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B_2 BRADYKININ RECEPTORS IN NG108-15 CELLS: cDNA CLONING AND FUNCTIONAL EXPRESSION

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Summary Two distinct cDNAs encoding bradykinin receptors (BKRs) were cloned from NG108-15 neuroblastoma-glioma hybrid cells. One was identical with rat uterus B_2 BKR, whereas the other one (mBKR) had 91 % amino acid homology to the rat B_2 BKR and 82 % homology to human B_2 BKR. Southern blot analysis and genomic DNA cloning revealed that mBKR is derived from the mouse genome. The mBKR, expressed in *Xenopus* oocytes and COS-7 cells, produced functional BKRs that exhibited the properties of smooth muscle type B_2 BKR. These results suggest that both the rat and mouse B_2 BKRs of the smooth muscle type are expressed in NG108-15 cells.

Bradykinin (BK), a nonapeptide, is involved in a variety of physiological and pathological processes, including increased vascular permeability, vasodilatation, smooth muscle contraction, nociception and cell proliferation (1, 2). These diverse responses to BK are mediated by at least two types of receptors, B_1 and B_2 (1, 2). At the cellular level, BK activates phospholipase C and A_2, producing biologically active phospholipid metabolites such as diacylglycerol, inositol-1, 4, 5-trisphosphate (IP_3), arachidonic acids, leucotrienes and prostaglandins (3, 4).

In NG108-15 cells, a mouse neuroblastoma-rat glioma hybrid cell line (5), BK elicits a hyperpolarization followed by a prolonged depolarization (6, 7). This biphasic change in membrane potential is accompanied by a rapid phosphatidylinositol-4, 5-bisphosphate (PIP_2) breakdown via a pertussis toxin-insensitive G protein (8). The initial hyperpolarization is due to the activation of Ca^{2+}-dependent K^+-conductance, presumably resulting from IP_3 and the subsequent release of intracellular Ca^{2+} (9). The depolarizing phase is due to inhibition of voltage-dependent K^+-conductance (6, 7). Furthermore, activation of BKRs facilitates acetylcholine secretion from NG108-15 cells at synapses with myotubes (10, 11). Thus, this cell line has served as an excellent model to study molecular mechanisms of BKR-mediated modulation of neuronal excitability and synaptic transmission.

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Although B_2 BKR cDNAs have been cloned from rat uterus (12), human lung (13) and human uterus (14), BKRs in NG108-15 cells have not been fully evaluated. In this study, we characterized BKRs in NG108-15 cells by cDNA cloning, sequencing and functional expression.

MATERIALS AND METHODS

Cloning and sequencing of cDNAs. A randomly-primed cDNA library was constructed in λgt10 using poly $(A)^+$ RNA prepared from NG108-15 cells (15). To obtain a probe, rat B2 BKR gene was amplified by polymerase chain reaction (PCR). Two primers, 5'-GGCTGCAGGCTGTCGTGGCTGTGTT-3' and 5'-CCGGATCCCTAGGATGCTTCAAGCT-3', were synthesized from the sequences corresponding to the nucleotides 323-342 and 1561-1580 (complementary strand) of the rat B2 BKR cDNA (12). The PCR contained 600 ng rat genomic DNA, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001 % (w/v) gelatin, 0.2 mM each of dATP, dCTP, dGTP, dTTP, 50 U/ml *Taq* I DNA polymerase (Perkin-Elmer-Cetus), 1 μM of each primer and 10 mM Tris-HCl (pH 8.3), in a total volume of 50 μl. After 95 °C for 5 min, 30 cycles of 92 °C for 1 min, 60 °C for 2 min and 72 °C for 3 min were performed. The amplified fragment was digested with *Pst*I and *Bam*HI and ligated to the *Bam*HI/*Pst*I-cleaved pBluescript SK(-) (Stratagene) to yield pBRBR. The *Bgl*II (583)/*Bam*HI (vector) fragment from pBRBR was then used to screen the NG108-15 cell cDNA library; restriction endnuclease sites are identified by numbers in parentheses indicating the 5'-terminal nucleotide generated by cleavage. Hybridization was performed at 37 °C in the presence of 30 % formamide and filters were washed at 50 °C in 0.5 x SSC containing 0.1 % SDS. The inserts of positive clones were subcloned into pBluescript SK(-) and sequenced on both strands (16).

Southern blot hybridization analysis. Genomic DNA (2 μg) extracted (17) from Sprague-Dawley rat liver, A/J mouse liver and NG108-15 cells was digested with various restriction enzymes, electrophoresed on a 0.75 % agarose gel, and blotted onto a nylon membrane (Zetabind, Cuno). To prepare the probe, the *Eco*RV (vector) /*Nco*I (1023) fragment from pBKR15 was ligated into the *Nco*I/*Eco*RV sites of pBSN (18) to yield pBRVN. The probe was synthesized (19) with [α-^{32}P] UTP (3000 Ci/mmol, New England Nuclear), using *Eco*RI-cleaved pBRVN as the template for transcription with T3 RNA polymerase (Stratagene). The blot was hybridized at 65 °C as previously described (20) and washed with 0.2 x SSC and 0.1 % SDS at 65 °C with RNase A treatment (19).

RNA blot hybridization analysis. Poly $(A)^+$ RNA (3 μg) from NG108-15 cells, separated on a 1.1 % agarose-2.2 M formaldehyde gel, was transferred onto a nylon membrane (Zetabind). The subsequent procedures and probe were the same as for Southern blot analysis.

Isolation of genomic clone. A mouse genomic library in EMBL3 (Clontech) was screened with ^{32}P-labeled mBKR cDNA under the same hybridization condition as for cDNA library screening.

cDNA expression in *Xenopus* oocytes and electrophysiological measurements. A recombinant plasmid carrying the mBKR cDNA was constructed as follows. The *Bam*HI (-108) /*Nco*I (417) fragment from pBKR11, the 0.49 kb *Nco*I (1023) /*Sma*I (vector) fragment from pBKR15, and the 3.0 kb *Sma*I /*Bam*HI fragment from pSPAX (15) were ligated to yield pSPMBRΔN. The *Nco*I (417) /*Nco*I (1023) fragment was obtained by PCR using randomly-primed cDNA synthesized from NG108-15 cell poly $(A)^+$ RNA and primers corresponding to nucleotide residues 400-420 and 1182-1199 (complementary strand). The amplified fragment was digested with *Nco*I and ligated to the *Nco*I-cleaved pSPMBRΔN to yield pSPMBR. The insert was confirmed by DNA sequencing. The mBKR-specific mRNA was synthesized *in vitro* using *Xho*I-cleaved pSPMBR as described (15). *Xenopus laevis* oocytes were injected with mBKR-specific mRNA; the concentration was 3 ng/μl and the average volume injected was ~50 nl per oocyte. Whole-cell currents were recorded after 1-3 days with a conventional two-microelectrode voltage clamp amplifier (Axoclamp 2A) as previously described (15). The two pipettes were filled with 3 M KCl. The chamber was perfused with normal frog Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES, pH 7.2). The current records were low-pass filtered at 3 kHz and sampled at 1.6 kHz.

cDNA expression in COS-7 cells and radioligand binding assay. pSPMBR was cleaved with *Sma*I, ligated to the *Hin*dIII linker dCAAGCTTG and cleaved with *Hin*dIII. The resulting 1.6 kb fragment was cloned into the *Hin*dIII site of pKNH (21) to yield pKMBR. The DEAE-dextran

method was used for the transfection of COS-7 cells (22). After 48 hours, cells were harvested by scraping into phosphate-buffered saline and centrifuged for 5 min at 500 x g. The cell pellets were resuspended in 25 mM TES buffer containing 1 mM 1,10-phenanthroline and homogenized with a Polytron for 10 to 15 sec. The homogenized membranes were centrifuged twice at 50,000 x g for 10 min and then were rehomogenized in fresh buffer and resuspended in assay buffer (25 mM TES, pH 6.8, 1 mM 1,10-phenanthroline, 140 μg/ml bacitracin, 5 μM lisinopril and 5 μM cilazaprilat) and 0.1 % bovine serum albumin. Protein concentration was determined by the method of Bradford (23) using bovine serum albumin as the standard. Binding assays were performed using previously described methods (24). For saturation binding studies, membranes (10 μg of protein) were incubated with increasing concentration of [^{3}H] BK (102 Ci/mmol, New England Nuclear) in a total volume of 200 μl for 60 min at 25 °C. Nonspecific binding was determined in the presence of 1 μM BK and represented less than 5 % of the total binding at 25 pM [^{3}H] BK. For competition binding assays, membranes were incubated with [^{3}H] BK (25 pM) and increasing concentrations of various BK compounds for 60 min at 25 °C. Assays were terminated by filtration over glass-microfiber filters (Whatmann GF/B) presoaked in 0.1 % aqueous polyethylenimine. The filters were washed twice with 2 ml of ice-cold 25 mM TES and radioactivity was quantitated by liquid scintillation counting. The data were analyzed with the program LIGAND (25).

RESULTS

A cDNA library prepared from NG108-15 cells was screened with a probe derived from the rat B_2 BKR genome. From ~1.5 x 10^6 plaques, 15 hybridizable clones were isolated. Among these, nucleotide sequences of clones pBKR1 and pBKR2 were identical with that of the rat uterus B_2 BKR (12). Two overlapping clones, pBKR11 and pBKR15, exhibited sequences distinct from the rat B_2 BKR (12). Fig. 1 shows the cDNA sequence determined with pBKR11 and pBKR15, together with the deduced amino acid sequence. The translational initiation site was tentatively assigned to the ATG triplet (nucleotides 1-3) that appears downstream of the nonsense codon found in-frame (nucleotides -120 to -118). The nucleotide sequence surrounding this ATG triplet agrees better with the consensus sequence (26) than the upstream ones (nucleotides -78 to -76 and -30 to -28) in that the nucleotide at position -3 is G. Thus the mBKR protein is composed of 366 amino acid residues (including the initiating methionine) with a calculated molecular weight of 41,556. The amino acid sequence of mBKR has 91 % homology to rat uterus B_2 BKR (12) and 82 % homology to human lung B_2 BKR (13).

Since NG108-15 is a hybrid cell line between mouse N18TG-2 neuroblastoma and rat C6BU-1 glioma cells (5), we examined the species origin of the mBKR by Southern blot analysis. Under high stringency condition, single hybridizable bands observed in the differently digested NG108-15 cell genomic DNA were consistent with those observed in mouse genomic DNA (Fig. 2). In contrast, no hybridizable band was detectable in rat genomic DNA (Fig. 2). We then screened a mouse genomic library using mBKR cDNA. Two positive clones isolated possessed the entire protein-coding sequence identical with that of mBKR cDNA (data not shown), indicating that the mBKR gene is derived from the mouse genome.

Blot hybridization analysis of poly (A)$^+$RNA from NG108-15 cells identified RNA species of ~8100, ~6200 and ~4000 nucleotides hybridizable with the RNA probe prepared from mBKR cDNA as shown in Fig. 3.

To verify the functional properties, we injected *Xenopus* oocytes with mBKR-specific mRNA and examined the current responses to ligand application. The application of BK induced

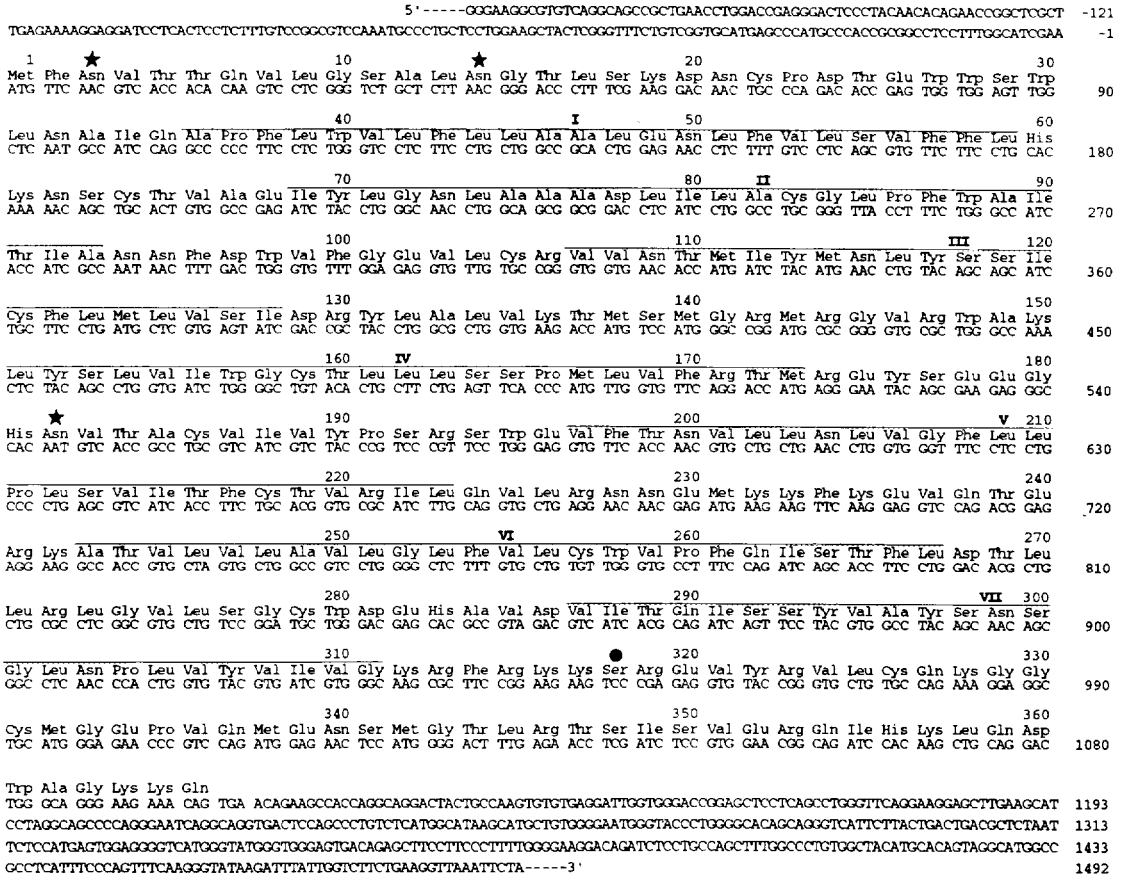

Fig. 1. Nucleotide sequence of cloned cDNA encoding NG108-15 cell BKR (mBKR) and the deduced amino acid sequence. The nucleotide sequence was determined using clones pBKR11 (carrying nucleotides -182 to 557) and pBKR15 (547-1492). Positions of the putative transmembrane segments I -VII are overlined. Three potential *N*-glycosylation sites (asterisks) and one potential phosphorylation site by protein kinase A (closed circle) are indicated.

an oscillatory inward current at a holding potential of -60 mV, indicating the activation of Ca^{2+}-dependent Cl^- channels (27) (Fig. 4 A-D). The application of [des-Arg^9] BK, a B_1 receptor agonist, did not induce response in any of the 30 oocytes tested, which did respond to the subsequent application of BK (Fig. 4A). The BK-induced current was inhibited by B_2 receptor antagonists, [Thi^{5,8}, D-Phe^7] BK (Fig. 4B), HOE140 (Fig. 4C) and D-Arg^0 [Hyp^3, Thi^{5,8}, D-Phe^7] BK (Fig. 4D).

The binding specificity of the mBKR was characterized by transfecting the expression plasmid (pKMBR) or vector DNA (pKNH) into COS-7 cells. The membranes from COS-7 cells transfected with pKMBR showed [^{3}H] BK binding in a saturating manner (Fig.5A), whereas those from COS-7 cells transfected with pKNH showed no specific binding of [^{3}H] BK (data not shown). A Scatchard analysis of [^{3}H] BK binding revealed a single set of sites with a K*d* of 570 pM and a B*max* of 4.1 pmol/mg protein (Fig. 5A). Agonists and antagonists competed for the binding of [^{3}H] BK with an order of potency typical of binding to the smooth muscle type B_2 BKR, with HOE140 being the most potent, followed by BK, Lys-BK, Met-Lys-BK, D-Arg^0

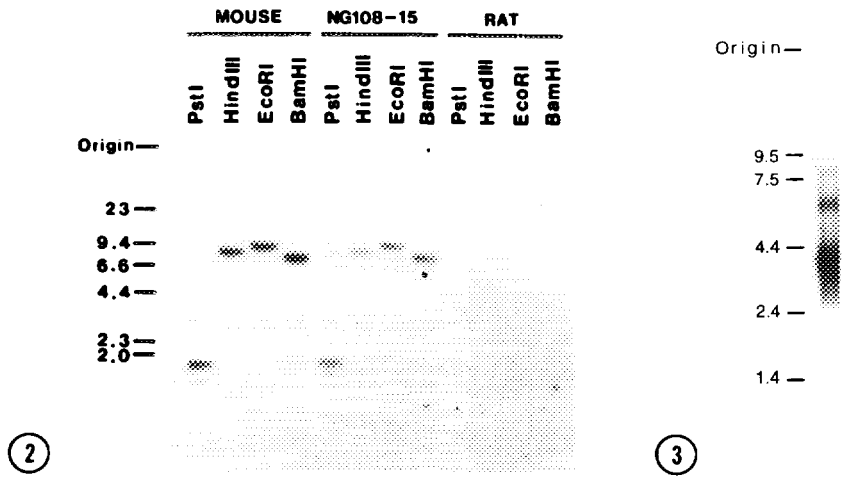

Fig. 2. Southern blot analysis of mouse, NG108-15 cell and rat genomic DNA. Genomic DNA isolated from A/J mouse liver, NG108-15 cells, and Sprague-Dawley rat liver was digested with indicated enzymes. The blot was hybridized with a ^{32}P-labeled RNA probe prepared from mBKR cDNA. Autoradiography was performed for 2 days at -70 °C with an intensifying screen. The size markers used were *Hind*III-digested λ DNA (sizes in kilobases).

Fig. 3. Blot hybridization analysis of poly (A)$^+$ RNA from NG108-15 cells. The probe used was a ^{32}P-labeled RNA probe prepared from mBKR cDNA. Autoradiography was for 20 hours at -70 °C with an intensifying screen. An RNA ladder (Bethesda Research Laboratories) was used as size markers (sizes in kilobases).

[Hyp^3, Thi^{5,8}, D-Phe^7] BK and [Thi^{5,8}, D-Phe^7] BK (Fig. 5B). These data, together with the expression study in *Xenopus* oocytes, indicate that mBKR should be classified as a BKR of the B2 smooth muscle type.

DISCUSSION

In this study on NG108-15 cells, we identified two B2 BKRs, one originating from mouse and one originating from rat. Codominant expression of both mouse and rat genes in NG108-15 cells has also been reported in the case of Histone H1 and H2B (28). On the basis of the expression studies in *Xenopus* oocytes and COS-7 cells, these two BKRs can be classified as being of the B2 smooth muscle type, although NG108-15 cells are derived from neuronal tissues. Phillips *et al.* (29) demonstrated that approximately 4.5 kb mRNA extracted from NG108-15 cells directed the synthesis of functional B2 BKR in *Xenopus* oocytes. The most abundant 4 kb transcript detected by RNA blot hybridization analysis (Fig. 3) reasonably agrees with this report. These results suggest that in NG108-15 cells both the rat and mouse B2 BKRs of the smooth muscle type, at least in part, mediate the BK-induced PIP_2 breakdown (8, 10) and thereby modulate the ionic conductances (6, 7) and acetylcholine release (10, 11).

More recently, McIntyre *et al.* (30) have cloned a mouse BKR gene, which differs from our mBKR by 8 amino acids and exhibits both B1 and B2 properties in *Xenopus* oocytes and COS-7 cells. In contrast, we could not detect any significant effect of [des-Arg^9] BK, a B1 agonist, in both *Xenopus* oocytes and COS-7 cells expressing mBKR. The precise reason for the

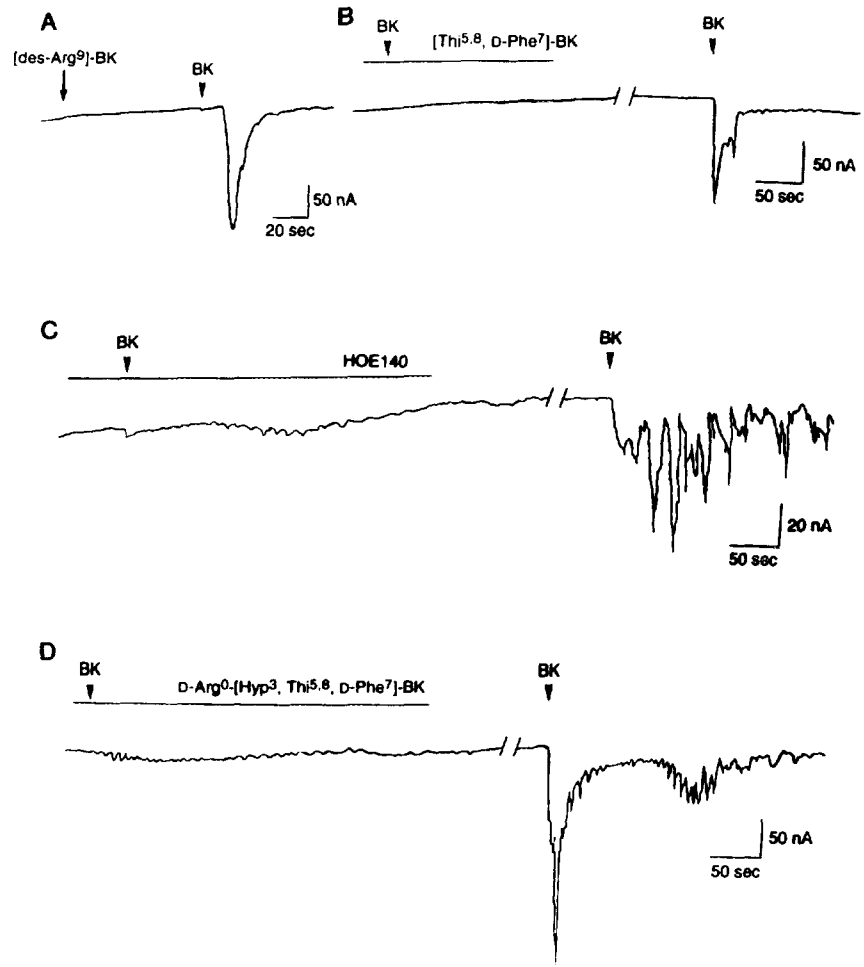

Fig. 4. Functional properties of the mBKR expressed in 4 *Xenopus* oocytes. A. Membrane current evoked by 3 µl, 10 µM [des-Arg^9] BK (arrow) and 3 µl, 1 µM BK (arrowhead). Inward currents elicited by application of 3 µl, 1 µM BK (arrowheads) in the presence of 10 µM [Thi^{5,8}, D-Phe^7] BK (B), 7.5 µM HOE140 (C) or 10 µM D-Arg^0 [Hyp^3, Thi^{5,8}, D-Phe^7] BK (D), and BK-responses in the same oocytes 20-90 min after washing (B-D). Downward reflection indicates inward current.

difference remains to be elucidated. From a mouse genomic DNA library, we isolated two independent clones carrying protein-coding sequences identical with mBKR cDNA. We therefore conclude that the mBKR gene in NG108-15 cells has not undergone any artificial mutation through the process of establishing the cell line.

While this manuscript was in preparation, Hess *et al.* (31) also reported a mouse genomic DNA clone whose protein-coding sequence is identical with that of the mBKR cDNA from NG108-15 cells. This BKR gene, like ours, showed pure B_2 phenotype. The uniformity of these independent results strongly confirms this finding.

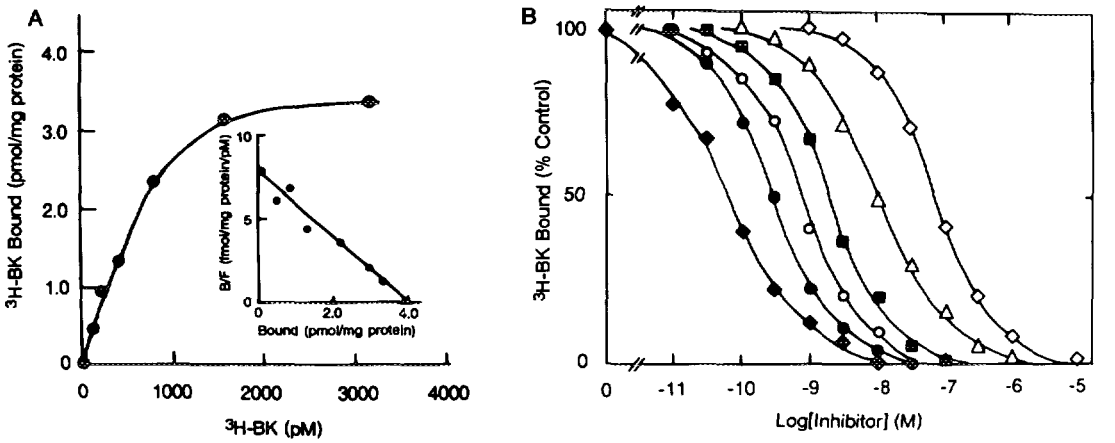

Fig. 5. Pharmacological profile of the mBKR expressed in COS-7 cells. A. The specific saturation binding of [^{3}H] BK to the membrane fractions from COS-7 cells transfected with the mBKR expression plasmid. The inset shows Scatchard plot analysis of the data. B. Competition analysis of various BK compounds for specifically bound [^{3}H] BK to the mBKR. BK compounds used were as follow: HOE140 (closed diamonds); BK (closed circles); Lys-BK (open circles); Met-Lys-BK (closed squares); D-Arg^0 [Hyp^3, Thi^{5,8}, D-Phe^7] BK (open triangles) and [Thi^{5,8}, D-Phe^7] BK (open diamonds). Each point represents the mean of duplicate determinations.

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