation (MTT assay) of human prostate cells

	Percent change from vehicle±S.E.M.			
	human prostate stroma	human prostate epithelium	PC-3	LNCaP
10 nM bradykinin	6.9±2.6[a]	−0.3±6.6	−9.8±6.1	2.1±2.0
1 μM bradykinin	18.6±1.9[b]	1.1±4.0	−9.1±3.7	−8.4±8.9
10 nM lysophosphatidic acid	6.1±2.2[a]	7.5±4.8	−12.7±5.7	−8.3±4.8
1 μM lysophosphatidic acid	−61.0±0.8[b]	−39.2±1.9[b]	−6.2±3.9	−61.5±2.4[b]

A minus sign indicates decrease in cell growth.
[a] $P<0.05$.
[b] $P<0.001$.

4. Discussion

This paper describes the pharmacological and functional characterization of bradykinin receptors in human prostate cells. Human prostate epithelial cells did not express functional bradykinin receptors whereas human prostate stromal cells possessed functional bradykinin B_2 receptors but not bradykinin B_1 receptors. Although both human prostate stromal and epithelial cells expressed mRNA for bradykinin B_1 and bradykinin B_2 receptors, we could only demonstrate a function associated with bradykinin B_2 receptors in stromal cells. This is similar to the observation by Walden et al. (1999) that stromal cultures from human prostates diagnosed with benign prostate hyperplasia solely expressed functional bradykinin B_2 receptors while the role of bradykinin B_1 receptors remained elusive, although membrane binding studies indicated bradykinin B_1 receptor expression in epithelial cells. We were also unable to demonstrate a function (with regards to intracellular calcium changes) of bradykinin B_1 receptors in canine prostate, wherein likewise to the human counterpart, bradykinin B_2 receptor-mediated signaling dominated canine prostate stromal cells (Srinivasan et al., 2004). Contrary to the human prostate epithelial cells, canine prostate epithelial cells also expressed functional bradykinin B_2 receptors (Srinivasan et al., 2004). However, it is possible that in primary cultures, bradykinin B_1 receptor expression is repressed or their coupling efficiency is lost and, hence, we and others have failed to demonstrate a function associated with this receptor subtype in prostate cells. In this regard, it is worth mentioning that bradykinin B_1 receptors are often 'induced' as a result of tissue injury and repair whereas bradykinin B_2 receptors are generally considered to be constitutively expressed (Blaukat, 2003). One possibility is to study changes in expression of the bradykinin B_1 receptor subtype following cellular insult. For example, in human lung fibroblasts, bradykinin B_1 receptor expression was up-regulated by interleukin pretreatment (Phagoo et al., 2001). Hence, although we clearly demonstrated presence of bradykinin B_2 receptors in human prostate cells, absence of functional bradykinin B_1 receptors should be cautiously extrapolated in both normal and diseased human prostate and their (bradykinin B_1 receptor) absence can be confirmed only after performing radioligand binding experiments. Whether bradykinin B_1 receptor is induced during prostatic hyperplasia (benign and/or malignant) remains to be studied. Our results along with those of Walden et al. (1999) only suggest that both normal and diseased human prostate stromal cells expressed functional bradykinin B_2 receptors.

Bradykinin behaved as a potent agonist in inducing intracellular calcium flux in human prostate stromal cells. The potency (pEC_{50}) of bradykinin in the human prostate stromal cells was 8.8 ± 0.2, comparable to that obtained in canine prostate stromal cells (Srinivasan et al., 2004). Unlike the canine prostate epithelial cells, wherein potency of bradykinin was 8.7 ± 0.06 (Srinivasan et al., 2004), we were not able to demonstrate any functional reserve of bradykinin receptors in human prostate epithelial cells. We ascertained the coupling of the bradykinin receptor subtype in human prostate stromal cells to the $G_{\alpha q}$ pathway by measuring accumulation of total inositol phosphate. The bradykinin B_2 receptor-selective agonist bradykinin was again more potent (8.2 ± 0.2) than the bradykinin B_1 receptor-selective agonist in the inositol phosphates accumulation assay, suggesting that the bradykinin receptor subtype was bradykinin B_2 subtype. It is worth noting that the potency of bradykinin was higher in the FLIPR assay than in the inositol phosphates assay, perhaps predictably, as the former reflects intracellular calcium changes, a biochemical event that is downstream and therefore more amplified than inositol phosphate accumulation alone. Presence of bradykinin B_2 receptors in the human prostate stromal cells was also confirmed by the ability of increasing concentrations of HOE-140, but not [des-Arg^{10}]HOE-140, to attenuate bradykinin-induced calcium changes. However, [des-Arg^{10}]HOE-140 induced a consistent potentiation of maximal response to bradykinin in the prostate stromal cells the effect was not concentration-dependent (Fig. 4C). [des-Arg^{10}]HOE-140 did not display any agonism in the human prostate stromal cells and in CHO cells expressing the human bradykinin B_2 receptor (data not shown). Furthermore, the potentiation of bradykinin responses in the presence of the bradykinin B_1 receptor-selective antagonist was also not observed in similar experiments performed on the CHO cells suggesting a phenomenon restricted to the human prostate stromal cells under the nonequilibrium experimental conditions present in FLIPR. Another possible explanation could be that the peptide antagonist [des-Arg^{10}]HOE-140 is an allosteric modulator of bradykinin B_1 receptors in the stromal cells and not in a cell system (such as the CHO cells) that was engineered to overexpress only the bradykinin B_2 receptor. Because the potentiation was only observed at relatively high concentrations of bradykinin, at which it may activate bradykinin B_1 receptors, it may be speculated that [des-Arg^{10}]HOE-140 acts as a positive allosteric modulator of the orthosteric ligand (bradykinin) for bradykinin B_1 receptors.

We did determine the estimate of apparent affinity for HOE-140 at the human prostate stromal cells to be 1.7 ± 0.4 nM, which was comparable to the affinity of HOE-140 at the recombinant human bradykinin B_2 receptor (1.0 ± 0.2 nM). In this regard, it is worth noting that, under experimental conditions such as in FLIPR, where the agonist never achieves true equilibrium with the receptor and the antagonist, we were still able to achieve a Schild slope of 0.88 ± 0.1 that was not different from unity in human prostate stromal cells. Likewise, the Schild slope was 0.85 ± 0.05 in the CHO cells and although this slope deviated from unity, we believe that is a reflection of an 'apparent' equilibrium status of bradykinin with bradykinin B_2 receptors and HOE-140. Nonetheless, the affinity

estimate of HOE-140 in human prostate stromal cells was also comparable to that obtained in canine prostate cells (Srinivasan et al., 2004). A significant difference existed in this study and that reported by Simpson et al. (2000) in the nature of antagonism by HOE-140 at the recombinant human bradykinin B_2 receptor. While we observed surmountable antagonism, Simpson et al. (2000) failed to surmount the effect of HOE-140 by increasing bradykinin concentrations. On the other hand, in human prostate stromal cells, we did observe significant suppression of the maximal bradykinin response in the presence of high HOE-140 concentrations. We corrected for the insurmountability using the modified Gaddum's analysis (Kenakin, 1997) and determined the affinity estimate of HOE-140 to be about 0.35 nM, about five times more potent than that estimated by Schild regression. Nonetheless, the pharmacology of HOE-140 in the human prostate stromal cells, along with the estimates of HOE-140 at bradykinin B_2 receptors, is a very strong indicator of the presence of bradykinin B_2 receptors in these cell types.

Lysophosphatidic acid is a known mitogen in prostate cells (Daaka, 2002). We therefore used lysophosphatidic acid as a positive control in FLIPR assays as receptors for lysophosphatidic acid can couple to $G_{\alpha q}$ (Fukushima and Chun, 2001) to increase intracellular calcium flux. Although we failed to demonstrate the presence of bradykinin receptor subtypes in prostate epithelial cells by FLIPR, lysophosphatidic acid was able to mobilize intracellular calcium in a concentration-dependent manner. Lysophosphatidic acid was also a potent agonist in human prostate stromal, BPH-1, and PC-3 cell types. BPH-1 cell line is an epithelial cell line generated from a patient clinically diagnosed with benign prostate hyperplasia (Hayward et al., 1995). Interestingly, in BPH-1 cells, we did observe bradykinin mediated calcium changes (pEC_{50} 6.1 ± 0.5), although the maximal bradykinin response was lower than that observed in human prostate stromal cells. In the presence of lithium chloride (which inhibits breakdown of inositol phosphate), the potency (pEC_{50} 7.1 ± 0.2) and maximal response elicited by bradykinin in the BPH-1 cell line was increased (data not shown). Therefore, it is possible that bradykinin B_2 receptors may be present in prostate epithelial cells as well, but only under uncontrolled growth conditions, such as observed during benign prostate hyperplasia. It is also possible that, during diseased conditions, bradykinin receptors may couple to signaling cascades that are not amenable to be studied by either the FLIPR or the inositol phosphate assay. In light of this speculation, it is interesting to point that although both PC-3 and LNCaP prostate cancer cells express bradykinin B_2 receptors at the protein level (Chen et al., 2002), we, along with Baba and Yamaguchi (2001), were unable to generate bradykinin-induced calcium mobilization in these cells.

In light of the aforementioned discussion of assay sensitivity, we studied bradykinin-dependent cellular proliferation in human prostate cells. Bradykinin caused cellular proliferation of human prostate stromal cells, but not of the human prostate epithelial, PC-3, or LNCaP cells. Human prostate stromal cell proliferation was mediated via activation of bradykinin B_2 receptors that activated the ERK pathway. ERK activation was attenuated by bradykinin B_2 receptor antagonism. In that regard, although we have not tested any inhibitors of ERK in our experiments, the data strongly suggests that bradykinin activated ERKs in prostate stromal cells. In human prostate epithelial cells, there was increased basal activation of ERK-1 and ERK-2 proteins as compared to that observed in human stromal cells. It is possible that this increased activity of ERK pathway may mask any effect of bradykinin-induced human prostate epithelial cell proliferation. Lack of bradykinin-induced proliferation in PC-3 cells is in sharp contrast to reports of bradykinin-induced mitogenicity in these cell types (Barki-Harrington and Daaka, 2001; Barki-Harrington et al., 2003). The difference could be due to low passage number (32–42) used in our study as compared to the higher passage numbers (130–150) used in the literature. A plausible explanation is that expression of bradykinin receptors in the malignant PC-3 cells could be dependent on passage number and culture conditions. Cell passage in culture condition has been known to modulate receptor expression patterns in other systems (Boselli et al., 2002).

Lysophosphatidic acid, on the other hand, seemed to inhibit growth of prostate stromal, prostate epithelial, and LNCaP cells only at a high concentration (1 μM). In PC-3 cells, lysophosphatidic acid was without any effect at the two concentrations tested. We understand that androgen receptors may modulate lysophosphatidic acid-dependent growth in human prostate cell lines as LNCaP cells are androgen-dependent while PC-3 cells are androgen-independent. Furthermore, both the prostate epithelial and stromal cells were derived from young donors and are therefore likely to express androgen receptors. Whether androgen receptors also modulate bradykinin-induced cell proliferation remains to be studied. In conclusion, we have demonstrated that bradykinin B_2 receptors in normal human prostate stromal cells induced cellular proliferation via activation of ERK proteins, raising the possibility of therapeutic intervention of this subtype of bradykinin receptor during uncontrolled prostatic growth associated with benign prostate hyperplasia and prostate cancer.

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