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ARTICLES

Regulation of Inducible Bradykinin B1 Receptor Gene Expression Through Absence of Internalization and Resensitization

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Abstract Rapid induction and down-regulation of bradykinin B1 receptor (BKB1R) gene expression is tightly regulated at the transcriptional and mRNA levels (Zhou et al. [1998] Biochem. J. 330:361–366; Zhou et al. [1999] Mol. Cell Biol. Res. Commun. 1:29–35). Here we explore regulation of BKB1R expression at the protein level. To make this inducible gene express constitutively, we utilized a bicistronic mammalian expression vector (pCMin) for stable transfection of the BKB1R gene into human lung fibroblasts, IMR90SV40. The BKB1R displayed a high affinity and specificity (K_d = 0.5 nM) for desArg^{10}-kallidin. The receptor mediated such signaling events as arachidonic acid (ARA) release, phosphoinositide (PI) turnover and Ca^{2+}-flux. The receptor function proved differentially desensitized. For example, after initial exposure to desArg^{10}-kallidin, a second stimulation with desArg^{10}-kallidin did not induce further Ca^{2+}-flux or ARA-release while PI-turnover continued unabated. Unlike most of the G-protein coupled receptors, the BKB1R did not internalize within 60 min of exposure to 10 nM desArg^{10}-kallidin. It also did not resensitize. Thus, the duration and signal capacity of the BKB1R at the protein level is regulated through lack of internalization, an absence of resensitization and a lack of desensitization for certain events such as PI turnover. In fact, the absence of BKB1R resensitization is likely a very important contributor to the rapid disappearance of this inducible receptor. J. Cell. Biochem. 78:351–362, 2000.

Key words: G-protein coupled receptor; inducibility; bradykinin B1 receptor; differential desensitization; internalization; resensitization; arachidonic acid release; phosphoinositide turnover; Ca^{2+}-flux; bicistronic mammalian expression vector

The G-protein coupled bradykinin B1 receptor (BKB1R) is induced by inflammatory processes and bacterial infection [Dray and Perkins, 1993]. The receptor gene has been cloned at both the cDNA and genomic levels [Menke et al., 1994; Yang and Polgar, 1996]. The BKB1R is expressed only minimally under normal, basal conditions. Upon exposure to inflammatory cytokines, BKB1R gene expression is upregulated rapidly [Dray and Perkins, 1993]. Its transient expression is regulated through transcriptional activation and mRNA destabilization [Zhou et al., 1998, 1999]. Regulation of this receptor at the protein level could prove important in the short-lived expression of this gene. However, at this time, the regulation and duration of BKB1R action has been studied only sparsely. This is at least in part due to the very low constitutive expression of this gene. Human embryo lung fibroblasts, IMR90, and IMR90SV40 (IMRSV) express functional BKB1R after exposure to IL-1β [Zhou et al., 1998; Menke et al., 1994]. We generated a stable IMRSV cell line that constitutively expressed BKB1R. The generation of this transfectant was facilitated by expression in a bicistronic (pCMin) vector.

The response of G-protein coupled receptors (GPCR) to agonists is tightly regulated through desensitization and resensitization processes [Dohlman et al., 1991; Hausdorff et al., 1990]. Desensitization prevents cells from uncon-

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trolled stimulation, and resensitization allows cells to recover and maintain responsiveness [Bohm et al., 1997]. The current paradigm is based largely on studies of a limited number of receptors, such as the G_s-coupled β-adrenergic receptor [Ferguson et al., 1995; Freedman et al., 1995; Fredericks et al., 1996; January et al., 1997]. In contrast, relatively little is known about the nature and role of these regulatory mechanisms within the G_i and G_q coupled receptor families.

Results presented here demonstrate that the various signals mediated by BKB1R are regulated through selective desensitization, meaning that while Ca^{2+} flux and arachidonic acid (ARA) release are desensitized upon addition of a second dose of ligand, desArg^{10}-kallidin, phosphoinositide (PI) turnover is not. Perhaps more importantly, BKB1R is not internalized or resensitized, insuring that the receptor's life span is limited. This is in contrast to the constitutively expressed BKB2R which is rapidly internalized and resensitized [Prado et al., 1997, 1998].

MATERIALS AND METHODS

Bicistronic Mammalian Expression Vector, pCMin

The bicistronic vector, pCMin, was constructed in our laboratory to allow expression of two genes from one mRNA.[1] The first open reading frame is designed to contain the gene of interest. Its translation is carried out by conventional cap dependent ribosome entry. The second open reading frame contains the antibiotic-resistance gene and is translated due to insertion of a viral internal ribosome entry site (IRES) which allows the efficient mRNA cap-independent entry of the translational apparatus in eukaryotic cells [Kim et al., 1992]. pCMin is comprised of a vector backbone from pcDNA3 (Invitrogen, Carlsberg, CA) which spans from the neomycin gene to the CMV promoter. The multiple cloning sites (MCS) were constructed for insertion of the gene of interest. The IRES from encephalomyocarditis virus (EMCV) was inserted between the MCS and the neomycin gene to permit the translation of two open reading frames.

[1]For patent and technical information on pCMin, contact P. Polgar.

Cell Culture and Transfection

IMRSV cells were grown at 37°C in minimal essential medium, containing 10% fetal bovine serum supplemented with 50 units/ml penicillin and 50 ug/ml streptomycin. Rat-1 cells were grown in Dulbecco's modified Eagle's medium, containing 5% fetal bovine serum, 50 unit/ml penicillin and 50 ug/ml streptomycin. The BKB1R cDNA was produced as follows. Total RNA from IMR 90 cells, that were treated with IL-1β for 2 h, was converted to single stranded cDNA by reverse transcription and then amplified by PCR with 5′-CTGTGCATGGCATCATCCT GGCCC-3′ as the sense primer and 5′-CAATGCTGTTTTAATTCCGCCA-3′ as the antisense primer. The PCR product was cloned into the TA-cloning vector from Invitrogen Inc. It was then cloned into the bicistronic mammalian expression vector, pCMin. The fidelity of the constructs was confirmed through sequencing by the DNA-Protein Core of our Medical Center with an automatic DNA sequencer (Applied Biosystem Inc., Foster City, CA, Model 370A). For transfection, two solutions were prepared. One contained 2 ug of DNA in 20 ul of OPTI-MEM (Life Technologies, Inc., Bethesda, MD). The other contained 6 ul of Lipofectamine (Life Technologies, Inc.) and 14 ul of OPTI-MEM medium. The solutions were combined and incubated at room temperature for 15 min. The DNA-liposome complex was added to 1 ml of OPTI-MEM and added to each well. Sixteen hours later, the transfection medium was replaced with complete medium. The next day, transfected IMRSV were detached with trypsin and seeded in a 60-cm^2 dish containing complete medium and 500 μg/ml of Geneticin (G418, Life Technologies, Inc.) for selection.

Northern Blot Analysis

Total RNA was extracted from cells with guanidinium/ thiocyanate-phenol/chloroform according to the method described previously [Taylor et al., 1992]. RNA was quantitated by ultraviolet absorbance at 260 nm. Ten μg of denatured RNA was electrophoresed on a 1% agarose/formaldehyde denaturing gel and transferred to nylon membranes (DuPont–NEN, Boston, MA). The blots were hybridized with ^{32}P-labelled cDNA probes, at 65°C in a Rapid-hyb buffer (Amersham Life Science, Amersham, UK) and washed as described previ-

ously [Taylor et al., 1992]. All incubations were done at least in duplicate. The cDNA probe for BKB1R was generated as described previously [Zhou et al., 1998]. The cDNA probe for c-fos was obtained from Dr. Karin (School of Medicine, University of California, San Diego, La Jolla) as a generous gift.

Receptor Binding Assay

Receptor binding studies of BKB1R and BKB2R in intact IMRSV were carried out as described previously [Prado et al., 1997]. Confluent cell monolayers in 24-well plates (Costar, Cambridge, MA) were incubated in binding buffer containing various concentrations of [^{3}H]-BK (for BKB2R) or [^{3}H]-desArg^{10}-kallidin (for BKB1R) in the absence (total binding) or the presence of 10^{-7}M unlabeled ligand (nonspecific binding) for 2 h at 4°C. Cells were washed three times with ice-cold buffer and then solubilized with 0.2% sodium dodecyl sulfate. Radioactivity was determined in a LKB β counter. The affinity and the number of binding sites were then determined.

Arachidonic Acid Release

IMRSV were prelabeled with [^{3}H]-arachidonate (0.2 uCi/well) for 18 h as described previously [Prado et al., 1997]. Cells were washed with MEM containing 2 mg/ml bovine serum albumin, and incubated with stimulant for 20 min at 37°C. Medium was removed and centrifuged at 800*g*. Radioactivity in the supernatant was determined in an LKB β counter. Desensitization of ARA release was done as described previously [Prado et al., 1998]. Cells were pretreated with MEM containing saturating doses of BK or desArg^{10}-kallidin, followed by an intervening wash and then restimulated with second doses of BK or desArg^{10}-kallidin. Radioactivity in the supernatant was determined as described above.

Phosphoinositide Turnover

IMRSV were incubated with 1 μCi/ml of myo-[^{3}H]-inositol in 1 ml of growth medium for 18 h and the levels of inositol phosphate was determined 1 day later as previously described [Prado et al., 1997]. Desensitization of PI-turnover was done as described previously [Austin et al., 1997]. Briefly, the myo-[^{3}H]inositol-labeled cells were exposed to BK or desArg^{10}-kallidin and the total inositol phosphate was determined at 0 min, 30 min, and 60 min after exposure.

Receptor Internalization

Receptor internalization in intact cells was carried out as described previously [Prado et al., 1997]. Briefly, cells were incubated with 100 nM BK (for BKB2R) or desArg^{10}-kallidin (for BKB1R) for different times at 37°C. Cells were washed with ice-cold buffer and acid-stripped by incubating the cell for 30 s with 0.2 M acetic acid, pH 3.0, containing 0.5 M NaCl. The number of binding sites was determined by performing a binding assay as above. Data are expressed as the percentage of specific binding remaining after an acid wash.

Calcium Mobilization Assays

Mobilization of Ca^{2+} was determined as reported previously [Prado et al., 1998]. IMRSV were detached and washed once in MEM medium containing 10% FBS. The cells were then washed a second time in physiological buffer solution (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 0.9 mM $CaCl_2$, 15 mM HEPES, 0.1% BSA). The IMRSV were resuspended at 1×10^7 cells/ml and incubated with FURA-2AM for 30 min (2 μM final concentration). After 30 min, the cell suspension was diluted 10× with physiological buffer solution and incubated for another 30 min. Cells were pelleted and resuspended at 1×10^7 cells/ml. Ca^{2+} mobilization experiments were performed using a Perkin Elmer Fluorescence Spectrophotometer LS-3B. Intracellular calcium increase in the presence or absence of desArg^{10}-kallidin, BK, or desArg^9-bradykinin was measured as described previously [Ricupero et al., 1997]. Data were analyzed using the program, FURA (Perkin-Elmer, Oak Brook, IL).

To examine desensitization, cells were allowed to equilibrate at 37°C for 5 min and then stimulated sequentially with 10 nM then 100 nM of BK or desArg^{10}-kallidin at 2-min intervals. To confirm the results obtained from this desensitization procedure, a second method was used in all instances, which includes the first dose of 10 nM ligand, followed by a rapid wash and a second dose of 100 nM ligand. Resensitization was examined by exposing cells to a desensitizing dose of 10 nM BK or desArg^{10}-kallidin for various times at 37°C. The cells were then washed with physiological

TABLE I. Stable Transfection Efficiency of Bicistronic Vector pCMin vs. pcDNA3[a]

	pCMin Vector		pcDNA3 Vector	
	Clone	Receptor per cell	Clone	Receptor per cell
Stable transfection of BKB2R in Rat-1 cells	C	280 (98.5)	C	280 (98.5)
	1	10,598 (2347)*	1	3,437 (469)*
	2	14,459 (1395)*	2	212 (151)
	3	17,487 (2355)*	3	13,111 (1257)*
	4	10,628 (2460)*	4	430 (227)
	5	21,166 (1196)*	5	341 (101)
	6	8,524 (705)*	6	197 (97)
	7	15,877 (5025)*	7	179 (110)
	Mix	14,209 (975)*	Mix	314 (132)
Stable transfection of BKB1R in IMRSV	C	1,014 (406)		
	1	17,607 (2048)*		
	2	13,755 (528)*		
	3	11,714 (416)*		
	4	7,993 (454)*		
	5	11,158 (890)*		
	Mix	13,899 (554)*		

[a]Rat-1 cells were stably transfected with pCMin/BKB2R or pcDNA3/BKB2R constructs as described (upper panel). IMRSV were stably transfected with pCMin/BKB1R construct (lower panel). Both single cell clones and mass cultures were obtained and the specific binding for BKB2R and BKB1R were measured as described. "C" represents the untransfected cells; "Mix" represents the mixed population; The value in the brackets is the standard deviation.
*Indicates significant difference ($P < 0.05$) from corresponding C (untransfected control).

buffer and resuspended at 1×10^7 cells/ml. Calcium mobilization assays as described above were carried out by stimulating cells with a second dose of 10 nM BK or desArg^{10}-kallidin after the desensitization.

RESULTS

Efficiency of the pCMin Plasmid

With conventional mammalian expression vectors, such as pcDNA3, separate promoters are used to drive the expression of a resistance gene such as neomycin and the gene of interest. The cells form distinct colonies upon transfection and selection. It is then necessary to isolate single cells that express the gene of interest. Generally this is a lengthy and difficult process. To make the stable transfection into a routine step, we constructed a bicistronic vector that makes cell cloning following transfection unnecessary. To test the efficacy of the procedure, we inserted the BKB2R gene into the pCMin/neomycin vector and transfected this construct into the Rat-1 cells. Binding of BK to seven isolated clones is shown in Table I. As expected, all isolated clones bound BK at approximately the same level. Importantly, the original transfected mass culture, grown to confluence, bound BK. Conversely, only two of the seven pcDNA3/BKB2R transfectant colonies bound BK. The transfected mass culture, in this case, did not bind BK.

Because the IMRSV do not form individual colonies following transfection, we generated a mass culture of IMRSV transfected with the pCMIN/neomycin/BKB1R. The mixed population bound desArg^{10}-kallidin as illustrated in Table 1. To insure that all cells within this mass culture expressed the BKB1R, each of the five clones that originated from a single cell dilution in a 96-well plate were then isolated. All clones bound desArg^{10}-kallidin as shown. The pcDNA3/BKB1R transfected IMRSV mass culture did not bind desArg^{10} kallidin (Data not shown). No further dilution cloning procedures were carried out with this transfectant.

Characterization of Stable BKB1R Transfected IMRSV

IMRSV stably transfected with pCMin/Neomycin/BKB1R construct ("pCMinB1" cells)

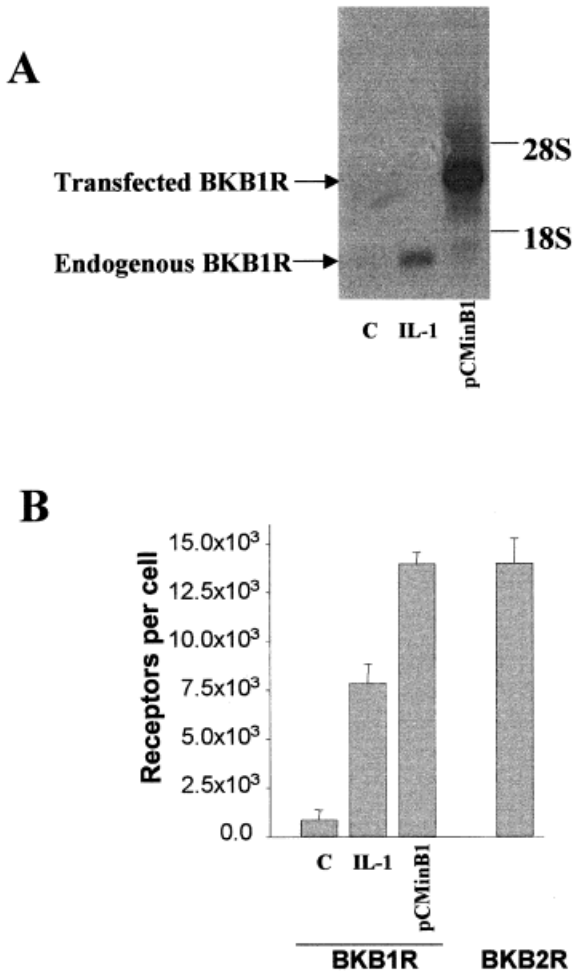


Fig. 1. Characterization of the pCMinB1 stable cell line. **A**: Total RNA was isolated from pCMinB1 cells and untransfected IMRSV cells that were stimulated with 100 pg/ml IL-1β or vehicle alone for 2 h. Northern blot was performed as described. The positions of transfected and endogenous BKB1R mRNA and the ribosomal 18 S and 28 S are indicated. **B**: Binding assays were performed on pCMinB1 cells and untransfected IMRSV cells that were stimulated with 100 pg/ml IL-1β or vehicle alone for 2 h. [^{3}H]desArg^{10}-kallidin was used for BKB1R binding. [^{3}H]BK was used for BKB2R binding. The numbers of receptors per cell were calculated. Data is a representative of at least three independent experiments.

were characterized as illustrated in Figure 1. As shown in Figure 1A, the transfected BKB1R mRNA displayed a higher molecular weight than the endogenous BKB1R mRNA due to the inclusion of the neomycin resistance gene mRNA with the BKB1R mRNA. Treatment of IMRSV with IL-1β induced endogenous BKB1R expression. Introduction of the pCMin/Neo/BKB1R construct did not induce endogenous BKB1R expression (Fig. 1A).

Wild-type IMRSV constitutively express approximately 1.4×10^4 BKB2R/cell and 1×10^3 BKB1R/cell. Cytokines such as IL-1β or TNFα rapidly increase the number of BKB1R/cell [Zhou et al., 1998, 1999; Menke et al., 1994]. The low number of these receptors under basal conditions is illustrated in Figure 1B. Following treatment with IL-1β, the BKB1R number increases approximately eight-fold to 7.8×10^3/cell (Fig. 1B). The stable transfectant, pCMinB1, expressed approximately 1.39×10^4 BKB1R/cell. As illustrated, a similar number of BKB2R/cell were also present in the pCMinB1. The pCMinB1 thus provided us with a unique model to study the regulatory mechanisms of BKB1R stability and function, particularly in comparison to the closely related and constitutively expressed BKB2R.

To confirm that the BKB1R retained its binding characteristics within the pCMinB1 cells a Scatchard Plot analysis was performed on saturation binding curves of desArg^{10}-kallidin and BK. The K_d for desArg^{10}-kallidin is 0.5 nM and for BK is 2.6 nM. The ligand specificity for the receptors was evaluated by displacement of radioactively labeled ligands from receptors with various competitors. For BKB1R, the order of potency was desArg^{10}-kallidin⩾desArg^9-bradykininK>BK. For BKB2R, BK displaced [^{3}H]-BK effectively, whereas, both desArg^{10}-kallidin and desArg^9-bradykinin were not able to displace [^{3} H]-BK.

The potency of desArg^{10}-kallidin and desArg^9-bradykinin has been shown to be different for BKB1R from different species [Pesquero et al., 1996; Hess et al., 1996]. To confirm the order of ligand specificity for this human BKB1R at the functional level, we determined Ca^{2+} flux in response to various ligands in wild-type IMRSV and pCMinB1 cells. As shown in Figure 2, 100 nM desArg^9-bradykinin, 10 nM desArg^{10}-kallidin, but not 10 nM desArg^9-bradykinin, induced an increase of $[Ca^{2+}]_i$ in the pCMinB1 cells. For the IMRSV cells, no detectable increase of $[Ca^{2+}]_i$ in response to desArg^9-bradykinin or desArg^{10}-kallidin was observed. BK stimulation induced an increase of $[Ca^{2+}]_i$ in both cell types since BKB2R is constitutively expressed in these cells. These results confirmed that the transfected BKB1R was functionally active and desArg^{10}-kallidin has a higher ligand specific-

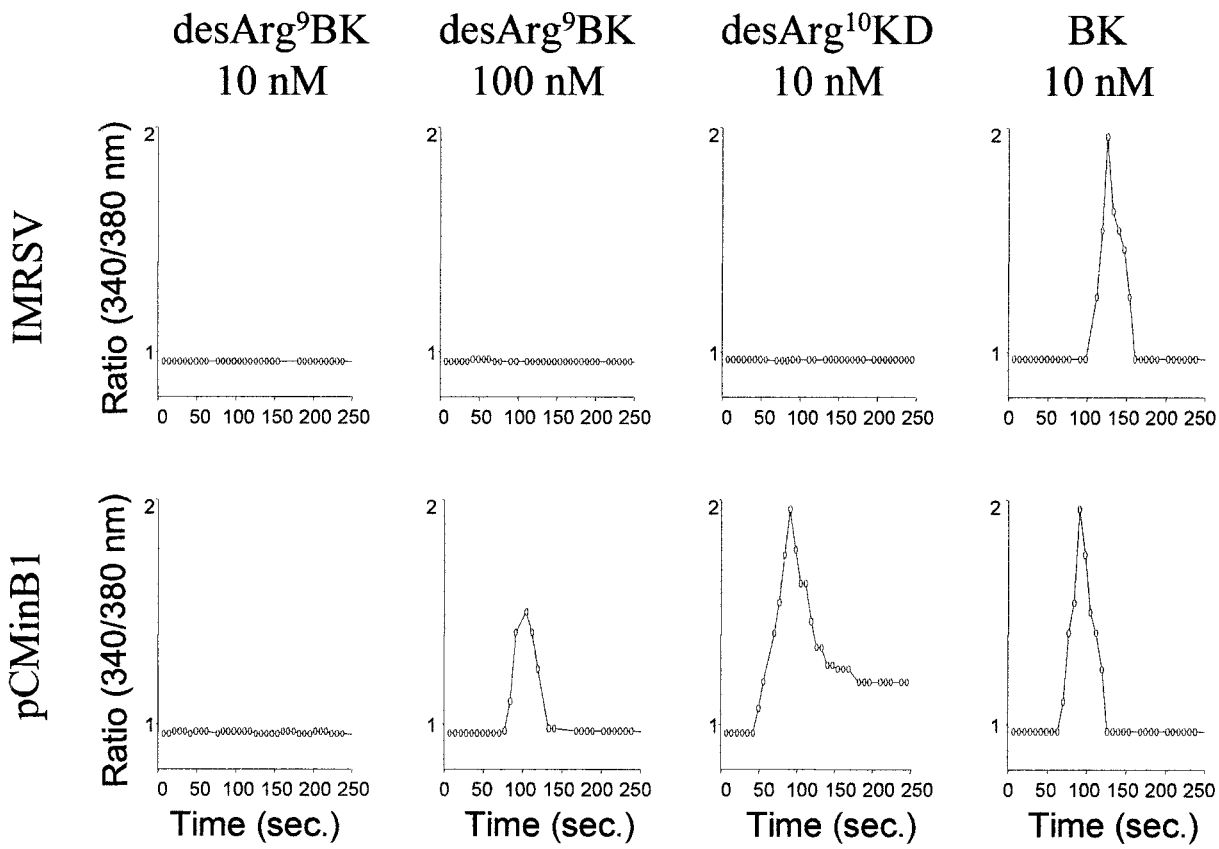


Fig. 2. Effect of various kinin effectors on intracellular calcium mobilization in IMRSV and pCMinB1 cells. Fura-2-loaded untransfected IMRSV cells (upper panel) and pCMinB1 cells (lower panel) were stimulated with 10 nM desArg^9-bradykinin, 100 nM desArg^9-bradykinin, 10 nM desArg^{10}-kallidin, or 10 nM BK. Ca^{2+} mobilization was measured as described in methods section. The data shown are representative of at least three independent experiments.

ity for this human BKB1R than desArg^9-bradykinin does.

Signaling by Ligand Activated BKB1R

The pCMinB1 cells were exposed to 10 nM desArg^{10}-kallidin or 10 nM BK. ARA release, PI turnover and calcium mobilization were determined. We also evaluated the ability of BKB1R to stimulate c-fos expression, since it was formerly determined that BKB2R upregulates the expression of this gene [El-Dahr et al., 1998]. As shown in Figure 3A, both desArg^{10}-kallidin and BK increased ARA release to a similar magnitude. However, desArg^{10}-kallidin displayed a considerably larger effect on PI turnover (Fig. 3B). As shown in Figure 3C, while BK triggered a rapid transient Ca^{2+} flux, desArg^{10}-kallidin triggered a biphasic flux with a rapid initial increase and a sustained elevated plateau. This biphasic pattern was also reported with desArg^9-bradykinin stimulation of rabbit superior mesenteric artery smooth muscle cells [Mathis et al., 1996] most likely affecting a related but not identical receptor. As illustrated in Figure 3D, activation of BKB1R also increased c-fos gene expression. In fact, activation by desArg^{10}-kallidin appeared to cause a considerably more pronounced increase in c-fos mRNA level than BK.

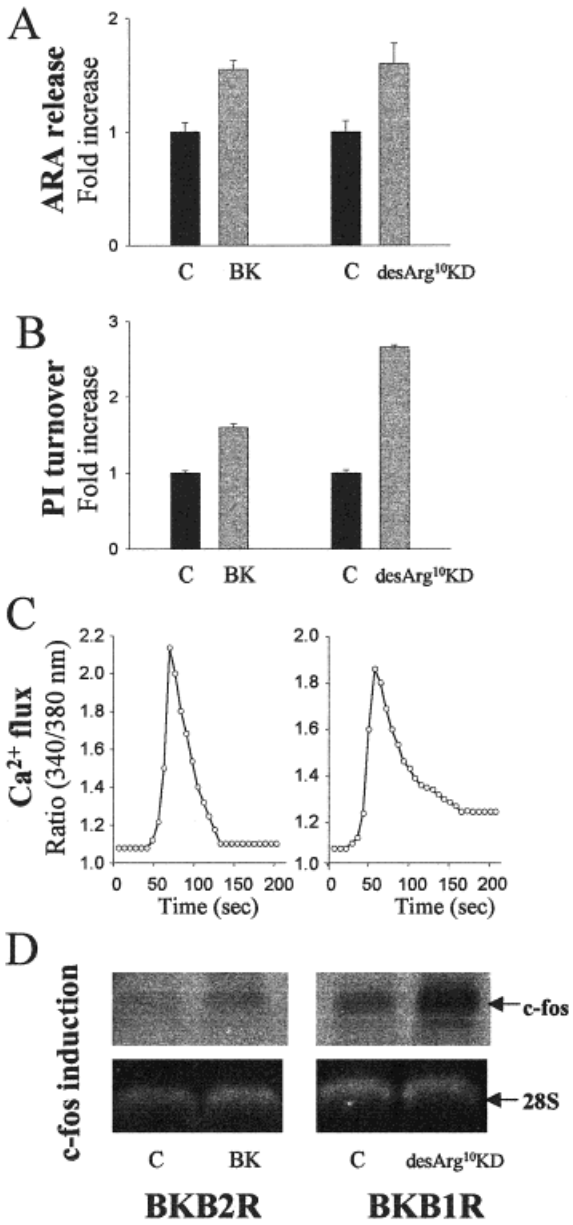
Decay of the Functional Receptor

To test the stability of the functional BKB1R, we treated the cells with 10 μg/ml cycloheximide (CHX). This CHX concentration was determined previously to completely inhibit protein synthesis in less than 5 min [Zhou et al., 1998]. We then tested the binding for either BKB1R or BKB2R at various time points. As

shown in Figure 4, 50% of the binding of desArg^{10}-kallidin was lost after 4 h CHX treatment. In contrast, there was no statistically significant loss of BK binding during the same time interval. It is tempting to suggest that compared to the BKB2R, the BKB1R plays a transient role which correlates with the rapid upregulation and downregulation of this receptor [Zhou et al., 1998].

Ligand Induced Internalization

A marked difference between BKB1R and BKB2R was also observed with regard to ligand-induced internalization. As illustrated in Figure 5, the BKB2R was internalized rapidly upon exposure to 10 nM BK with only 30% specific binding remaining after 30 min. However, unlike the BKB2R, ligand activated BKB1R displayed no statistically significant internalization within 60 min following binding.



Receptor Desensitization

Ligand-induced desensitization for the BKB1R was tested with regard to ARA release, PI turnover and Ca^{2+} flux. After initial exposure, a second dose of desArg^{10}-kallidin had no further effect on ARA release (Fig. 6A). Also, the time course of BKB1R mediated release of ARA reached a plateau within 30 min (inset). These results illustrate ligand-induced desensitization. However, with regard to PI turnover, desArg^{10}-kallidin mediated a linear accumulation of total IP over 60 min that suggested a lack of desensitization (Fig. 6B). With regard to Ca^{2+} flux, BKB1R did not induce further Ca^{2+} flux, after a second ligand exposure (Fig. 6C). Notably, the BKB1R mediated $[Ca^{2+}]_i$ increase sustained an elevated baseline following the initial transient peak. These results suggest that the various actions of the BKB1R are regulated selectively through desensitization.

Specificity of the Ca^{2+} Flux Desensitization

A lack of cross-desensitization between BKB2R and BKB1R has been shown using

Fig. 3. Functional comparison of the BKB1R and the BKB2R. **A**: The [^{3}H]arachidonate-labeled pCMinB1 cells were exposed to desArg^{10}-kallidin, BK, or vehicle alone and the arachidonic acid release was measured as described. The error bars represent standard deviations of three experiments. The increases over control (vehicle treated) are statistically significant ($P < 0.05$). **B**: The myo-[^{3}H]inositol-labeled cells were exposed to desArg^{10}-kallidin, BK or vehicle alone and the total inositol phosphate was determined as described in the methods section. The error bars represent standard deviations of three experiments. The increases over control (vehicle treated) are statistically significant ($P < 0.05$). **C**: The Fura-2-loaded cells were stimulated with 10 nM desArg[^{10}-kallidin, or BK and the Ca^{2+} was measured as described. Data represent three separate experiments. **D**: Cells were stimulated with desArg^{10}-kallidin, BK or vehicle alone for 2 h. Total RNA was isolated and the Northern blot was performed as described. The ethidium bromide stained formaldehyde gel for ribosomal 18 S was shown to demonstrate equal loading. Data is a representative of two independent experiments.

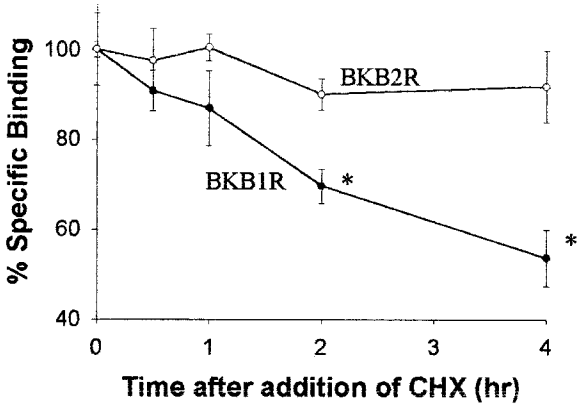


Fig. 4. Effect of CHX on BKB1R and BKB2R binding sites. The pCMinB1 cells were treated with 10 μg/ml CHX. The specific binding for BKB1R and BKB2R was determined at different time points after the addition of CHX as described in the methods section. The error bars represent standard deviations and the asterisks denote significant difference ($P < 0.05$) from time 0.

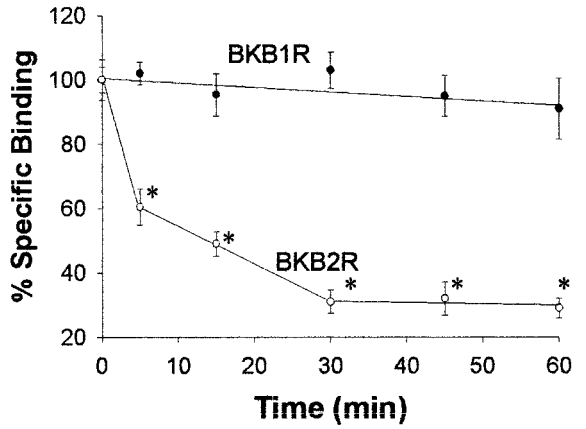


Fig. 5. Ligand induced internalization of BKB1R and BKB2R. The pCMinB1 cells were treated with 100 nM desArg^{10}-kallidin or BK for various periods of time. After acid stripping with buffer at pH 3.0 as described in the methods section to dissociate the receptor-ligand complex on the cell surface and three washes with the binding buffer, the specific binding for BKB1R and BKB2R was determined. The error bars represent standard deviations and the asterisks denote significant difference ($P < 0.05$) from time 0.

desArg^{9}-BK as the ligand for BKB1R [Bascands et al., 1993]. We tested cross-desensitization with desArg^{10}-kallidin as the ligand for BKB1R. After initial exposure to 10 nM BK, addition of 10 nM desArg^{10}-kallidin produced a second $[Ca^{2+}]_i$ peak (Fig. 7A). Similarly, after initial exposure to desArg^{10}-kallidin, addition of BK produced a second Ca^{2+} peak on top of the elevated Ca^{2+} baseline

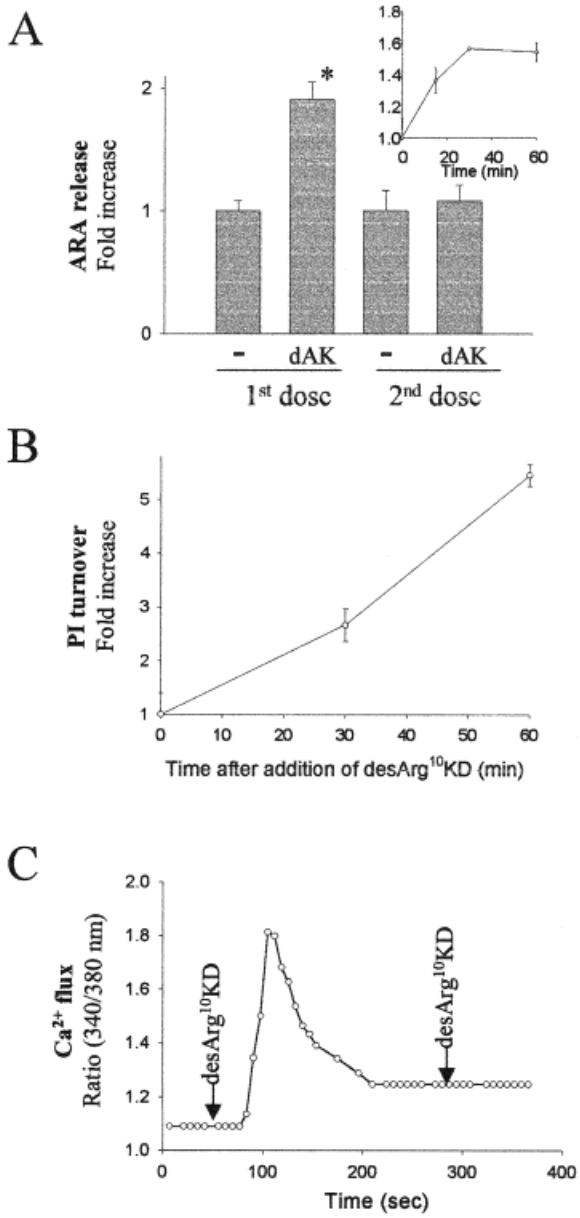


Fig. 6. Desensitization of the BKB1R and BKB2R. **A**: The [^{3}H]arachidonate-labeled pCMinB1 cells were treated with no stimulation (−) or desArg^{10}-kallidin (dAK). After an intervening wash, the stimulated cells were restimulated with a second dose of desArg^{10}-kallidin. The arachidonic acid release was measured as described. The asterisks denote significant difference ($P < 0.05$) from control. **Inset**: pCMinB1 cells were stimulated with desArg^{10}-kallidin and the arachidonic acid release was measured at 0 min, 15 min, 30 min, and 60 min after the stimulation. **B**: The myo-[^{3}H]inositol-labeled cells were exposed to desArg^{10}-kallidin and the total inositol phosphate was determined as described in the methods section at 0 min, 30 min, and 60 min after exposure. **C**: The pCMinB1 cells that were loaded with Fura-2 were stimulated with 10 nM desArg^{10}-kallidin, followed by a second dose of 10 nM desArg^{10}-kallidin. The Ca^{2+} flux was measured as described in the Materials and Methods section. Data is a representative of three independent experiments.

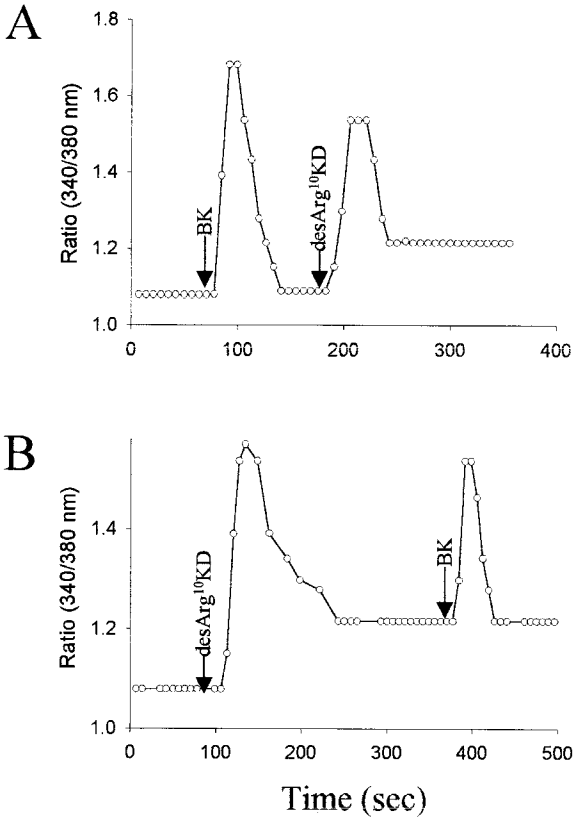


Fig. 7. Cross-desensitization of BKB1R and BKB2R. pCMinB1 cells loaded with Fura-2 were (**A**) stimulated with 10 nM BK followed by 10 nM desArg^{10}-kallidin, or (**B**) stimulated with 10 nM desArg^{10}-kallidin, followed by 10 nM BK. The Ca^{2+} flux was measured as described in the methods section. Data is a representative of three independent experiments.

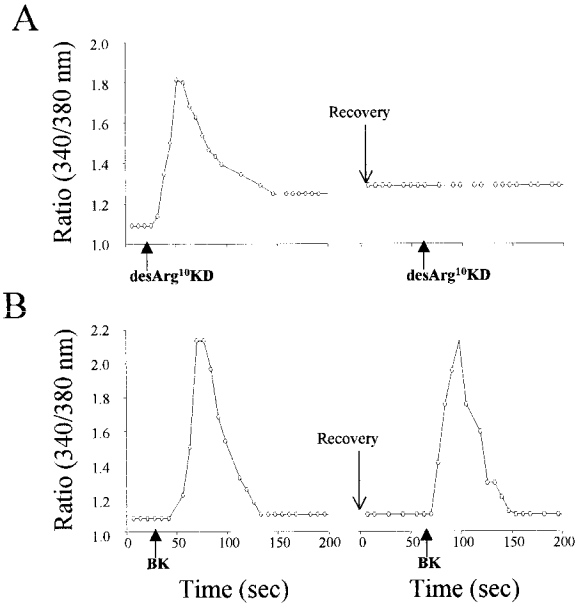


Fig. 8. Resensitization of BKB1R and BKB2R. **A**: The pCMinB1 cells were pretreated with 10 nM desArg^{10}-kallidin for 30 min (left). Cells were washed and allowed to recover at 37°C for 15 min and then stimulated again with 10 nM desArg^{10}-kallidin (right). **B**: The pCMinB1 cells were pretreated with 10 nM of BK for 30 min (left). Cells were washed and allowed to recover at 37°C for 15 min and then stimulated again with 10 nM BK (right). Ca^{2+} flux was then measured. Data is a representative of three independent experiments.

(Fig. 7B). Thus BKB1R and BKB2R are not cross-desensitized.

Resensitization

The pCMinB1 cells were tested for resensitization potential following a saturating dose of desArg^{10}-kallidin or BK. Cells were incubated with the ligand for 30 min and then washed with fresh medium and incubated in the same medium for 15 min. These cells were then stimulated with a second dose of 10 nM desArg^{10}-kallidin or BK. Calcium mobilization was used as determinant. As shown in Figure 8A, desArg^{10}-kallidin was unable to initiate a Ca^{2+} flux after the initial stimulation with desArg^{10}-kallidin. However, a second stimulation with BK induced a sizable increase in $[Ca^{2+}]_i$ (Fig. 8B). Thus unlike BKB2R, BKB1R is unable to regain responsiveness following desensitization. Furthermore, the lack internalization for BKB1R (Fig. 5) suggests that internalization and resensitization are linked.

DISCUSSION

One of the unique features of the BKB1R is its inducibility. This gene is upregulated rapidly, transcriptionally, within 4 hours, by inflammatory cytokines such as IL-1β [Zhou et al., 1998]. Its promoter contains a powerful, cell specific enhancing element which provides the gene with cell and perhaps species selectivity [Yang et al., 1998]. The BKB1R mRNA also contains a unique, very short 3′ tail whose action contributes to the rapid degradation of its message [Zhou et al., 1999]. In this communication we investigated the actions of BKB1R to determine whether the receptor itself provides mechanisms toward its rapid demise. The cytokine dependent upregulation of this gene has made it difficult to study the signaling capacity of this receptor free of the inducing agents which themselves exhibit a broad spec-

trum of signaling actions. To circumvent these complications, we stably transfected BKB1R into IMRSV. Importantly, this cell possesses native functional machinery for the induction and function of this receptor including responsiveness to the BKB1R promoter enhancer [Yang et al., 1998]. The transfected cells expressed a similar number of receptors for both BKB1R and BKB2R (13,900 and 14,000 receptors/cell, respectively). This provided us with an opportunity to study the actions of the functional BKB1R, free of cytokine stimulation, in conjunction with the normally constitutively expressed BKB2R.

The ligand potency of desArg^{10}-kallidin and desArg^{9}-bradykinin has been shown to be different for BKB1R from different species [Pesquero et al., 1996; Hess et al., 1996]. For example, the order of ligand potency for human and rabbit BKB1R is desArg^{10}-kallidin$\gg$desArg^{9}-bradykinin$>$BK and for mouse BKB1R is desArg^{9}-bradykinin$>$ desArg^{10}-kallidin$\gg$BK [Pesquero et al., 1996]. Using the pCMinB1 cell, we confirmed the order of ligand potency for human BKB1R at the binding and functional level. We also demonstrated with this cell that the signaling actions of BKB1R are very similar to those of BKB2R with respect to activation of such events as PI turnover, ARA release, Ca^{2+} mobilization and the induction of the immediate early gene, c-fos.

Importantly, the functional BKB1R is short lived, particularly when compared to the BKB2R. Following treatment with CHX to abolish protein synthesis, 50% of the binding of desArg^{10}-kallidin was lost within 4 h. Comparatively, the BKB2R showed no binding loss within the same 4 h segment. This illustrates that downregulation of BKB1R is indeed also determined at the protein level.

Receptor desensitization is an essential step in regulating receptor action [Bohm et al., 1997]. We showed previously that the activated BKB2R is rapidly desensitized [Prado et al., 1998]. Here we illustrate that desArg^{10}-kallidin activated PI turnover proceeds unimpeded for at least 60 min. However, BKB1R mediated ARA release and Ca^{2+} flux are short lived (Fig. 6), with a very similar pattern as that seen with BKB2R. This phenomenon which we term selective desensitization was also observed with the G-protein coupled gonadotropin-releasing hormone receptor, GnRHR [Anderson et al., 1995; McArdle et al., 1996]. Anderson and coworkers showed that IP production induced by this receptor does not undergo rapid desensitization following stimulation with gonadotrophin releasing hormone, while GnRHR mediated Ca^{2+} flux desensitized rapidly [Anderson et al., 1995]. Interestingly, unlike ligand activated BKB2R, which internalizes rapidly, the activated BKB1R is internalized minimally with no significant receptor uptake observed within 60 min of exposure to desArg^{10}-kallidin (Fig. 5). Similar results on BKB1R internalization were reported by Austin et al. [1997]. They proposed that the lack of desArg^{10}-kallidin stimulated PI turnover desensitization was caused by lack of receptor internalization. However, Prado et al. [1998], using various BKB2R point mutants, illustrated that internalization and desensitization are separate processes. Thus, in the case of the BKB1R, two desensitization processes appear to be taking place. A rapid desensitization, seen with Ca^{2+} flux and ARA release, and a slow desensitization seen with PI turnover. This rapid process has been linked to receptor phosphorylation [Prado et al., 1998; Blaukat et al., 1996; Smith et al., 1998]. The slow process could be receptor uptake linked. This differential desensitization would enable BKB1R, and perhaps other inducible receptors, to manifest both transient and longer lasting signals which link to both transient and longer lasting biological functions by BKB1R [Dray and Perkins, 1993; Regoli et al., 1993].

Our results further indicate that the internalization of BKB1R and its resensitization are linked. When resensitization was measured using Ca^{2+} flux, BKB1R, which was only minimally internalized even after 60 min, showed no resensitization. On the other hand, BKB2R, which internalizes rapidly, exhibited pronounced resensitization. We described previously a single point mutant BKB2R where the T^{137} within the second intracellular loop was converted to P^{137} [Prado et al., 1998]. This mutant receptor functioned normally with respect to activating ARA release, PI turnover, and Ca^{2+} mobilization. However, it lacked the capacity to internalize. Interestingly, unlike Wild-type BKB2R, after desensitization, this mutant receptor was not resensitized. This is consistent with our present findings showing

that BKB1R is not internalized within 60 min of activation and is also not resensitized within that time scale. Interestingly, inhibitors which block the internalization of BKB2R and the β2-adrenergic receptor also hinder receptor dephosphorylation [Blaukat et al., 1996; Pippig et al., 1995]. This action may be required for the ultimate resensitization of these receptors. It remains to be resolved which motif(s) determine internalization, which determine desensitization, and what correlation exists between these two actions.

In summary, we developed a model system to study the longevity and action of BKB1R. The various signaling events mediated by BKB1R proved to be regulated differentially through selective desensitization. By this means the BKB1R may be producing both transient and longer lasting signaling events. The apparent lack of internalization and resensitization limits the activity of the receptor at the protein level, providing further, stringent, functional control of this inducible receptor.

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