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Characterization of dual agonists for kinin B1 and B2 receptors and their biased activation of B2 receptors

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ABSTRACT

Kinin B1 and B2 receptors (kB1R and kB2R) play important roles in many physiological and pathological processes. In some cases, kB1R or kB2R activation can have overlapping or complementary beneficial effects, thus an activator of both receptors might be advantageous. We found that replacement of the C-terminal Arg in the natural kB2R activators bradykinin (BK) or kallidin (KD) with Lys (K^9-BK or K^{10}-KD) resulted in agonists that effectively stimulate the downstream signaling of both the kB1R and kB2R as measured by increased inositol turnover, intracellular calcium, ERK1/2 phosphorylation, arachidonic acid release and NO production. However, K^9-BK and K^{10}-KD displayed some characteristics of biased agonism for kB2Rs as indicated by the rapid kinetics of ERK1/2 phosphorylation induced by K^9-BK or K^{10}-KD compared with the prolonged response mediated by BK or KD. In contrast, kinetics of ERK phosphorylation stimulated by K^{10}-KD activation of the kB1R was the same as that induced by known kB1R agonist des-Arg^{10}-KD. Furthermore, the endocytosis of kB2Rs mediated by K^9-BK and K^{10}-KD was remarkably less than that induced by BK and KD respectively. K^{10}-KD stimulated kB1R and kB2R-dependent calcium responses and ERK1/2 phosphorylation in bovine endothelial cells. In cytokine-treated human endothelial cells, K^{10}-KD stimulated ERK1/2 phosphorylation and a transient peak of NO production that was primarily kB2R-dependent. K^{10}-KD also stimulated prolonged NO production that was both kB1R and kB2R-dependent. These data provide the first examples of dual agonists of kB1R and kB2R, and a biased agonist of kB2R and may provide useful clues for developing dual modulators of kB1Rs and kB2Rs for potential therapeutic use.

1. Introduction

Bradykinin (BK) or kallidin (KD) have 9 or 10 amino acids and are released from kininogen precursors by plasma or tissue kallikrein that selectively activate the kinin B2 receptor (kB2R) [1,2]. The C-terminal Arg of BK or KD can be cleaved by membrane carboxypeptidase M (CPM) or plasma carboxypeptidase N (CPN) to generate des-Arg^9-BK (DABK) or des-Arg^{10}-KD (DAKD), which are not kB2R agonists, but instead specifically activate the kinin B1 receptor (kB1R) [2–7]. Thus, the presence or absence of the basic C-terminal Arg of BK and KD is a key determinant of their selectivity for the two kinin receptors. The kB1R and kB2R have 36% sequence identity in humans and belong to the family of seven transmembrane receptors, coupling to G protein subtypes Gαq and Gαi [2,7].

kB1R and kB2R play key roles in many pathological or physiological processes including nociception, inflammation, and cardiovascular and renal diseases [2,8–11]. For example, kB2R knockout mice have hypertension and exaggerated blood pressure increases induced by angiotensin II and chronic salt loading [12]. kB2R knockout mice develop age-related heart failure due to left ventricular remodeling [13]. kB1R signaling reduces blood pressure in kB2R knockout mice although kB1R knockout alone does not alter normal blood pressure [14]. Diabetic nephropathy was also enhanced in kB2R knockout mice [15] and renal injury due to ischemia/reperfusion was markedly worse in kB1R and kB2R double knockout mice compared with kB2R knockout or wild type mice [16]. These data indicate beneficial roles for kB1R and kB2R signaling in the cardiovascular and renal systems. In contrast, kB1R and kB2R activation can have deleterious effects, causing pain and inflammation. For example, both kB1R and kB2R are involved in nociception as kB2R agonist BK induced thermal hyperalgesia in kB1R knockout mice and kB1R agonist DABK induced thermal

Abbreviations: CP, carboxypeptidase; kB1R, kinin B1 receptor; kB2R, bradykinin B2 receptor; $[Ca^{2+}]_i$, intracellular calcium concentration; PBS, phosphate buffered saline; DMEM, Dulbecco's Modified Eagle's Medium; GPCR, G protein-coupled receptor; BK, bradykinin; KD, kallidin; DABK, des-Arg^9-bradykinin; DAKD, des-Arg^{10}-kallidin; K^9-BK, Lys^9-bradykinin; K^{10}-KD, Lys^{10}-kallidin; DALKD, des-Arg^{10}-Leu^9-kallidin; HEK, human embryonic kidney; FBS, Fetal bovine serum; CHO, Chinese hamster ovary; PI, phosphoinositide; IP3, inositol triphosphate; RIPA, radio-immunoprecipitation assay; B9430, D-Arg-[Hyp^3, Igl^5, D-Igl^7, Oic^8]-bradykinin; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase.

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hyperalgesia in kB2R knockout mice [17]. kB1R also regulates pain by facilitating the nociceptive spinal reflex, as it was decreased in kB1R knockout mice [18]. The kB1R and kB2R also regulate the recruitment and infiltration of inflammatory cells into local tissues. For example, in experimental autoimmune encephalomyelitis, leukocytes infiltrating into the central nervous system were decreased in kB2R knockout mice whereas T helper type 17 cells were reduced in kB1R knockout mice [19,20].

Because of the potential roles of these receptors in various diseases, numerous agonists and antagonists of kB1R or kB2R have been developed [21–24], with most of them being derivatives of the natural kinin peptides [2], but some low molecular weight non-peptide ligands have also been produced [2,24]. These ligands generally have high selectivity for either the kB1R or kB2R [2]. However, in cases where kB1R and kB2R activation results in overlapping or complementary responses [12,14,17,19,20], ligands that bind to both kB1R and kB2R might be more therapeutically efficacious. B9430 (D-Arg-[Hyp^3, Igl^5, D-Igl^7, Oic^8]-BK) was developed as a dual antagonist of both the kB1R and kB2R [25], but dual agonists of kB1R and kB2R have not been described. Although KD can bind with a relatively high affinity to both human receptors [2], it is either much less potent or inactive in stimulating kB1R-dependent responses [3,26–29]. A complicating factor in studies assessing the activity of KD on kB1Rs is the possible conversion of added KD to the potent kB1R agonist DAKD by cellular carboxypeptidases such as the widely expressed carboxypeptidase M [4,30,31]. In fact, we found that in transfected HEK cells stably expressing only kB1Rs, KD was inactive in stimulating a calcium response, but became a potent kB1R agonist in cells co-expressing CPM and kB1Rs [3,27].

G protein-coupled receptor activation by agonists was initially thought to convert the receptor from an "off" state to a fully "on" state that would then activate all associated downstream pathways. However, more recently it has been discovered that some agonists may activate only a subset of possible receptor responses, which has been termed "biased agonism" or "ligand bias" [32,33]. Furthermore, agonists may exhibit bias in their ability to stimulate receptor desensitization and endocytosis [32,33]. Biased agonists have been well characterized for several G protein-coupled receptors, but have not been identified for the kinin receptors.

Because the presence or absence of the C-terminal Arg of BK and KD is a key switch for selectivity with the kB1R or kB2R, we tested the agonist activity of BK and KD in which the C-terminal Arg was replaced with lysine (K^9-BK and K^{10}-KD). Surprisingly, we found that K^9-BK and K^{10}-KD effectively stimulate both kB1R and kB2R signaling, but had biased effects on kB2R-mediated ERK activation. These findings provide promising clues for development of dual kB1R and kB2R agonists that could be useful therapeutic agents for treatment of cardiovascular diseases.

2. Materials and methods

2.1. Materials

Low-glucose Dulbecco's Modified Eagle's Medium (DMEM) and HAM's F-12 medium were obtained from GIBCO/Life Technologies. Fetal bovine serum (FBS) was from Atlanta Biologicals. HOE140, des-Arg^9-HOE140, BK, des-Arg^9-BK, des-Arg^{10}-KD and des-Arg^{10}-Leu^9-KD were from Sigma. KD was from Bachem. K^9-BK and K^{10}-KD were synthesized by Chi Scientific. [^{3}H]-Des-Arg^{10}-KD was from PerkinElmer. [^{3}H]-BK was from Amersham Biosciences. The primary sequence of peptides used is summarized in Table 1. [^{3}H]-Arachidonic acid and myo-[^{3}H]inositol were from American Radiolabeled Chemicals, Inc. Fura-2/AM was from Molecular Probes. Anti-ERK1/2 and anti-phosphorylated ERK1/2 antibodies were from Cell Signaling. Goat anti-mouse and anti-rabbit IgG conjugated-HRP was from Pierce. Common chemicals were from Fisher Scientific.

Table 1
Sequences of peptides used.

Peptide	Amino acid sequence
Bradykinin (BK)	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Lys^9-bradykinin (K^9-BK)	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Lys
Des-Arg^9-bradykinin (DABK)	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe
Kallidin (KD)	Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Lys^{10}-kallidin (K^{10}-KD)	Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Lys
Des-Arg^{10}-kallidin (DAKD)	Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe
Des-Arg^{10}-Leu^9-kallidin (DALKD)	Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Leu
HOE 140[a]	D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-Tic-Oic-Arg

[a] Hyp, *trans*-4-hydroxy-Pro; Thi, β-(2-thienyl)-Ala; Tic, [D]-1,2,3,4-tetrahydroisoquinolin-3-yl-carbonyl; Oic, (3as,7as)-octahydroindol-2-yl-carbonyl.

2.2. Cells

Human embryonic kidney (HEK293) and Chinese hamster ovary (CHO) cells were from the American Type Culture Collection. Cells were maintained in DMEM or F-12 containing 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FBS. Bovine pulmonary artery endothelial cells (BPAEC) were from Genlantis and were maintained in DMEM containing 100 U/ml penicillin, 100 mg/ml streptomycin and 15% FBS. Primary human lung microvascular endothelial cells (HLMVEC) were from Lonza and cultured in flasks coated with 0.1% gelatin in Endothelial Cell Basal Medium (EBM®-2, Lonza) supplemented with EGM®-2 SingleQuots® kit (Lonza) and 10% fetal bovine serum (Atlanta Biologicals). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and HLMVEC between passages 3 and 6 were used for assay.

2.3. Generation of receptor constructs

The cDNA for human kB1R was a gift from Dr. Fredrik Leeb-Lundberg (University of Lund, Sweden) and for human kB2R was from Syntex Co. The cDNA of human CPM was cloned by our lab [34]. Wild type kB1R, kB2R or CPM was cloned into pcDNA3 or pcDNA6 vectors (Invitrogen) for expression in mammalian cells. kB1R was also cloned into pIRES (Clontech) at the Nhe I/Xho I sites, together with EGFP at the Sal I/Not I sites, to achieve the coexpression of kB1R and GFP separately, but at the same time in the same cells [3]. kB2R-YFP was generated as described by Chen et al. [35]. All the PCR fragments used were amplified using high fidelity Taq DNA polymerase. Constructs were verified by DNA sequencing performed by the DNA Services Facility of the Research Resources Center, University of Illinois at Chicago.

2.4. Transfection and establishment of stable cell lines

HEK293 cells, at 70–80% confluence in 6-well plates, were transfected with SuperFect (Invitrogen) reagent containing 5 μg DNA according to the manufacturer's instructions. After 48 h, cells were transferred to selective medium containing G418 (500 μg/ml) or blasticidin (5 μg/ml) according to the resistance gene contained in the vector. The cells were cultured for 15–30 days in selective medium, and then diluted for single clone selection. For kB1R and kB2R selection, the increase in $[Ca^{2+}]_i$ stimulated by their specific agonist (DAKD or DABK, respectively) was evaluated for each clone [3]. The cDNA of kB2R-YFP was transfected into CHO cells, and the appropriate clones were selected in HAM's F-12 medium supplemented with blasticidin (5 μg/ml). Cells that highly expressed YFP were separated by fluorescence-activated cell sorting with an Elite ESP cell sorter (Coulter Corp.). The function of kB2R-YFP was confirmed by measuring arachidonic acid generation after stimulation with BK [35]. For CPM selection, the enzyme activity was determined as described [3] and expression was confirmed by Western blotting.