Requirement for direct cross-talk between B1 and B2 kinin receptors for the proliferation of androgen-insensitive prostate cancer PC3 cells

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Stimulation of endogenous kinin receptors promotes growth of androgen-independent prostate cancer PC3 cells via activation of the mitogenic extracellular-signal-regulated kinase (ERK) pathway. In the present study, we show that kinin-mediated mitogenic signalling and prostate-cell growth involves two subtypes of bradykinin (BK) receptors, B1R and B2R. Specific stimulation of either B1R or B2R by their respective agonists des-Arg⁹-BK and Lys-BK promoted ERK activation and cell growth, whereas selective blockade with specific antagonists des-Arg⁹-[Leu⁸]BK and Hoe 140 respectively obliterated this effect, indicating the presence of both receptor subtypes. However, blockade of B1R also inhibited B2R-mediated ERK activation and cell growth, and, similarly, antagonism of B2R inhibited the B1R-mediated response. Furthermore, both B1R and B2R agonists promoted internalization of B1R, whereas both receptor antagonists blocked this effect. The B1R ligands des-Arg⁹-BK and des-Arg⁹-

[Leu⁸]BK had no effect on the binding of BK to B2R, as demonstrated by radioligand competitive binding studies. However, blockade of either B1R or B2R impaired the ability of the reciprocal receptor to produce inositol phosphates, suggesting that the interaction between B1R and B2R is proximal to activation of phospholipase C. These results provide evidence for the existence of B1R–B2R complexes in prostate cancer PC3 cells and demonstrate that antagonism of one receptor interferes with the signalling ability of the other, possibly at the level of receptor–G α_q protein coupling. Selective inhibition of B1R, which is up-regulated in injured and cancerous tissue, may be beneficial for the treatment of advanced prostate cancer.

Key words: bradykinin, G-protein-coupled receptor, mitogen-activated protein kinase (MAPK), receptor dimerization.

INTRODUCTION

Prostate cancer is the second most common cause of cancer death affecting males in the U.S.A. [1]. Androgens, in the form of testosterone and dihydrotestosterone, are considered the primary growth promoters of normal and malignant prostate cells. However, pathological growth of the prostate persists even in the continued presence of androgen ablation therapy, suggesting androgen-independent prostate cancer-cell growth [2,3]. Potential mechanisms involved in growth of androgen-independent prostate carcinomas include amplification and over-expression of androgen receptors, which could compensate for lower androgen levels in the serum [4], and androgen-receptor mutations, which may alter the responsiveness of androgen receptors to anti-androgens and other steroids [5]. However, in one third of all advanced prostate tumours, no discernible androgen-receptor mutation or amplification has been noted, implying a potential role for other growth factors.

G-protein-coupled receptors (GPCRs) are expressed in normal epithelial prostate cells and have been shown to mediate growth of androgen-independent prostate cancer cells *in vitro* [6–8]. Expression of GPCRs, such as the prostate-specific PSGR [9] and endothelin 1A receptor [10], is increased in advanced prostate cancer specimens. Furthermore, enzymes that regulate the expression of GPCR ligands, such as lysophosphatidylcholine acyltransferase [11] and human kallikrein 2 [12,13], are also increased in advanced prostate cancer. Importantly, human kallikrein 2 is a kininogenase [14] that produces bradykinin

(BK), suggesting that kinin receptors could be activated in advanced prostate cancer.

Kinins exert their effects on target cells through the activation of their cognate B1R and B2R GPCRs [15]. These two receptor subtypes demonstrate low sequence similarity and differ in their rank order of potency towards specific agonists and antagonists. B1R is usually expressed at very low levels in normal tissues, but undergoes marked up-regulation following injury or inflammation [16], whereas B2R is ubiquitously expressed and is thought to mediate most of the physiological actions of BK. Both receptor subtypes couple primarily to G α_q to elicit cellular responses [15].

Recently, we demonstrated that stimulation of endogenous BK receptors induces the growth of androgen-independent prostate cancer PC3 cells via activation of the extracellular-signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) pathway [8]. In the present paper, we demonstrate that kinin-induced ERK activation and subsequent PC3 cell growth requires direct cross-talk between B1R and B2R, and that a blockade of either B1R or B2R affects the ability of the other receptor to signal.

MATERIALS AND METHODS

Materials

BK (B2R-selective agonist), des-Arg⁹-BK (B1R-specific agonist), Lys-BK (B2R-specific agonist), des-Arg⁹-[Leu⁸]BK (B1R-specific antagonist) and Hoe 140 (B2R-specific antagonist) were pur-

Abbreviations used: AT1R, angiotensin II type 1 receptor; BK, bradykinin; B1R, BK receptor 1; B2R, BK receptor 2; EGF, epidermal growth factor; ERK, extracellular-signal-regulated kinase; GPCR, G-protein-coupled receptor; MAPK, mitogen-activated protein kinase.

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chased from Sigma. The concentrations of each agonist and antagonist were determined empirically for each assay so that maximal, but specific, responses were obtained. [^3H]BK was purchased from New England Nuclear and *myo*-[^3H]inositol was purchased from Amersham. WST-1 was purchased from Roche Molecular Biochemicals. Anti-(phospho-ERK) antibody was obtained from Cell Signaling, anti-ERK 2 was from Upstate Biotechnology, anti-FLAG M2 was from Sigma and horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch. Cell culture media and supplements were purchased from Gibco-BRL. All other reagents were standard laboratory grade.

Peptides encompassing human B1R residues 317–353 and human B2R residues 310–364 were synthesized and used to immunize female New Zealand White rabbits. Unconjugated peptides were used for initial antigen injection with Freund's complete adjuvant, and this was followed by two booster injections with Freund's incomplete adjuvant. Immunoglobulins were purified using Protein A resin as described by the manufacturer (Pierce).

Cell culture and growth

Bone metastasis human prostate cancer PC3 cells were obtained from the American Type Culture Collection and used as a model for the study of androgen-independent prostate cancer. PC3 and chimpanzee ovarian sarcoma COS-7 cells were maintained in RPMI 1640 and Dulbecco's modified Eagle's medium respectively, containing 10% (v/v) foetal calf serum and antibiotics (100 $\mu\text{g}/\text{ml}$ penicillin/streptomycin). For growth, high passage (passage 130–150) PC3 cells were seeded in 96-well plates at a density of 2000 cells/well in standard culture medium for 24 h to allow attachment. The next day, the culture medium was replaced with serum-free RPMI 1640 containing 100 nM of either the B1R-specific des-Arg⁹-BK or the B2R-specific Lys-BK agonist. For antagonist experiments, 1 μM of either B1R-specific des-Arg⁹-[Leu⁸]BK or B2R-specific Hoe 140 was added to cells 30 min before stimulation with agonist. The cell-proliferation reagent WST-1 was added to each sample 48 h after treatment and cells were incubated for an additional 30 min at 37 °C. Attenuance of samples (D_{450}) was determined using a scanning multiwell spectrophotometer [8]. Absolute attenuance was normalized to the attenuance of unstimulated cells in each plate and was expressed as a percentage of the control value.

Expression of B1R and B2R

PC3 cells were washed with ice-cold PBS, scraped into TE buffer (10 mM Tris/HCl, pH 8, and 1 mM EDTA), and were disrupted by Dounce homogenization [17]. Nuclei and cell debris were removed by centrifugation at 500 *g* for 5 min at 4 °C. The supernatant was re-centrifuged at 14000 *g* for 20 min at 4 °C and the pellet was collected and diluted in lysis buffer [150 mM NaCl, 50 mM Tris/HCl, pH 8.0, 5 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate, 1% (v/v) Nonidet P40, 0.5% (w/v) deoxycholate, protease inhibitor tablet (Boehringer Mannheim), 1 mM PMSF and 100 μM Na₃VO₄]. B1R and B2R expression was analysed by immunoblotting using a 1:1000 dilution each of B1R- or B2R-specific antibodies. The specificity of the antibodies was determined by including synthetic B1R- and B2R-neutralizing peptides. Immune complexes on nitrocellulose were detected using a 1:7000 dilution of anti-rabbit horseradish peroxidase-conjugated secondary antibody and were visualized by enhanced chemiluminescence.

ERK activation

Cells were serum-starved for 24 h before exposure to agonist at 37 °C for 10 min. Antagonists were added 30 min before agonist stimulation. Reactions were terminated by direct addition of lysis buffer to cell monolayers and protein concentrations were determined using the Bradford method [17a]. Equal amounts of protein from each sample were separated by SDS/PAGE (4–20% gels) and were transferred on to nitrocellulose filters. ERK phosphorylation was detected and quantified exactly as described in [6,8].

Internalization assays

COS-7 cells in 10 cm tissue culture plates were transiently transfected with 1 μg of FLAG epitope-tagged B1R (pcDNA3-FLAG-B1R), using LIPOFECTAMINETM (Invitrogen), according to the manufacturer's instructions. After overnight incubation, transfected cells were seeded equally into 24-well culture plates and allowed to grow for additional 24 h. FLAG-B1R internalization was determined using ELISA, exactly as described in [18]. Antagonists were added 30 min before stimulation. Cells were stimulated with agonists for 30 min at 37 °C before the addition of anti-FLAG M2 antibodies. Receptor internalization was defined as the fraction of total cell surface receptors that was removed from the plasma membrane following agonist stimulation.

Ligand-binding studies

Binding assays were performed using confluent PC3 cells in 24-well plates. Reactions were carried out in 0.25 ml of binding buffer [20 mM Hepes, pH 7.4, 17 mM NaCl, 5.4 mM KCl, 0.34 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 0.21 mM MgSO₄, 0.63 mM CaCl₂, 0.1% (w/v) BSA], containing 3 nM [^3H]BK and various concentrations of competing ligands (Lys-BK, des-Arg⁹-BK, des-Arg⁹-[Leu⁸]BK, Hoe 140) on ice for 4 h [19]. Non-specific binding was determined in the presence of a 1000-fold excess of unlabelled BK and subtracted from total binding. Reactions were terminated by three rapid washes with ice-cold binding buffer. Cells were solubilized in 0.1 M NaOH and the amount of bound [^3H]BK was determined by scintillation counting. Data were analysed by non-linear least squares regression equations using the Prism computer program (version 3.0; GraphPad Software Inc., San Diego, CA, U.S.A.).

Inositol phosphate measurements

PC3 cells were grown in 12-well plates and incubated for 18–24 h with 4 μCi of *myo*-[^3H]inositol/ml in serum-free growth medium. After labelling, cells were washed twice with PBS and pre-incubated with PBS/LiCl in the presence or absence of antagonists for 30 min at 37 °C. Next, cells were stimulated for 1 h with agonists. Reactions were terminated by the addition of an equal volume of 0.8 M perchloric acid, and total inositol phosphates were assayed by anion exchange chromatography on Dowex AG1-X8 columns, as described in [20].

RESULTS

Stimulation of endogenous B1R or B2R by their selective agonists, des-Arg⁹-BK and Lys-BK respectively, induced a 1.5-fold increase in the proliferation rate of androgen-independent prostate cancer PC3 cells (Figure 1). This effect was receptor-mediated, since pre-incubation of cells with specific B1R (des-

Figure 1 Endogenous B1R and B2R kinin receptors mediate growth of PC3 cells

PC3 cells were seeded in 96-well plates and were serum-starved overnight. B1R antagonist des-Arg⁹-[Leu⁸]BK (1 μ M) and B2R antagonist Hoe 140 (1 μ M) were added 30 min before addition of B1R agonist des-Arg⁹-BK (100 nM) or B2R agonist Lys-BK (100 nM). Cell proliferation was determined as described in the Materials and methods section. Results are means $\pm$ S.D. for five independent experiments. *, $P < 0.01$ compared with the control; **, $P < 0.05$ compared with the agonist-stimulated signal.

Arg⁹-[Leu⁸]BK) or B2R (Hoe 140) antagonists abrogated the signal initiated by their respective receptors. Unexpectedly, pre-incubation of cells with the antagonist of one receptor also inhibited the ability of the other receptor to induce cell growth: pre-incubation of cells with des-Arg⁹-[Leu⁸]BK abolished B2R-mediated cell proliferation and, similarly, pre-incubation of cells with Hoe 140 abolished the B1R-mediated effect (Figure 1). In control experiments, treatment with epidermal growth factor (EGF) induced growth of the PC3 cells (1.5–1.7-fold above basal levels), and that response was not affected by pre-treatment of cells with antagonists of either B1R or B2R (results not shown). These results suggest that both B1R and B2R are present in the prostate cancer PC3 cells, and that they communicate with one another to promote cell growth.

The expression of B1R and B2R in PC3 cells was confirmed by the use of specific antibodies against B1R and B2R. Figure 2(A) shows that immunoblotting with the anti-B1R antibody detected one major band that migrated with an apparent molecular mass of 40 kDa. This protein band was not detected in the presence of a neutralizing B1R synthetic peptide, but was visible in the presence of the B2R synthetic peptide. Immunoblotting with the anti-B2R antibody demonstrated expression of two prominent proteins, which migrated with apparent molecular masses of 40 kDa and 65 kDa (Figure 2B). These protein bands were detected in the presence of the B1R peptide, but disappeared in the presence of competing B2R peptide. The size of B2R obtained is similar to that found in smooth muscle cells [21] and platelets [22]. Expression of the B1R and B2R in PC3 cells was also confirmed by immunostaining, where plasma membrane localization was noted for both receptors. Interestingly, the cloned PC3 cells used in this study demonstrated a heterologous expression pattern for B1R in that the majority, but not all, of the PC3 cells expressed the protein (R. Guo and Y. Daaka,

Figure 2 PC3 cells express B1R and B2R proteins

Crude membranes were obtained from the PC3 cells by differential centrifugation following cell homogenization. Equal amounts of proteins were analysed for expression of B1R (A) or B2R (B) in the presence, or absence, of competing B1R or B2R neutralizing peptides. Arrows indicate specific reactive protein bands and molecular mass markers are given in kDa.

unpublished work). The reason for this is not clear and may reflect the property of cell transformation [23]. Together, these results demonstrate endogenous expression of both B1R and B2R in prostate cancer PC3 cells.

Advanced human prostate tumours express elevated levels of activated ERK MAPKs compared with benign specimens [24,25], suggesting a potential role for ERK in the pathological growth of the prostate. Using *in vitro* model systems, we previously showed that the BK-induced PC3 cell growth requires activation of the ERK pathway [8]. In the present study, we tested whether the cross-talk between B1R and B2R, which was required for PC3 cell growth (Figure 1), was also required for the kinin-receptor-mediated activation of ERK. As demonstrated in Figure 3, stimulation of PC3 cells with specific agonists for either B1R or B2R caused a similar 8-fold increase in the phosphorylation state of ERK. Similar to the effect on cell proliferation, blockade of the B1R with des-Arg⁹-[Leu⁸]BK abrogated the ability of both B1R and B2R to activate ERK. The same was observed for B2R where B2R-regulated activation of ERK was inhibited by the antagonist for either B1R or B2R. In all cases, simultaneous addition of both agonists or antagonists did not have an additive effect (results not shown). The levels of ERK 2 remained unaffected by the different treatments,

Figure 3 Effect of stimulation of B1R and B2R on activation of ERK

(A) B1R and B2R stimulation induces activation of ERK. Serum-starved PC3 cells were stimulated with 10 nM BK, des-Arg⁹-BK or Lys-BK for 10 min and lysates were separated by SDS/PAGE (4–20% gels). For antagonist treatment, des-Arg⁹-[Leu⁸]BK (1 μ M) or Hoe 140 (1 μ M) was added 30 min before agonist stimulation. Proteins were transferred on to nitrocellulose filters and immunoblotted for phosphorylated ERK (p-ERK). Filters were stripped of immunoglobulins and reblotted for ERK2 to demonstrate equal loading of protein in each lane. (B) Quantitative data from five independent experiments.

indicating that the observed changes stemmed from differences in ERK activation, and not in expression levels.

To demonstrate that Lys-BK and des-Arg⁹-BK are specific agonists for B2R and B1R respectively, we used HEK-293 cells, which do not express detectable kinin receptors. Cells were examined for their potential to activate ERK following stimulation with des-Arg⁹-BK, Lys-BK or EGF (used as control). Stimulation of wild-type HEK-293 cells with des-Arg⁹-BK or Lys-BK did not induce detectable ERK phosphorylation. On the other hand, stimulation of HEK-293 cells expressing B1R with des-Arg⁹-BK, but not Lys-BK, induced ERK phosphorylation (results not shown). Furthermore, stimulation of the HEK-293 cells expressing B2R with Lys-BK, but not des-Arg⁹-BK, induced ERK phosphorylation (results not shown). In all cases, stimulation with EGF promoted ERK phosphorylation. These results demonstrate kinin-receptor specificity of each ligand: des-Arg⁹-BK activates B1R, but not B2R, and Lys-BK activates B2R, but not B1R.

An important aspect of GPCR activity and regulation is the internalization of receptor into intracellular membrane compartments following exposure to agonist [26]. We employed this characteristic to confirm further the existence of the interaction between B1R and B2R. Receptor-internalization experiments were performed in COS-7 cells that endogenously express only

Figure 4 Effects of B1R and B2R agonists and antagonists on internalization of FLAG-B1R

COS-7 cells transiently expressing FLAG-B1R were seeded equally into 24-well tissue culture plates and serum-starved for 18 h. Cells were exposed to B1R agonist des-Arg⁹-BK (100 nM) or B2R agonist Lys-BK (100 nM) for 30 min at 37 °C. B1R antagonist des-Arg⁹-[Leu⁸]BK (10 μ M) and B2R antagonist Hoe 140 (10 μ M) were added 30 min before stimulation with agonist. Cells were analysed for their plasma membrane content of the FLAG-B1R by ELISA. Results are means $\pm$ S.D. for three independent experiments performed in duplicate.

B2R. FLAG-epitope-tagged B1R was expressed in these cells and internalization was assessed using ELISA, as described in the Materials and methods section. As depicted in Figure 4, direct stimulation of B1R with its selective agonist des-Arg⁹-BK resulted in sequestration of the FLAG-B1R from the plasma membrane, an effect that was abolished following pre-treatment of cells with the selective B1R antagonist des-Arg⁹-[Leu⁸]BK. However, the blockade of endogenous B2Rs with Hoe 140 also attenuated the des-Arg⁹-BK-induced sequestration of FLAG-B1R, whereas stimulation of endogenous B2R with Lys-BK caused internalization of the receptor. HEK-293 cells expressing FLAG-B1R were used to demonstrate specificity of Hoe 140. Pre-treatment of the HEK-293-B1R cells with Hoe 140 did not interfere with des-Arg⁹-BK-induced sequestration of FLAG-B1R (results not shown). Taken together, these results suggest that B1R and B2R are likely to be complexed with one another in PC3 cells.

To determine whether the observed effect of B1R antagonists on B2R signalling, and vice versa, is due to interference with ligand binding, we performed competitive radioligand-binding analyses on PC3 cells endogenously expressing both receptors using the B2R agonist [³H]BK in the presence of B1R and B2R competitors. Figure 5 shows that specific binding of [³H]BK to PC3 cells was, as expected, displaced by increasing concentrations of the B2R agonists BK and Lys-BK and the B2R antagonist Hoe 140. However, the B1R-selective agonist des-Arg⁹-BK and antagonist des-Arg⁹-[Leu⁸]BK had no effect on the binding of [³H]BK to PC3 cells, indicating that the biological consequence of the cross-talk between B1R and B2R occurs at a step distal to

Figure 5 Competitive binding of [^3H]BK to PC3 cells

PC3 cells in 24-well plates were serum-starved overnight and exposed to 3 nM [^3H]BK and the indicated concentrations of competing ligands for 4 h on ice. Reactions were terminated by rapid washing. Cells were solubilized and the amount of bound [^3H]BK was determined by scintillation counting. Results were analysed by non-linear least squares regression equations using the Prism computer software package.

Figure 6 Effects of B1R and B2R stimulation on accumulation of inositol phosphates

PC3 cells in 12-well plates were serum-starved overnight and labelled with *myo*-[^3H]inositol for 18 h. Cells were pre-incubated for 30 min with the indicated antagonist, followed by stimulation with agonist for 1 h, as described in the Materials and methods section. Reactions were terminated and total inositol phosphates were assayed by anion exchange chromatography. *, $P < 0.01$ compared with the control; **, $P < 0.01$ compared with Lys-BK- and des-Arg 9 -BK-induced values. CN, non-stimulated.

ligand binding. Further, these results also demonstrate that the concentrations of agonists and antagonists used in the other assays displayed receptor-specific effects.

Since most of the metabolic effects of kinin receptors are attributed to G_{α_q} -mediated activation of phospholipase C [15], we next measured the effects of B1R and B2R antagonism on agonist-mediated inositol phosphate formation. As depicted in Figure 6, both agonists produced a 1.5-fold increase in formation of total inositol phosphates, an increase that is consistent with other results obtained in response to stimulation of endogenous kinin receptors [27]. Inositol phosphate production mediated by B1R was inhibited in the presence of either B1R or B2R antagonists and, similarly, the B2R-mediated effect was obliterated in the presence of either B2R or B1R antagonists. Taken together, these results suggest the existence of cross-talk, at the receptor-G-protein coupling level, between B1R and B2R, and that this interaction is required for PC3 cell growth.

DISCUSSION

Despite the long-held view that GPCRs function as monomeric signalling units (i.e. a single receptor couples to a G-protein upon ligand binding), a growing body of evidence suggests that these receptors form higher oligomeric structures through direct interactions among either identical or non-identical family members [28–30]. However, the underlying mechanisms involved in receptor oligomerization and the exact role of oligomerization in GPCR function remain largely unknown. In the present paper, we suggest a novel role for B1R–B2R interaction in mitogenic signalling by demonstrating that the two BK receptor subtypes interact with each other and that this interaction is critical for the proliferative response of prostate cancer PC3 cells.

Our results show that the specific blockade of either B1R or B2R is sufficient to cause inhibition of kinin-mediated cell proliferation, demonstrating that antagonist occupancy of B1R negatively affects the signalling properties of B2R. Similar results were obtained for B2R, where antagonism of B2R obliterated the B1R-regulated cellular response. Since both receptors seem to be equally important in promoting ERK activation and prostate cancer cell proliferation, a critical role may be given to inducible B1R in these processes. Although B2Rs are ubiquitously expressed, B1Rs are expressed at low levels in healthy tissue and undergo marked up-regulation in response to inflammatory [16] and proliferative conditions such as gastric neoplasms [31]. Indeed, analysis of human prostate biopsies shows that, whereas the B2R is constitutively expressed in both benign and malignant epithelial prostate cells, B1R is expressed only in malignant, and not benign, prostate lesions (R. Guo and Y. Daaka, unpublished work). Taken together, these results lead us to propose that the occurrence of B1R–B2R complexes increases under pathological conditions and leads to altered mitogenic signalling by either receptor.

The existence of direct interactions between various GPCRs has been demonstrated by a variety of biochemical, physiological and pharmacological approaches [28,29]. Specifically, B2R was recently shown to form a complex with angiotensin II type 1 receptor (AT1R) in HEK-293 cells [21]. The AT1R–B2R heterodimer displayed increased efficacy and potency of angiotensin signalling, but a decreased BK signalling capacity [21]. In the present study, existence of B1R–B2R complexes in prostate cells is supported by receptor internalization properties. FLAG–B1R internalizes into intracellular compartments following the direct stimulation of either B1R or B2R. Moreover, internalization of B1R is regulated by B2R, since blockade of B2R with its selective antagonist inhibits internalization of FLAG–B1R, even in the

presence of direct activation by its own ligand. These results are in agreement with results showing that internalization of β 2-adrenergic receptors following isoprenaline stimulation is attenuated in cells that co-express κ opioid receptors, which do not internalize in response to agonist stimulation [32].

Formation of receptor–receptor complexes can modulate signal transduction at multiple levels, including ligand binding [32,33], as well as efficacy and potency of signalling [21]. Binding experiments performed on endogenous B1R and B2R in our system showed that B1R agonists and antagonists do not bind to the active site of B2R, confirming their receptor specificity and indicating that the point of interaction between the two receptors is distal to ligand binding. These results are similar to findings regarding β 2-adrenergic and the δ opioid receptors, where heterodimerization did not alter ligand binding to either receptor, but affected downstream signalling properties [32].

The inhibition of phospholipase C-mediated inositol phosphate formation following receptor blockade suggests that the point of interaction between B1R and B2R is at the level of receptor coupling to the $G\alpha_q$ protein. Since blockade of one receptor impaired inositol phosphate formation by the other, it is possible that the role of the unstimulated receptor is to form a platform that enables efficient coupling of the stimulated receptor to the G-protein. Indeed, heterodimerization between the B2R and the AT1R enhances coupling of the AT1R to $G\alpha_q$, thus enabling angiotensin II-mediated signalling [21]. This possibility is supported by observations of the crystal structure of G-protein transducin that show a large receptor-facing interface [34], which may suggest that the functional unit activating a G-protein is a receptor dimer [35]. Further experiments are required to confirm this assumption and to identify the points of interaction.

Despite the accumulating evidence for direct receptor–receptor interaction, little is known about the physiological role of this form of interaction. Recently, GPCR heterodimerization was shown to have clinical ramifications when heterodimerization between B2R and AT1R was demonstrated to increase in women with pre-eclampsia compared with women with normal blood pressure [22]. In the present study, we showed that a direct interaction between two GPCRs, B1R and B2R, is required for the proliferation of androgen-independent prostate cancer PC3 cells. Furthermore, the interaction between the receptors is most probably at the receptor/G-protein interface. Dimerization of B1R–B2R may therefore have a fundamental role in prostate tumorigenesis, and selective targeting of these complexes may provide new venues for the treatment of advanced prostate cancer.

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REFERENCES

- Jemal, A., Thomas, A., Murray, T. and Thun, M. (2002) Cancer statistics, 2002. *Ca Cancer J. Clin.* **52**, 23–47.
- Djakiew, D. (2000) Dysregulated expression of growth factors and their receptors in the development of prostate cancer. *Prostate* **42**, 150–160.
- Raj, G. V., Barki-Harrington, L., Kue, P. F. and Daaka, Y. (2002) Guanosine phosphate binding protein coupled receptors in prostate cancer: a review. *J. Urol.* **167**, 1458–1463.
- Abate-Shen, C. and Shen, M. M. (2000) Molecular genetics of prostate cancer. *Genes Dev.* **14**, 2410–2434.
- Zhao, X. Y., Malloy, P. J., Krishnan, A. V., Swami, S., Navone, N. M., Peehl, D. M. and Feldman, D. (2000) Glucocorticoids can promote androgen-independent growth of prostate cancer cells through a mutated androgen receptor. *Nat. Med.* **6**, 703–706.
- Kue, P. F. and Daaka, Y. (2000) Essential role for G proteins in prostate cancer cell growth and signaling. *J. Urol.* **164**, 2162–2167.
- Guo, C., Luttrell, L. M. and Price, D. T. (2000) Mitogenic signaling in androgen sensitive and insensitive prostate cancer cell lines. *J. Urol.* **163**, 1027–1032.
- Barki-Harrington, L. and Daaka, Y. (2001) Bradykinin induced mitogenesis of androgen independent prostate cancer cells. *J. Urol.* **165**, 2121–2125.
- Xu, L. L., Stackhouse, B. G., Florence, K., Zhang, W., Shanmugam, N., Sesterhenn, I. A., Zou, Z., Srikantan, V., Augustus, M., Roschke, V. et al. (2000) PSGR, a novel prostate-specific gene with homology to a G protein-coupled receptor, is overexpressed in prostate cancer. *Cancer Res.* **60**, 6568–6572.
- Gohji, K., Kitazawa, S., Tamada, H., Katsuoka, Y. and Nakajima, M. (2001) Expression of endothelin receptor associated with prostate cancer progression. *J. Urol.* **165**, 1033–1036.
- Faas, F. H., Dang, A. Q., White, J., Schaefer, R. and Johnson, D. (2001) Increased prostatic lysophosphatidylcholine acyltransferase activity in human prostate cancer: a marker for malignancy. *J. Urol.* **165**, 463–468.
- Haese, A., Graefen, M., Steuber, T., Becker, C., Pettersson, K., Piironen, T., Noldus, J., Huland, H. and Lilja, H. (2001) Human glandular kallikrein 2 levels in serum for discrimination of pathologically organ-confined from locally advanced prostate cancer in total PSA-levels below 10 ng/ml. *Prostate* **49**, 101–109.
- Finlay, J. A., Evans, C. L., Day, J. R., Payne, J. K., Mikolajczyk, S. D., Millar, L. S., Kuus-Reichel, K., Wolfert, R. L. and Rittenhouse, H. G. (1998) Development of monoclonal antibodies specific for human glandular kallikrein (hK2): development of a dual antibody immunoassay for hK2 with negligible prostate-specific antigen cross-reactivity. *Urology* **51**, 804–809.
- Charlesworth, M. C., Young, C. Y., Miller, V. M. and Tindall, D. J. (1999) Kininogenase activity of prostate-derived human glandular kallikrein (hK2) purified from seminal fluid. *J. Androl.* **20**, 220–229.
- Hall, J. M. (1997) Bradykinin receptors. *Gen. Pharmacol.* **28**, 1–6.
- Phagoo, S. B., Reddi, K., Anderson, K. D., Leeb-Lundberg, L. M. and Warburton, D. (2001) Bradykinin B1 receptor up-regulation by interleukin 1 β and B1 agonist occurs through independent and synergistic intracellular signaling mechanisms in human lung fibroblasts. *J. Pharmacol. Exp. Ther.* **298**, 77–85.
- Lin, F. T., Krueger, K. M., Kendall, H. E., Daaka, Y., Fredericks, Z. L., Pitcher, J. A. and Lefkowitz, R. J. (1997) Clathrin-mediated endocytosis of the β -adrenergic receptor is regulated by phosphorylation/dephosphorylation of β -arrestin 1. *J. Biol. Chem.* **272**, 31051–31057.
- Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.
- Parent, J. L., Labrecque, P., Orsini, M. J. and Benovic, J. L. (1999) Internalization of the TXA₂ receptor α and β isoforms. Role of the differentially spliced COOH terminus in agonist-promoted receptor internalization. *J. Biol. Chem.* **274**, 8941–8948.
- Pizard, A., Marchetti, J., Allegrini, J., Alhenc-Gelas, F. and Rajerison, R. M. (1998) Negative cooperativity in the human bradykinin B2 receptor. *J. Biol. Chem.* **273**, 1309–1315.
- Daaka, Y., Luttrell, L. M., Ahn, S., Della Rocca, G. J., Ferguson, S. S., Caron, M. G. and Lefkowitz, R. J. (1998) Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. *J. Biol. Chem.* **273**, 685–688.
- Abdalla, S., Lother, H. and Quitterer, U. (2000) AT1-receptor heterodimers show enhanced G-protein activation and altered receptor sequestration. *Nature (London)* **407**, 94–98.
- Abdalla, S., Lother, H., el Massiery, A. and Quitterer, U. (2001) Increased AT₁ receptor heterodimers in preeclampsia mediate enhanced angiotensin II responsiveness. *Nat. Med.* **7**, 1003–1009.
- Lefkowitz, R. J., Pierce, K. L. and Luttrell, L. M. (2002) Dancing with different partners: protein kinase A phosphorylation of seven membrane-spanning receptors regulates their G protein-coupling specificity. *Mol. Pharmacol.* **62**, 971–974.
- Gioeli, D., Mandell, J. W., Petroni, G. R., Frierson, Jr, H. F. and Weber, M. J. (1999) Activation of mitogen-activated protein kinase associated with prostate cancer progression. *Cancer Res.* **59**, 279–284.
- Price, D. T., Rocca, G. D., Guo, C., Ballo, M. S., Schwinn, D. A. and Luttrell, L. M. (1999) Activation of extracellular signal-regulated kinase in human prostate cancer. *J. Urol.* **162**, 1537–1542.
- Ferguson, S. S. (2001) Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol. Rev.* **53**, 1–24.
- Yang, C. M., Tsai, Y. J., Pan, S. L., Lin, C. C., Wu, W. B., Wang, C. C., Huang, S. C. and Chiu, C. T. (1999) Inhibition of bradykinin-induced phosphoinositide hydrolysis and Ca²⁺ mobilisation by phorbol ester in rat cultured vascular smooth muscle cells. *Cell. Signalling* **11**, 899–907.
- Bouvier, M. (2001) Oligomerization of G-protein-coupled transmitter receptors. *Nat. Rev. Neurosci.* **2**, 274–286.
- Devi, L. A. (2001) Heterodimerization of G-protein-coupled receptors: pharmacology, signaling and trafficking. *Trends Pharmacol. Sci.* **22**, 532–537.
- Jordan, B. A. and Devi, L. A. (1999) G-protein-coupled receptor heterodimerization modulates receptor function. *Nature (London)* **399**, 697–700.

-
- 31 Sawant, S., Snyman, C. and Bhoola, K. (2001) Comparison of tissue kallikrein and kinin receptor expression in gastric ulcers and neoplasms. *Int. Immunopharmacol.* **1**, 2063–2080
- 32 Jordan, B. A., Trapaide, N., Gomes, I., Nivarthi, R. and Devi, L. A. (2001) Oligomerization of opioid receptors with β 2-adrenergic receptors: a role in trafficking and mitogen-activated protein kinase activation. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 343–348
- 33 Rocheville, M., Lange, D. C., Kumar, U., Sasi, R., Patel, R. C. and Patel, Y. C. (2000) Subtypes of the somatostatin receptor assemble as functional homo- and heterodimers. *J. Biol. Chem.* **275**, 7862–7869
- 34 Noel, J. P., Hamm, H. E. and Sigler, P. B. (1993) The 2.2 Å crystal structure of transducin- α complexed with GTP γ S. *Nature (London)* **366**, 654–663
- 35 Hamm, H. E. (2001) How activated receptors couple to G proteins. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 4819–4821
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