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Expression cloning of a rat B_2 bradykinin receptor

(oocyte expression/hormone receptor/pain/G protein-coupled receptor/rat uterus)

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ABSTRACT **A cDNA encoding a functional bradykinin receptor was isolated from a rat uterus library by a clonal selection strategy using *Xenopus laevis* oocytes to assay for expression of bradykinin responses. The predicted protein is homologous to the seven transmembrane G protein-coupled superfamily of receptors. Bradykinin and its analogs stimulate a Cl^- current in oocytes expressing the receptor with the rank order of potency: bradykinin ≈ Lys-bradykinin > [Tyr^8]-bradykinin >> [Phe^6]bradykinin. This is the rank order of potency observed for these compounds in competitive binding assays on soluble receptor from rat uterus. Des-Arg^9-bradykinin (10 μM) elicits no response when applied to oocytes expressing the receptor; thus, the cDNA encodes a B_2 type bradykinin receptor. [Thi^{5,8},DPhe^7]bradykinin, where Thi is β-(2-thienyl)-alanine, is a very weak partial agonist and inhibits the bradykinin-mediated ion flux, suggesting the cDNA encodes a smooth muscle, rather than a neuronal, B_2 receptor subtype. Receptor message has a distribution consistent with previous reports of bradykinin function and/or binding in several tissues and is found in rat uterus, vas deferens, kidney, lung, heart, ileum, testis, and brain. Receptor subtypes are a possibility because several tissues contain two or three message species (4.0, 5.7, and 6.5 kilobases). Southern blot high-stringency analysis demonstrated that the rat, guinea pig, and human genomes contain a single gene. As bradykinin is a key mediator of pain, knowledge of the primary structure of this receptor will allow a molecular understanding of the receptor and aid the design of antagonists for pain relief.**

The nonapeptide bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) is a mediator of pain, inflammation, vascular permeability, smooth muscle tone in vascular and other tissues, and gastrointestinal function (1–5). Bradykinin can serve as a growth factor (2, 6, 7). Bradykinin binds to G protein-coupled receptors that activate phospholipase C or phospholipase A_2 and increases synthesis of inositol trisphosphate or arachadonic acid (8–10).

Bradykinin receptors have been classified as two major subtypes, B_1 and B_2 (1). The bradykinin metabolite des-Arg^9-bradykinin is a B_1 receptor agonist with potency greater than bradykinin, whereas it is inactive at B_2 receptors. B_2 receptors have been subdivided into two subtypes, a "neuronal" form, which is fully activated by [Thi^{5,8},DPhe^7]bradykinin, where Thi is β-(2-thienyl)-alanine, and a "smooth muscle" form, which is weakly activated by [Thi^{5,8},DPhe^7]bradykinin (11, 12). Other subtypes of the B_2 receptor have also been suggested (13, 14).

The design of clinically useful bradykinin antagonists would be facilitated by knowledge of the primary structure of the bradykinin receptor. Because bradykinin has high affinity for both its receptor and its degradative enzyme, angiotensin converting enzyme, a full characterization of the receptor protein has been difficult (ref. 15; S.B. and K.J., unpublished work). Thus, we have used a clonal selection technique to isolate a cDNA clone encoding a smooth muscle, B_2 bradykinin receptor (16–18).¶

MATERIALS AND METHODS

RNA Isolation. Total RNA was isolated from cells grown in culture by a modification of the method of Chomczynski and Sacchi (19) by using the reagent RNAzol (Cinna/Biotecx Laboratories, Friendswood, TX). Total tissue RNA was prepared as in Cathala *et al.* (20). Poly(A)$^+$ RNA was prepared by two passes over a column of oligo(dT) cellulose, type III (Collaborative Research).

Oocyte Preparation, Injection, and Electrophysiological Measurements. mRNA or cRNA was injected into collagenase-treated *Xenopus laevis* oocytes (usually 1 ng/nl for mRNA and library pools and 0.0075 ng/nl per clone 60; 40 nl per oocyte) as described in Zagotta *et al.* (21). Whole-cell bradykinin-induced currents were measured 1–4 days later with a conventional two-microelectrode voltage clamp (Axoclamp 2A with virtual ground headstage; Axon Instruments, Burlingame, CA). Cells were continuously perfused, except when indicated, with 96 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/1 mM $MgCl_2$/5 mM Hepes (pH 7.6) and 2.5 mM sodium pyruvate at room temperature. Membrane voltage was clamped at −60 mV to avoid rectification of the Ca^{2+}-dependent Cl^- current at more hyperpolarized potentials (22). The clamp current recorded through the ten-times attenuated virtual ground was low-pass filtered with an eight-pole Bessel filter (Frequency Devices, Haverhill, MA), digitized at 10 Hz.

cDNA Library Construction. Rat uterus poly(A)$^+$ RNA was sucrose-gradient fractionated (23) and then assayed after injection into oocytes for bradykinin-induced currents. Fraction C, enriched in 28 S RNA, contained the most activity and thus was used for cDNA library construction. The library was constructed in Lambda Zap II (Stratagene) by using an oligo(dT)-*Not* I primer and *Eco*RI adapters.

cRNA Preparation and Library Fractionation. cRNA was prepared from 10 μg of *Not* I-linearized λ DNA by using T7 RNA polymerase in the presence of cap analog (Pharmacia). The library was initially divided into five pools of ≈20,000 individuals each. In subsequent rounds of division the total number of individuals to be screened was calculated by using the Poisson distribution (ref. 23, p. 225; $P = 0.95$).

DNA Sequencing. Fluorescence-based DNA sequences were obtained by using a 373A DNA sequencer (Applied Biosystems).

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Thi, β-(2-thienyl)-alanine.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M59967).

Dose–Response Curves, Binding Assays, and Data Analysis. Bradykinin responses could be elicited from oocytes injected with as little as 30 pg of cRNA, and there was a dose-dependent increase in response with injections as large as 3 ng. To avoid achieving a plateau in the dose–response curves as a consequence of saturation of downstream elements in the second-messenger cascade, 300 pg of cRNA was injected per oocyte in most experiments.

Oocytes injected with cRNA transcribed from the full-length clone 60 were treated with at least four different concentrations of each compound, and the peak current was measured. Data were subjected to simple descriptive statistics, frequency distribution plots, and Shapiro-Wilk statistics with Statistical Analysis Software (24). The data were best modeled by a logarithm-normal distribution. Mean errors and nonlinear regression (25, 26) were computed in the logarithm domain; data shown are reconverted to the linear domain ± SEs.

Binding assays were done on 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)-solubilized (27) rat uterus membranes (28). Binding assays were done in mixtures containing membrane protein at 0.3 mg/ml and buffer composed of 0.5 mM CHAPS, 1 μM leupeptin, 100 μM phenylmethylsulfonyl fluoride, 10 μM captopril, 1 mM 1,10-phenanthroline, 10 mM KH_2PO_4 (pH 6.8), 1 mM EDTA, and 150,000 dpm of [^{3}H]bradykinin (specific activity, 96 Ci/mmol; 1 Ci = 37 GBq) with or without various concentrations of unlabeled bradykinin or analog; the total assay volume was 550 μl (29). Nonspecific binding was ≈20% of the total binding. Data were analyzed by nonlinear regression, and the SDs are given.

Sequence Alignments. The scoring alignment was determined by the algorithm of Needleman and Wunsch, as implemented in the University of Wisconsin Genetics Computer Group suite of programs (30). The computer-generated alignment was further adjusted by eye to maximize alignment of residues that are believed to be functionally equivalent. The consensus is calculated as the majority vote of the receptor sequences in Fig. 2 and several other peptide receptors not shown: two tachykinin receptors [rat neuromedin K (31) and rat substance P receptors (17)] and a protein of unknown function (doggpcr1), which is 23.8% identical to the Rat-Bkr (32).

RESULTS AND DISCUSSION

Several receptors that activate phospholipase C have been cloned by expression in *X. laevis* oocytes (16–18). We sought an adequate source of mRNA from which to construct a cDNA library. Oocytes were injected with poly(A)$^+$ mRNA (40 ng) isolated from a number of tissues and cell lines and then assayed for bradykinin-induced currents by using a two-electrode voltage clamp. The observed currents are consistent with activation of endogenous Ca^{2+}-dependent Cl^- channels. mRNA from rat uterus reproducibly gave bradykinin-stimulated ion fluxes (median 34 nA; range 4–366 nA; 45/51 oocytes responding, Fig. 1*A*); other tissues did not give as reproducible signals. The response was mediated by bradykinin B_2 receptors because [Thi^{5,8},DPhe^7]bradykinin blocked the response and des-Arg^9-bradykinin had no effect.

Based on the tissue distribution studies, a cDNA library was prepared in Lambda Zap II from sucrose-gradient size-fractionated poly(A)$^+$ mRNA. The library of ≈100,000 individuals was screened by using six rounds of clonal selection to obtain a single clone, RUC15495-60 (Fig. 1 *B* and *C*, Fig. 3*A Inset*). The 4111-base insert was sequenced, and an open reading frame of 1266 nucleotides with methionine codons at nucleotides 78 and 168 was deduced. The second methionine codon conforms best to a consensus translation-start sequence (33) and is presumed to be the first amino acid of the primary translation product. The entire open reading frame is contained within a 1.9-kb *Eco*RI fragment of the cDNA. Bradykinin responses in oocytes injected with cRNA from

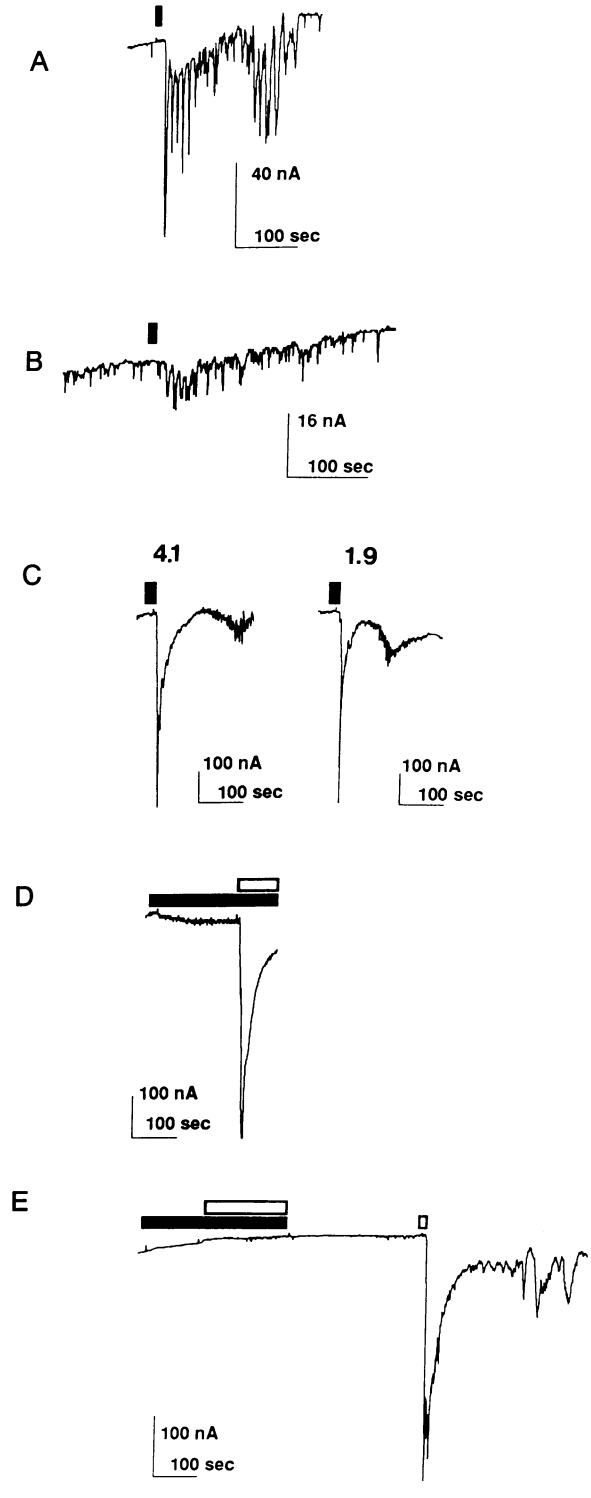

FIG. 1. Bradykinin-induced responses from oocytes injected with mRNAs. (*A*) Poly(A)$^+$ RNA from rat uterus. (*B*) cRNA from the first library division. (*C*) cRNA made from either the full 4.1-kb clone (clone 60) or the 1.9-kb subclone. (*D–E*) cRNA made from clone 60. (*A–C*) Oocytes were continuously perfused, and 1 μM bradykinin was applied during the time indicated by the solid bar. (*D*) Response of an oocyte to treatment with 10 μM des-Arg^9-bradykinin (solid bar) and 10 nM bradykinin (open bar). (*E*) Response of an oocyte to treatment with 1 μM [Thi^{5,8},DPhe^7]bradykinin (solid bar) and 10 nM bradykinin (open bar); finally the cell was washed, and 10 nM bradykinin was applied (small open bar).

the 1.9-kilobase (kb) fragment were identical to those seen in oocytes injected with the full-length clone (Fig. 1*C*).

The predicted protein sequence of 366 amino acids has a molecular mass of 41,696 Da and is homologous to members of the seven transmembrane G protein-coupled family of receptors (Fig. 2). Hydrophobicity analysis reveals seven putative transmembrane domains consistent with a seven transmembrane structure (40). The largest overall degree of homology, 25% identity, is to the canine histamine-2 receptor (39). Reasonably strong homology, ≈23%, is observed to the neurotensin receptor and tachykinin receptors (substance P, substance K, and neuromedin K) and to the carboxyl-half of the luteinizing hormone/human chorionic gonadotropin receptor (17, 18, 31, 37). Homology to the muscarinic receptors and adrenergic receptors and the visual rhodopsins is evident but less pronounced, 23–19% (Fig. 2). The sequence contains three potential N-linked glycosylation sites in predicted extracellular domains, two in the putative N-terminal extracellular domain, and one adjacent to a conserved cysteine (residue 186) in the second extracellular loop. This cysteine is a feature of most seven transmembrane receptors identified to date. In the β_2-adrenergic receptor and bovine rhodopsin (41, 42) the conserved cysteine in extracellular loop 2 is thought to be disulfide-bonded to a conserved cysteine in extracellular loop 1 (residue 105 in the bradykinin receptor). Cys-326 closely following transmembrane region 7 may anchor the receptor carboxyl tail to the plasma membrane through its palmitoylation because a homologous cysteine occurs in many members of this family and has been shown to be palmitoylated in both adrenergic receptors and rhodopsins (43, 44).

Several workers have demonstrated that bradykinin receptor function is regulated by bradykinin, other hormones, second messengers, and their analogs (8, 45, 46). In oocytes expressing clone 60, a second bradykinin bolus applied 3–5 min after a preceding identical dose yielded a smaller response; the second response to 10 nM bradykinin was 18 ± 6% (n = 10) of the first response, and the second response to 1 μM bradykinin was 7 ± 3% (n = 15) of the first response. This desensitization may involve several of the potential sites of receptor modification encoded by the cDNA, particularly those found in the third cytoplasmic loop and carboxyl tail.

Dose–response curves of bradykinin and its analogs were obtained by measurement of bradykinin- and analog-induced ion fluxes in oocytes injected with cRNA made from the 4.1-kb clone after verifying that downstream elements of the

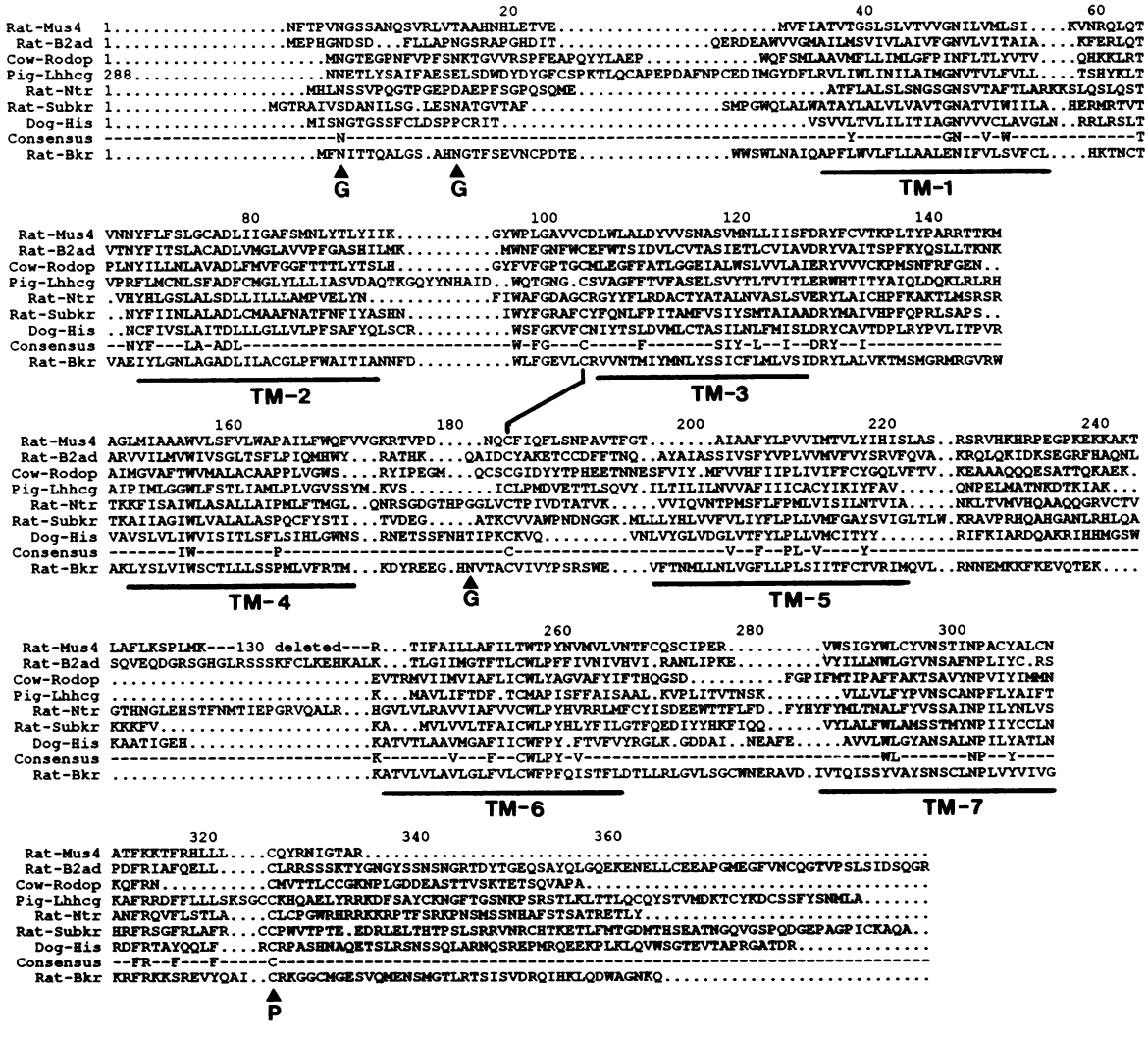

FIG. 2. Predicted amino acid sequence of the rat bradykinin receptor aligned with other G protein-coupled receptors. Sequences are arranged in order of increased identity to the rat bradykinin receptor. Rat-Mus4, rat muscarinic-4 receptor (34); Rat-B2ad, rat β-2 adrenergic receptor (35); Cow-Rodop, cow rhodopsin (36); Pig-Lhhcg, pig luteinizing hormone/human chorionic gonadotropin receptor (37); Rat-Ntr, rat neurotensin receptor (18); Rat-Subkr, rat substance K receptor (38); Dog-His, dog histamine receptor (39); Rat-Bkr, rat bradykinin receptor. G, putative asparagine-linked glycosylation sites in extracellular domains; P, putative palmitoylation site. Line connecting cysteines 105 and 186 represents a putative disulfide bond. Solid bars represent approximate positions of transmembrane (TM) regions. The numbering is correct for the bradykinin receptor.

second-messenger cascade were not saturated. The high affinity of expressed receptor for bradykinin ($EC_{50} \approx 3$ nM) is similar to that seen for contraction of rat uterus, ($EC_{50} \approx 12$ nM) (47) (Fig. 3*A* and Table 1). The rank order of potency is bradykinin ≈ Lys-bradykinin > [Tyr[8]]bradykinin >> [Phe[6]]bradykinin and is very similar to that determined from competitive binding of these analogs to bradykinin receptor in CHAPS-solubilized rat uterus membranes (Table 1). Des-Arg[9]-bradykinin (10 μM) had no effect on oocytes expressing the receptor (Fig. 1*D*); thus, the cDNA encodes a B_2 rather than a B_1 bradykinin receptor.

Bradykinin stimulation of oocytes expressing the receptor was blocked by the antagonist [Thi[5,8],DPhe[7]]bradykinin with an IC_{50} of 400 ± 240 nM (Fig. 3*B* and Fig. 1*E*), which is similar to its pA_2 of 6.3 (500 nM) measured for the inhibition of bradykinin-induced uterus contraction (47) and to its IC_{50} of 170 ± 60 nM measured in competitive binding assays to CHAPS-solubilized receptor from rat uterus membranes. [Thi[5,8],DPhe[7]]bradykinin was a very weak partial agonist: 10 μM or 1 mM produced 2–5% of the response of 10 nM bradykinin (data not shown). The weak agonism of this compound indicates that clone 60 probably does not encode the subtype observed in the nerves of the rat vas deferens (11) or on neuroblastoma N1E-115 cells (12), where [Thi[5,8],DPhe[7]]-bradykinin is a full agonist. Thus, clone 60 appears to encode the smooth muscle subtype where this compound acts as a very weak partial agonist.

Table 1. Correlation of potencies of bradykinin and several analogs in stimulating current in injected oocytes with binding to the bradykinin receptor

	Induced currents in oocytes, EC_{50} (nM)	Rat uterus receptor binding, IC_{50} (nM)
Bradykinin	2.85 ± 0.2	1.1 ± 0.6
Lys-bradykinin	1.9 ± 0.8	12 ± 3
[Tyr[8]]bradykinin	17.4 ± 2	167 ± 10
[Phe[6]]bradykinin	≈10,000	3600 ± 1200

The agonists did not significantly differ in the maximum response achieved (100–400 nA). Data were pooled from at least three separate experiments to include 3–62 oocytes per concentration. A full dose–response curve was not collected for [Phe[6]]bradykinin. Specific responses were not seen in clone 60-injected oocytes when the following substances were applied (at 1 μM, unless indicated): substance P (n = 12); xenopsin (n = 3); Arg-vasopressin (n = 6); ranatensin (n = 3); bombesin (n = 7); carbachol (100 μM; n = 5); and serotonin (n = 5). Substances that interact with angiotensin converting enzyme, the inhibitor SQ20,881 (10 μM; n = 13), and the substrate angiotensin I (n = 6) were ineffective.

The tissue distribution of mRNA encoding the bradykinin receptor was determined by Northern (RNA) analysis (Fig. 4*A*). These data suggest that bradykinin receptor message is found in the uterus, vas deferens, kidney, ileum (data not shown), heart, lung, testis, and brain. This distribution is

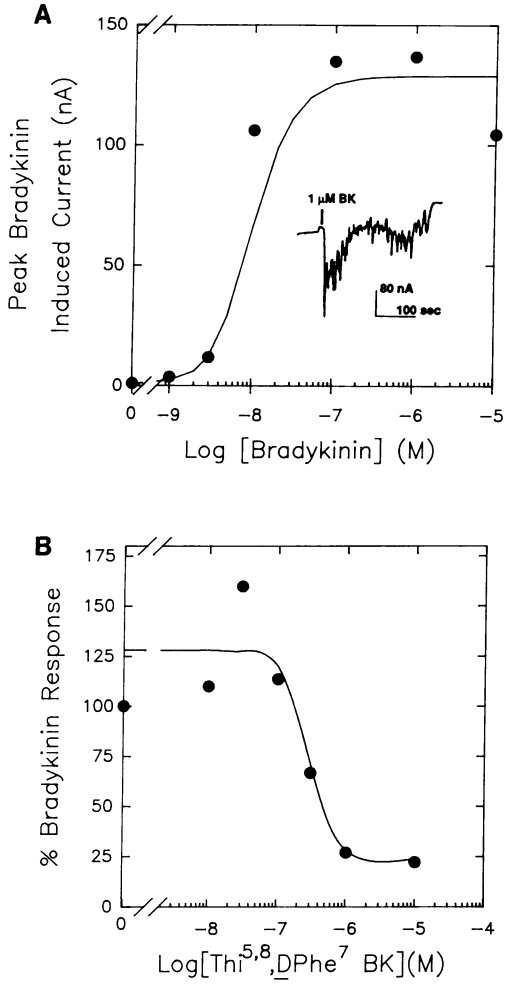

FIG. 3. Pharmacology of bradykinin receptor expressed in oocytes injected with cRNA derived from clone 60. (*A*) Peak responses (nA) were measured to application of bradykinin at various concentrations. The data are the mean responses of 3–62 oocytes per point for eight separate experiments. (*Inset*) Bradykinin (1 μM) was applied at bar. (*B*) [Thi[5,8],DPhe[7]]bradykinin blocks the response to 10 nM bradykinin. Oocytes were treated for 2 min with the indicated concentrations of [Thi[5,8],DPhe[7]]bradykinin and then challenged with 10 nM bradykinin mixed with the indicated concentration of [Thi[5,8],DPhe[7]]bradykinin. Data are the means of 5–45 oocytes per concentration pooled from five separate experiments.

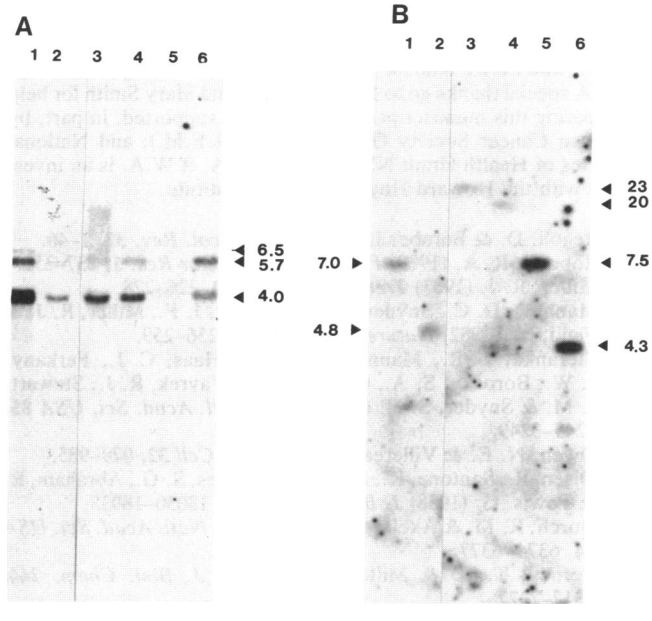

FIG. 4. Tissue distribution of mRNA encoding the bradykinin receptor (*A*) and the genomic organization of a bradykinin receptor gene (*B*). (*A*) Northern blot of poly(A)$^+$ mRNA from various rat tissues. The prominent bands are 4.0 kb, 5.7 kb, and 6.5 kb. Lanes: 1, uterus; 2, heart; 3, lung; 4, kidney; 5, testis; 6, brain. Ten micrograms of RNA was loaded in every lane except lane 1 where 2 μg was loaded. The blots (*A* and *B*) were washed at 0.2× standard saline citrate (SSC) at 55°C. The probe used was a ^{32}P random-primed 1.9-kb *Eco*RI fragment of clone 60 that includes the complete open reading frame. (*B*) Southern blot of genomic DNA; human (lanes 1 and 2), guinea pig (lanes 3 and 4), and rat (lanes 5 and 6). The DNA was digested with *Eco*RI (lanes 1, 3, and 5) and *Hin*dIII (lanes 2, 4, and 6).

similar to that observed in guinea pig (28). The rat uterus has several times more receptor message than any other tissue or cell line tested (Fig. 4*A*). mRNAs of 4.0, 5.7, and 6.5 kb were seen (Fig. 4*A*). A 4.0-kb message is the most prominent in the uterus and is present in all tissues. A 6.5-kb band occurs only in uterus and kidney. All tissues except heart and testis have a 5.7-kb message. The existence of multiple mRNA species could be due to splicing precursors, variable length of polyadenylylation, alternate selection of polyadenylylation sites, or different subtypes of the receptor. The 4111-base cDNA is apparently a full-length clone of the 4-kb message.

A single gene highly homologous to the rat cDNA was seen in DNA from rat, human, and guinea pig by Southern blot analysis (Fig. 4*B*). *Drosophila melanogaster* and *X. laevis* DNA showed no genes highly homologous to the rat bradykinin receptor.

The following lines of evidence suggest that a bradykinin receptor of the B_2 smooth muscle subtype has been isolated: the rank order of potency of bradykinin and its analogs is similar to that reported for B_2 receptors, des-Arg^9-bradykinin is not an agonist, and [Thi^{5,8},DPhe^7]bradykinin is a weak partial agonist and blocks bradykinin-mediated responses. In addition, mRNA tissue distribution studies are consistent with the described cDNA encoding a bradykinin receptor. The primary sequence of the described receptor places it in the seven transmembrane G protein-coupled receptor superfamily. The availability of this cDNA encoding a bradykinin receptor will allow better definition of coupling mechanisms, aid the identification of receptor subtypes, and allow investigation of structure–function relationships with the goal of designing specific and potent antagonists for the relief of pain.

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