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Molecular Cloning and Functional Characterization of a Mouse Bradykinin B1 Receptor Gene[1]

João B. Pesquero,*,† Jorge L. Pesquero,‡ Suzana M. Oliveira,† Adelbert A. Roscher,§ Rainer Metzger,¶ Detlev Ganten,† and Michael Bader†,[2]

**Department of Biophysics, Escola Paulista de Medicina, Rua Botucatu 862, 04032-062 São Paulo, Brazil; ‡Department of Physiology and Biophysics, Universidade Federal de Minas Gerais-ICB, Av. Antonio Carlos 6627, 31270-010 Belo Horizonte, Brazil; §Department of Clinical Chemistry and Biochemistry, Children's Hospital, University of Munich, Lindwurmstr. 4, D-80337 Munich, Germany; ¶Cell Control Biomedical Laboratories, Am Klopferspitz 19, D-82152 Martinsried, Germany; and †Max-Delbrück-Center for Molecular Medicine (MDC), Wiltbergstr. 50, D-13125 Berlin-Buch, Germany*

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The gene encoding a putative mouse bradykinin B1 receptor was cloned from a genomic library by low stringency screening. Analysis of two isolated clones revealed a region which contains an open reading frame uninterrupted by introns and encodes a 334 amino acid protein, which exhibits seven potential transmembrane domains and is 68% identical to the human and rabbit bradykinin B1 receptors. Lipopolysaccharide-treatment induces B1 receptor transcripts in the heart, liver and lung. Stable expression of the coding region in COS-7 cells resulted in high levels of binding sites for the specific B1 ligand des-Arg^{10} kallidin (K_d = 1.3 nM; B_{max} = 51 fmol/mg protein). The rank order of affinity of the receptor for the agonists and antagonists was: des-Arg^{9}BKdes-Arg^{9}Leu^{8}BKdes-Arg^{10}kallidin≫Hoe-140 = bradykinin. Functional coupling of the cloned receptor was demonstrated by the dose-dependent effect of des-Arg^{9}BK on the extracellular acidification rate in stably transfected COS-7 cells. This effect was not produced by bradykinin and could be blocked by the B1 antagonist des-Arg^{9}Leu^{8}BK.

The processing of kininogens by the serine proteases kallikreins present in the blood and tissues give rise to a set of small peptides known collectively as kinins. These short lasting molecules initiate many biological processes related to the cardiovascular system as well as to inflammation (1–3). The pharmacological activity of kinins is mediated through the activation of at least two subtypes of receptors, namely B1 and B2. Whereas B2 receptors are widely distributed throughout mammalian tissues, the B1 subtype is very weakly expressed under normal conditions but induced in the course of inflammation and trauma (3). Among the various kinin peptides generated BK and KD are known to bind with high affinity to the B2 receptor, whereas the degradation products des-Arg^{9}BK and des-Arg^{10}KD are better ligands for the B1 receptor subtype (1). The interconversion of B2 into B1 agonists takes place on the cell membrane and in the plasma and is accomplished by arginine-carboxypeptidases with kininase I activity (4,5).

The B1 receptor was first described as a separate entity in *in vitro* experiments with isolated rabbit aortic strips submitted to prolonged time of incubation (1). This postulated *de novo* synthesis of the B1 receptor has also been reported in other systems *in vivo* following treatment with bacterial LPS, muramyl dipeptide or cytokines (6–8) and this up-regulation may be mainly directed by cytokines (9).

Recently the cDNAs for the BK-B1 receptor were cloned and characterized from the human embryonic cell line IMR90 (10) and from rabbit aortic smooth muscle cells (11). The described B1

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[2] Author for correspondence. Fax: (49) 30 94172510. E-mail: bader@hypertonie.fgh.mdc-berlin.de.

Abbreviations: BK, bradykinin; KD, kallidin; LPS, lipopolysaccharide; RT, reverse transcription; PCR, polymerase chain reaction; MERGEPTA, D,L-2-mercaptomethyl-3-guanidinoethylthio-propanoic acid.

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receptors, like the B2 (12–15), belong to the family of the seven transmembrane domain containing G-protein coupled receptors but exhibit little homology to the B2 receptors.

The pharmacological properties of human and rabbit B1 receptors expressed in COS-7 cells are similar concerning the ligand des-Arg^{10}KD. However, they behave differently with regard to des-Arg^{9}BK (11). Nevertheless, both receptors present a much higher affinity for des-Arg^{10}KD as compared with des-Arg^{9}BK, which is in accordance to potencies observed in other cell cultures (16).

In this study, using the human BK-B1 receptor cDNA to screen a mouse genomic library, we isolated and characterized a gene which presents high homology (78%) to the recently published human and rabbit BK-B1 receptor cDNAs. The functional expression and pharmacological studies of this cloned receptor clearly demonstrate that it is the murine homologue of the BK-B1 receptors.

MATERIALS AND METHODS

Library screening. A 129SVJ mouse genomic library containing *Sau3AI* partial digests in λ FIXII (Stratagene) was screened with a B1 cDNA probe labeled by the random priming method (Prime-it II, Stratagene) with [α-^{32}P]dCTP (DuPont NEN). The probe was generated by PCR using human genomic DNA as template and primers designed based on the human B1 cDNA sequence described by Menke et al (10) (sense: CTACGGCCTGTGACAATGCTCC; anti-sense: GTTCAATGCTGTTTTAATTCCG). Hybridization was carried out overnight at 55°C in a solution containing 6 × SSC (1 × SSC = 150 mM NaCl, 15 mM Na-citrate, pH 7.5), 10 × Denhard's solution, 1% SDS and 250 μg/ml denatured salmon sperm DNA. The filters were washed four times for 5 min in 2 × SSC/0.5% SDS at room temperature and twice at 65°C in 1 × SSC/0.5% SDS for 15 min. Filters were dried and exposed to X-ray film with an intensifying screen at −80°C. Positive plaques were isolated and rescreened until pure preparations were obtained.

Restriction and DNA sequence analysis. DNA samples were digested with various restriction enzymes under single and double digestion conditions and were run through a 1% agarose gel. Ethidium bromide-stained gels were photographed under UV-light and the DNA was blotted onto nylon filters as described by Southern (17). Hybridizations were carried out with the labeled human BK-B1 cDNA fragment as described above. Appropriate restriction fragments were isolated from 1% agarose gels and subcloned into the pBluescriptIISK+ vector (Stratagene). DNA sequences were determined in both orientations by the dideoxynucleotide chain termination method (18) using a modified form of T7 DNA polymerase (Sequenase, USB). Part of the sequencing was carried out using an automatic sequencing machine (Vistra DNA sequencer 725, Molecular Dynamics) and Thermo Sequenase labelled primer cycle sequencing kit (Amersham Life Science).

Expression cell line. The putative mouse BK-B1 receptor coding region (−7 to +1251 relative to the ATG start codon) was subcloned in the expression vector Rc/RSV (Invitrogen) and transfected into COS-7 cells using Lipofectamin (Life Technologies). Forty-eight hours later, selection for transfectants was initiated by addition of 1 mg/ml G418 (Life Technologies) and resistant colonies were isolated after two weeks of treatment.

BK-B1 mRNA expression. Mice were anesthetized with thiobutabarbital sodium salt (100 mg/kg) and 50 μl of a 0.02 μg/μl LPS solution (SIGMA) was injected intravenously (7). After a 5 h treatment the animals were sacrificed and the organs removed. Control animals were injected with saline. Total RNA was isolated from different tissues with lithium cloride/urea according to the method of Auffray and Rougeon (19). The presence of intact RNA was verified on an ethidium bromide-stained agarose gel. 1 μg of the purified RNA was incubated with RNase-free DNase (RQ1-DNase, Promega) for 30 min at 37°C followed by incubation at 70°C for 10 min and then reverse transcribed in the presence of 1 μM random hexanucleotides, 1.25 mM of each dNTP, 40 units of RNasin (Promega) and 200 units of MMLV reverse transcriptase (Life Technologies) in 20 μl of PCR buffer containing 50 mM KCl, 20 mM Tris-HCl pH 8.4, 2.5 mM $MgCl_2$ and BSA 1 mg/ml. A PCR was performed using the RT reaction in a 50 μl reaction volume of PCR buffer containing 50 ng of sense (CGGAAGCCTGGGATCTGCTGTG, pos. +86 − +107 relative to the ATG start codon) and antisense (CTCAGGGAGGCCAGGATGTG, pos. +781 − +801) primers, 0.5 mM of each dNTP and 2 units of Taq polymerase (Life Technologies) for 30 cycles (95°C / 45 seconds, 58°C / 45 min, 72°C / 1 min) followed by a final 7 min extension at 72°C. The products were run through a 1% agarose gel and blotted. These Southern blots were hybridized with a 559 bp mouse BK-B1 probe (+190 to +749) radioactively labeled. Hybridization was carried out overnight at 65°C and membranes were washed as described above. Values of radioactivity from the specific BK-B1 band were determined in a Phosphoimager (Fuji) and normalized to the intensity of bands obtained in a PCR performed with the same RT reaction using β-actin specific primers.

Binding assay. All experiments were performed at 4°C with cell monolayers supported on a porous stainless steel platform covered with a film of ice water in an ice bath. Cell medium was removed and cells were washed twice with 1 ml ice-cold medium, as described elsewhere (20). Binding was initiated by incubating the cells for 90 min with 0.5 ml fresh medium containing the indicated concentrations of [^{3}H]des-Arg^{10}KD. Cells were then rinsed twice with buffer, incubated with 2 ml of 0.1% trypsin (10 min, 37°C), and quantitatively transfered to vials for radioassay after addition of 15 ml of aquasol (DuPont NEN). Protein content was determined according to Lowry et al. (21). For displacement studies, cells were incubated with 3.3 nM [^{3}H]des-Arg^{10}KD for 2h at 4°C and different BK-receptor agonists and antagonists were tested for

their potency to compete with the labelled ligand for specific binding. Thereafter, specific binding was measured as described above.

Extracellular acidification rate measurements. Mock and mouse BK-B1 receptor transfected COS-7 were removed from the culture flask with trypsin and plated into sterile capsule-cups (seated in 12-well plates) at a density of 3×10^5 cells/cup. The plates were placed in an incubator at 37°C/5% CO_2 and the cells were allowed to grow for more than 24 h before experimentation. Spacers (100 μm thick) were added to the center of cell capsules followed by capsule inserts. The assembled cup was then transfered to sensor chambers containing 1 ml of low buffered (1mM phosphate) DMEM without bicarbonate and serum but containing 2mM glutamine and additional 44 mM NaCl to replace bicarbonate and adjust osmolarity. The sensor chambers were placed on the Cytosensor (Molecular Devices) microphysiometer (22,23) and allowed to equilibrate for more than 30 min before the beginning of the experiment. The medium was run through the chambers at a rate of 100 μl/min at 37°C. For the measurement of the extracellular acidification rate, cells were stimulated over a period of 40 seconds with the agonists with or without preincubation for 9 min with the antagonists.

RESULTS AND DISCUSSION

Isolation and cloning of mouse BK-B1 receptor gene. A human BK-B1 receptor probe of approximately 1 kb was generated by PCR using human genomic DNA as template and used to screen a mouse genomic library under low stringency conditions. Six positive clones, spanning about 30 kb of genomic sequence were isolated and analyzed by restriction and Southern blot analysis. Two of the selected clones (λB5 and λB6) were shown to overlapp at their 5′ and 3′ ends, respectively. These 3 kb overlapping sequences contained the region which hybridized with the labelled BK-B1 receptor probe. These fragments were isolated by digestion with XbaI (clone λB5) or BamHI/SalI (clone λB6) and subcloned in pBluescriptIISK+ (Stratagene). The fragments were sequenced and shown to encompass a region of 1002 bp with an open reading frame (Fig. 1) which presents 68% amino acid identity to the recently isolated rabbit BK-B1 receptor (11), 67% to the human BK-B1 receptor (10), and 30% to the mouse BK-B2 receptor coding region (13). The cloned sequence predicts a protein with a length of 334 amino acids and molecular weight of 38 kD (Fig.1). A prominent difference between mouse and human or rabbit BK-B1 receptor sequence was found in the N-terminal part, where an insertion of 8 amino acids is present in the mouse receptor (Fig.1). Furthermore, one base pair insertion after 330 amino acids leads to a translational frame shift compared to the other published B1 receptors which results in a divergent C-terminus and a considerably shorter protein (Fig. 1). The coding sequence for the mouse BK-B1 receptor is not interrupted by introns. Sequencing of the genomic human BK-B1 receptor DNA isolated by PCR revealed 100% homology to the published cDNA sequence (10) showing that the human coding sequence is also intronless (data not shown). Thus, the gene structure of the BK-B1 receptor resembles a high number of other G-protein coupled receptor, e.g. the BK-B2 subtype (13,24,25), which all lack intervening sequences in their coding regions. The high degree of identity of DNA and amino acid sequence between our isolated receptor and the human and rabbit sequences suggests that the clone described here is the murine homologue of the BK-B1 receptor.

Expression of the BK-B1 mRNA in mouse tissues. Even by RT-PCR, we could detect only low levels of BK-B1 mRNA in tissues from untreated mice (Table 1). Therefore, mice were treated with LPS and after 5 h of treatment the animals were sacrificed and RNA was extracted from tissues and submitted to RT-PCR. Southern blot analyses using a specific mouse BK-B1 receptor probe showed that the amount of BK-B1 receptor transcripts was markedly increased by the LPS treatment in the heart, liver, and lung (Table 1). Thus, the BK-B1 receptor up-regulation produced by LPS in different systems *in vivo* and *in vitro* (1,6) seems to be caused by mRNA induction.

Binding characteristics of the mouse BK-B1 receptor. Functional studies were performed by cloning the total putative coding region of the mouse BK-B1 receptor into the expression vector pRc/RSV under the control of the Rous Sarcoma virus long terminal repeat promoter. This construct was stably transfected into COS-7 cells and the pharmacological properties of the expressed protein were determined. The presence of the BK-B1 receptor transcript in the total RNA extracted from these transfected cells was confirmed by RT-PCR (data not shown). Scatchard analysis of

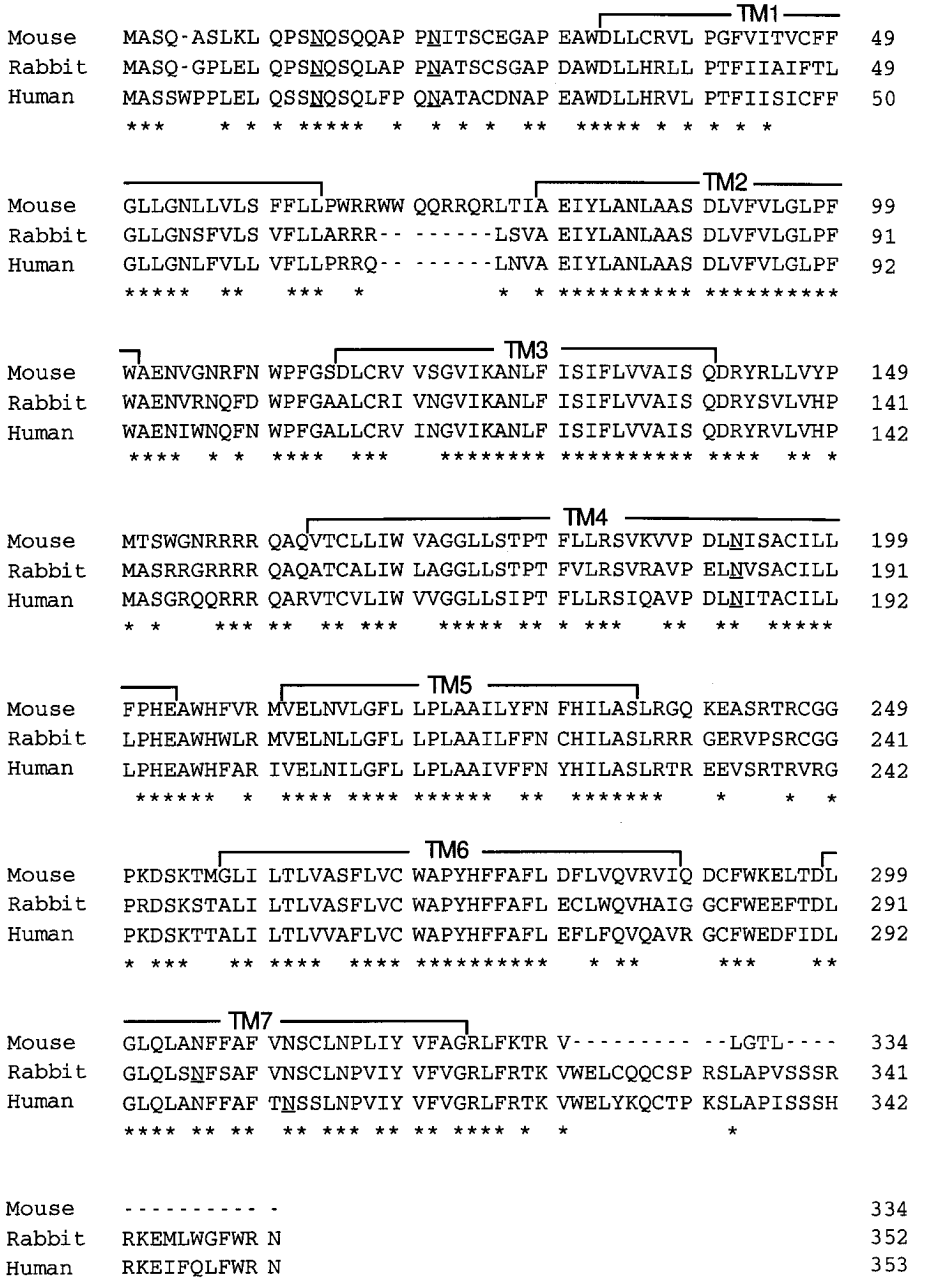

FIG. 1. Comparison of the amino acid sequence of the mouse, rabbit and human BK-B1 receptors. Asterisks (*) indicate identical residues in all three species. The marked regions correspond to the predicted seven transmembrane domains. Putative N-glycosylation sites are underlined.

saturation binding data with [^{3}H]des-Arg^{10} KD indicated a K_d value of 1.3 nM and a B_{max} value of 51 fmol/mg protein (Fig. 2A). Mock-transfected COS-7 cells did not exhibit any specific binding for the labelled peptide (data not shown). The ability of several BK receptor agonists and antagonists to displace [^{3}H]des-Arg^{10}KD from the cloned receptor was assessed. In these experiments, the following rank order of the apparent inhibition constants K_i (26) was obtained: des-Arg^{9}BK

TABLE 1
Tissue Distribution of Mouse BK-B1 Receptor mRNA

Tissue	Relative level of mRNA expression	
	Untreated	LPS-treated
Heart	+	+++++
Lung	+	+++
Kidney	+	+
Liver	++	+++

des-Arg^{9}Leu^{8}BK des-Arg^{10}KD ⩾ Hoe-140 = BK. The data obtained for the B1 agonist des-Arg^{9}BK and the B1 antagonist des-Arg^{9}Leu^{8}BK were very similar with K_i values of 0.32 and 0.38 nM, respectively. Des-Arg^{10}KD was slightly less effective ($K_i = 1.43$ nM). Similar relative potencies of des-Arg^{9}BK and des-Arg^{10}KD for the mouse BK-B1 receptor have been reported (2), whereas the human (10) and the rabbit (11) receptors display much higher affinities for des-Arg^{10}KD than for des-Arg^{9}BK. The distinct sequence differences we found between the mouse

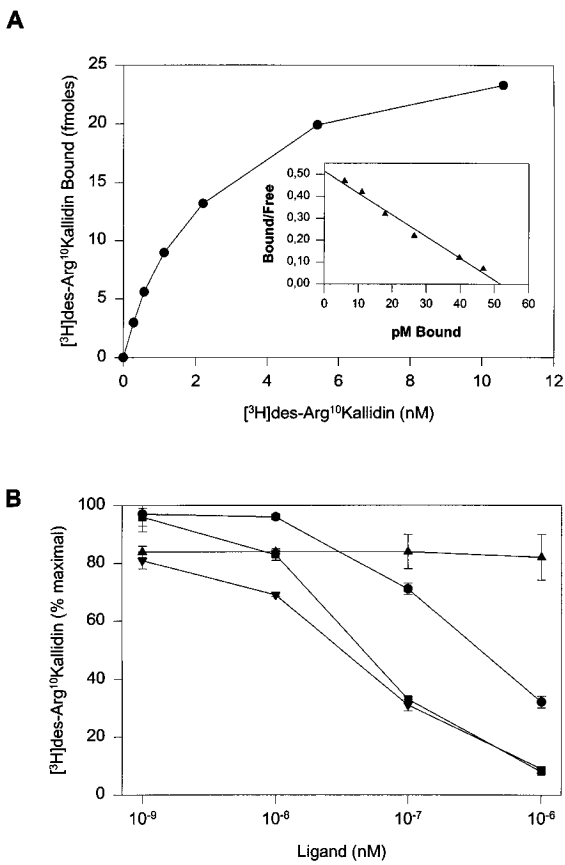

FIG. 2. (A) Saturation curve for specific binding of increasing concentrations of [^{3}H]des-Arg^{10}KD in intact COS-7 transfected with the putative BK-B1 receptor coding region. Nonspecific binding was less than 5% even at high concentrations. Inset shows the Scatchard analysis of the same data. Each point represents the mean of triplicate determinations. (B) Effect of MERGEPTA (10 μM) on the displacement by BK and des-Arg^{9}BK of [^{3}H]des-Arg^{10}KD (3.3 nM) bound to BK-B1 receptor transfected COS-7 cells. (●), BK; (▲), BK + MERGEPTA; (▼), des-Arg^{9}BK; (■), des-Arg^{9}BK + MERGEPTA.

protein and the human and rabbit receptors in the first intracellular loop and the C-terminus may cause this different pharmacological properties. Native BK (B2 agonist) was also able to displace the binding of [^{3}H] des-Arg^{10}KD to the mouse BK-B1 receptor. However, this effect was completely abolished by the kininase I inhibitor MERGEPTA, which prevents the conversion of BK to des-Arg^{9}BK (Fig. 2B). The B2 antagonist Hoe140 displaced des-Arg^{10}KD only ineffectively at very high concentrations above 1 μM.

Functionality of the mouse BK-B1 receptor. The functional coupling of the transfected BK-B1 receptor in COS-7 cells could be demonstrated by using the Cytosensor-microphysiometer system. This technique is able to measure small changes in cellular metabolism in response to ligand-receptor interactions (22,23). The addition of the BK-B1 agonist des-Arg^{9}BK to the cells produced a dose-dependent increase in the extracellular pH of transfected COS-7 cells, whereas the mock-transfected cells did not respond to the treatment (Fig. 3A). The effect could be blunted by the previous incubation of the cells with the specific B1 antagonist des-Arg^{9}Leu^{8}BK (Fig. 3B). BK was also able to stimulate the transfected COS-7 cells. This effect was, however, blocked by MERGEPTA, demonstrating that binding (see above) and stimulation of the B1 receptor by BK is dependent on its conversion to des-Arg^{9}BK by kininase I obviously present in the COS-7 cell membrane (data not shown).

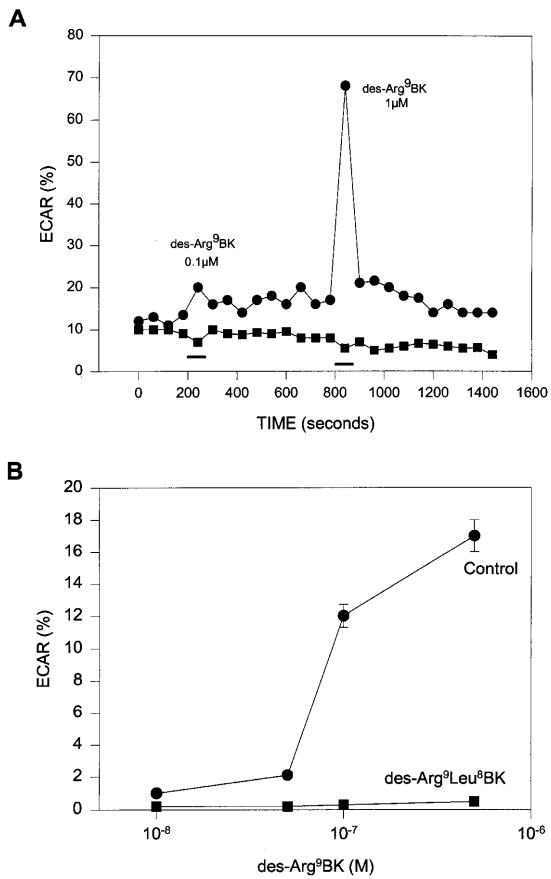

FIG. 3. Effect of des-Arg^{9}BK on the extracellular acidification rate (ECAR) measured by the Cytosensor system. (A) The time ranges of des-Arg^{9}BK additions to control (squares) and BK-B1 transfected (circles) COS-7 cells are depicted by bars. (B) Des–response curves for BK-B1 transfected COS-7 cells in the absence (circles) or in the presence (squares) of the BK-B1 receptor antagonist des-Arg^{9}Leu^{8}BK (1 μM).

In conclusion, these data are consistent with the hypothesis that the protein encoded by the cloned mouse genomic sequence is a BK-B1 receptor.

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