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Discrimination Between Putative Bradykinin B_2 Receptor Subtypes in Guinea Pig Ileum Smooth Muscle Membranes with a Selective, Iodinatable, Bradykinin Analogue

CLAUS LIEBMANN, ROGER BOSSÉ, and EMANUEL ESCHER

Institute of Biochemistry and Biophysics, Biological Faculty, University of Jena, D-07743 Jena, Germany (C.L.), and Department of Pharmacology, Faculty of Medicine, University of Sherbrooke, Sherbrooke, Quebec, J1H 5N4, Canada (R.B., E.E.)

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SUMMARY

We have synthesized a potent, selective, radioiodinated bradykinin (BK) analogue with high specific radioactivity (1000–1500 Ci/mmol). The new tracer, ^{125}I-[*p*-Phe^5]BK, was prepared carrier-free from the corresponding nitro precursor, [*p*-NO_2-Phe^5]BK, via catalytic hydrogenation and halodediazotation. This peptide bound to guinea pig ileum membranes in a biphasic pattern, with a high affinity dissociation constant of 3 pM (B_{max} = 22 fmol/mg of protein) and a low affinity dissociation constant of 192 pM (B_{max} = 245 fmol/mg of protein). The kinetically determined K_d values were 2 pM and 910 pM, respectively. The properties of the new tracer and of the peptide analogues [*p*-iodo-Phe^5]BK and [*p*-NO_2-Phe^5]BK were compared with those of [3,4-^{3}H(N)][2,3-prolyl]BK as label in both saturation and inhibition studies. The results indicated that [*p*-iodo-Phe^5]BK possessed increased affinity for the high affinity site and decreased affinity for the low affinity site, relative to BK. In rat myometrial membranes, in contrast, [*p*-iodo-Phe^5]BK failed to reveal a high affinity site and displayed reduced affinity for the low affinity site, compared with BK. The nitro precursor was a nonselective ligand with nanomolar affinity for all labeled binding sites in both membrane preparations. Measuring the influence of BK and its analogues on guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate binding to guinea pig ileum membranes, we showed that G proteins were separately activated via both binding sites, qualifying these sites as constituents of signal transduction pathways and, therefore, real membrane receptors. With the new tracer as label, the B_2 receptor antagonists D-Arg^0-[Hyp^3,Thi^5,D-Tic^7,Oic^8]BK and D-Arg^0-[Hyp^3,Thi^{5,8},D-Phe^7]BK recognized both binding sites with very high affinity in guinea pig ileum membranes, classifying these sites as B_2 receptors. The BK-induced contraction in guinea pig ileum is obviously mediated via the receptor with nanomolar affinity, but the physiological role of the high affinity receptor is still unknown.

The nonapeptide BK (RPPGFSPFR) displays numerous physiological as well as pathophysiological activities, including contraction and/or relaxation of smooth muscles, pain sensation, bronchoconstriction, inflammation, and modulation of neurotransmitter release. The membrane receptors that mediate these pleiotropic actions of BK have been pharmacologically classified as B_1, B_2, and B_3 subtypes. B_1 receptors have a higher affinity for des-Arg^9-BK and related peptides, compared with BK, whereas des-Arg^9-BK is inactive at B_2 and B_3 receptors. B_2 receptors with higher affinity for BK appear to be the most prevalent form mediating the diverse actions in various tissues. BK receptors mediating tracheal relaxation may not fall into the typical B_1 or B_2 subtypes and have been termed B_3 receptors (for review, see Refs. 1 and 2). Recently, Regoli *et al.* (3) postulated a further subdivision of B_2 receptors into B_{2A} (occuring in rabbit jugular vein) and B_{2B} (occuring in GPI) receptor subtypes. In addition, those authors defined a B_H site that may mediate BK-induced histamine release in rat mast cells (3).

Meanwhile, rat (4), human (5, 6), and mouse (7, 8) B_2 receptors have been cloned. They are members of the superfamily of seven-transmembrane domain G protein-coupled receptors. The pharmacology of BK receptors has been characterized extensively in binding studies by using [3,4-$^3H_2$][Pro^{2,3}]BK (9–12) as well as [*m*-^{125}I-Tyr^0]BK (13) or [*m*-^{125}I-Tyr^8]BK (14). However, for several tissues there are contradictory results postulating a single BK binding site (14–17) or two binding sites, one with a picomolar affinity constant and one with a

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ABBREVIATIONS: BK, bradykinin; I-BK, [*p*-iodo-Phe^5]bradykinin; NO_2-BK, [*p*-NO_2-Phe^5]bradykinin; NH_2-BK, [*p*-NH_2-Phe^5]bradykinin; B_{HA} receptor, high affinity B_2 receptor; B_{LA} receptor, low affinity B_2 receptor; GPI, guinea pig ileum; GTP[S], guanosine-5′-*O*-(3-thio)triphosphate; Hoe 140, D-Arg^0-[Hyp^3,Thi^5,D-Tic^7,Oic^8]bradykinin; NPC 349, D-Arg^0-[Hyp^3,Thi^{5,8}, D-Phe^7]bradykinin; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; DTE, dithioerythritol; BSA, bovine serum albumin; TFA, trifluoroacetic acid; HPLC, high performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

nanomolar affinity constant (9–12). Comparison among these studies is relatively difficult because of variations in the experimental conditions and because of the lack of selective, high affinity, radiolabeled ligands that might discriminate between the described multiple binding sites.

In the present paper, we report the synthesis of ^{125}I-BK, an analogue with higher specific radioactivity and greater selectivity for the B_{HA} site, compared with [^{3}H]BK. We have characterized the B_{HA} and B_{LA} binding sites (with the affinity constants K_H and K_L, respectively) from GPI smooth muscle membranes with this radioligand, with the corresponding nonradioactive I-BK, and with two different B_2 receptor antagonists. We have further compared the binding properties of ^{125}I-BK with those of [^{3}H]BK in the same tissue. The GPI was chosen because the BK receptors in this tissue have been well characterized as being of the B_2 type linked to phosphoinositide turnover (17) and mediating smooth muscle contraction. In addition, in some preliminary experiments the binding properties of I-BK in rat myometrial membranes were studied. To obtain more information about the functional importance of the detected BK binding sites, we determined both the biological potencies of I-BK and its nitro precursor NO_2-BK to contract the isolated GPI and their potencies to stimulate [^{35}S]GTP[S] binding to GPI membranes. Our results indicate that the new tracer confirms the presence of a B_{HA} site, presumably representing a B_2 receptor subtype in GPI smooth muscle membranes that occurs in addition to the well known B_{LA} site.

Experimental Procedures

Materials. ^{125}I-BK (1000–1500 Ci/mmol, carrier-free) was prepared from the corresponding nitro precursor NO_2-BK as described below. [3,4-$^3H_2$][Pro^{2,3}]BK (102 Ci/mmol) and [^{35}S]GTP[S] (1200–1400 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Unlabeled BK and GTP[S], des-Arg^9-BK, NPC 349, angotensin II, vasopressin, substance P, TES, HEPES, bacitracin, 1,10-phenanthroline, DTE, phenylmethylsulfonyl fluoride, EDTA, EGTA, polyethyleneimine, BSA, diethylstilbestrol, captopril, and GDP were obtained from Sigma Chemical Co. (St. Louis, MO). Hoe 140 was a gift from Prof. B. Schölkens (Hoechst AG, Frankfurt A. M. Germany). All other chemicals were of the purest form commercially available.

Preparation of NO_2-BK, I-BK, and ^{125}I-BK. Both NO_2-BK and I-BK were synthesized by solid-phase synthesis using the *N-tert*-butoxycarbonyl/TFA procedure. Peptides were cleaved from the resin with HF and purified by gel chromatography and medium-pressure preparative reverse phase chromatography (C_{18}). Chemical identities were assessed by fast-atom bombardment mass spectrometry, thin layer chromatography, and analytical HPLC (C_{18}). ^{125}I-BK was produced using a technique already published (18). Briefly, NO_2-BK (5–10 mg) was reduced by catalytic hydrogenation and then diazotized in 0.1 N H_2SO_4 at 0°. Introduction of 1 mCi of ^{125}I (Na^{125}I; specific radioactivity, 1500–2000 Ci/mmol; Amersham) into the diazonium BK derivative was performed using nascent Cu^0 as catalyst, for 12 hr at 4°. The reaction mixture was purified by HPLC and the radioactive peak was collected. The identity of the radiotracer was assessed by coelution in HPLC with the nonradioactive peptide (I-BK) in two solvent systems (0.05% TFA and 0.01% HCl/0.1% NaCl, with acetonitrile gradients).

Tissue preparation. A section of ileum approximately 15 cm long was removed from female Hartley guinea pigs (300–500 g) immediately after the animals were killed. The mucosa was scraped off and the muscle layers were homogenized in 25 mM TES buffer, pH 6.8, containing 1 mM 1,10-phenanthroline, with a Brinkman Polytron PT-10 homogenizer (Brinkman Instruments) at setting 7 for 20–30 sec. The homogenate was filtered through two layers of medical gauze, centrifuged at 100,000 × *g* for 20 min, and resuspended in preparation buffer. In an analogous procedure, myometrial membranes were prepared from uteri from female Wistar rats that had been treated with diethylstilbestrol (100 μg/kg, subcutaneously, 16 hr before the uterus was removed). For binding studies with [^{35}S]GTP[S], membranes were prepared as described above, with the exception that 20 mM Tris·HCl, pH 8.0, containing 1 mM EDTA, 1 mM DTE, and 1 mM phenylmethylsulfonyl fluoride was used as preparation buffer. Membranes were always freshly prepared. Protein content was determined according to the method of Lowry *et al.* (19).

Radioligand binding assays. The experimental conditions were identical for both [^{3}H]BK and ^{125}I-BK. Equilibrium binding assays were performed in polypropylene tubes, in a total volume of 2 ml, at 4° (on ice) for 60 min. Membranes (about 0.2–0.3 mg of protein/tube) were incubated with increasing tracer concentrations for saturation studies or with approximately 20–30 pM tracer for displacement experiments, in medium containing 25 mM TES, pH 6.8, 1 mM 1,10-phenanthroline, 140 μg/ml bacitracin, 10 μM captopril, 1 mM DTE, and 0.1% BSA. The incubation was terminated by filtration under reduced pressure through Whatman GF/C filters that had been pretreated with 0.1% (w/v) aqueous polyethyleneimine. The filters were washed with 3 ml of ice-cold 10 mM TES buffer, pH 6.8, transferred either into scinitillation vials (for [^{3}H]BK) or into polypropylene tubes (for ^{125}I-BK), and counted for radioactivity. Nonspecific binding was determined in the presence of 1 μM nonradioactive BK or I-BK as indicated. For both tracers, specific binding varied between 50 and 70%. Binding studies in physiological buffer were performed in the Krebs' solution used in the bioassay, supplemented with 20 mM HEPES and 1,10-phenathroline, bacitracin, captopril, DTE, and BSA in the concentrations indicated above. Under these conditions, specific binding of ^{125}I-BK of approximately 30% was obtained.

Association and dissociation experiments were performed by incubating membrane aliquots, as described above, for various periods of time with various concentrations of radioligand, as indicated in the figure legends. Dissociation was initiated by the addition of unlabeled BK (1 μM). Binding of [^{35}S]GTP[S] to GPI membranes was determined as described previously (20). The reaction mixture contained 0.2–0.4 nM [^{35}S]GTP[S], 1 μM GDP, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTE, 10 μM captopril, 50 mM Tris·HCl, pH 7.5, and approximately 3 μg of membrane protein, in a total volume of 200 μl. The incubation was started by addition of the membranes and was carried out in quadruplicate for 60 min at 4°. The reaction was terminated by filtration through Whatman GF/C glass fiber filters. The filters were washed three times with 5 ml of 10 mM Tris·HCl, pH 7.5, containing 5 mM $MgCl_2$. The filters were dried and counted for radioactivity in a toluene-based scintillator. Nonspecific binding was determined in the presence of 10 μM unlabeled GTP[S] and amounted to approximately 1% of added [^{35}]GTP[S].

Bioassays. The biological activities of BK, I-BK, and NO_2-BK were measured on GPI segments of 2-cm length. The muscle strips were suspended in a 10-ml organ bath containing oxygenated (95% O_2/5% CO_2) Krebs' solution (at 37°) of the following composition (in mM): NaCl, 117.5; KCl, 4.7; KH_2PO_4, 1.2; $CaCl_2 \cdot 6H_2O$, 2.5; $MgSO_4 \cdot 7H_2O$, 1.2; $NaHCO_3$, 25; and glucose, 5.5; pH 7.4. Atropine (2.2 μM) was added in all tests to eliminate the effects of BK-released acetylcholine at the muscarinic receptors. After the application of the peptides, changes of tension were recorded isometrically with a load of 1 g, using a physiograph (Grass model 7D). Cumulative concentration-response curves were determined to estimate the apparent affinity of each compound, which is expressed in terms of pD_2 (negative logarithm of the molar concentration of agonist needed to obtain 50% of the maximal response).

Data analysis. Both saturation and inhibiton curves were analyzed using the nonlinear regression program LIGAND (21). This program allows estimation of the binding parameters for any number of ligands reacting simultaneously with any number of receptors. The choice between the different models (e.g., one or two classes of binding sites) is based on the comparison of the residual mean square of each fit

using F statistics ($p < 0.01$) incorporated into the program. Data were weighted with the reciprocal of the variance. To verify the parameters obtained, the inhibition data were additionally analyzed with the linear optimization program (affinity spectra) of Tobler and Engel (22), which does not require initial estimates. Both methods yielded similar results.

The association rate was estimated from the plot of ln $(B_e/B_e - B)$ versus t, according to a pseudo-first-order reaction primarily yielding the observed association rate constant k_{obs}. B_e represents specific binding at equilibrium and B represents specific binding at time t. The dissociation rate constant k_{-1} was estimated from the negative slope of the plot of ln (B/B_0) versus t, where B_0 is the concentration of bound radioligand at time 0 (equal to binding at equilibrium, B_e) and B is bound tracer at time t. The real association rate constant k_{+1} could then be calculated according to the equation $k_{+1} = (k_{obs} - k_{-1})/L_T$, where L_T represents the total radioligand concentration.

Results

We have obtained ^{125}I-BK by the introduction of ^{125}I into the *para*-position of Phe^5 (18) of the BK molecule via hydrogenation followed by halodediazotation of NO_2-BK, with a yield of 40–75%, based on ^{125}I incorporation. A control synthesis with *p*-iodophenylalanine in the corresponding position was also performed and confirmed the correct structure of ^{125}I-BK (Fig. 1). The metabolic stability of ^{125}I-BK was assessed by HPLC after exposure of the radiolabel to GPI membranes under assay conditions. Before and after incubation, all of the tracer coeluted with I-BK, indicating its stability during the assay (data not shown).

^{125}I-BK binds to GPI membranes with higher affinity than does [^{3}H]BK. For both tracers, Scatchard plots as well as computer curve fitting of saturation data with the LIGAND program revealed two binding sites (Fig. 2). Using ^{125}I-BK, K_d values of 3 pM (K_H) and 192 pM (K_L) were calculated. Numbers of B_{HA} and B_{LA} sites were 22 and 245 fmol/mg of protein, respectively. The binding parameters calculated for [^{3}H]BK were 24 pM (K_H) and 200 pM (K_L), with corresponding receptor concentrations of 75 and 265 fmol/mg of protein. In both cases the data were better described by a two-site model than by a one-site model (for [^{3}H]BK, mean square root = 27.5, $F = 120$, $p \leq 0.001$; for ^{125}I-BK, mean square root = 18.1, F = 14.6, $p \leq 0.001$). Both association and dissociation kinetics of ^{125}I-BK binding to GPI membranes (Fig. 3) also dislayed biphasic

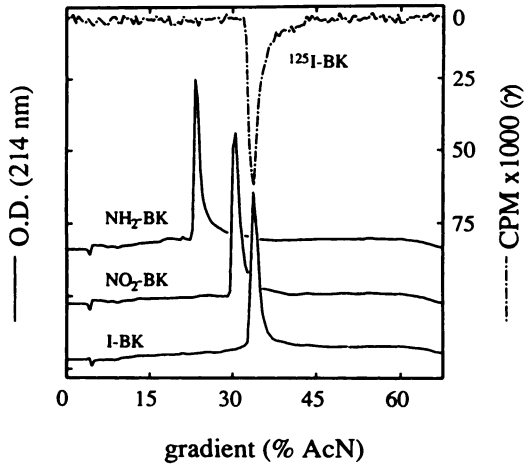

Fig. 1. HPLC elution patterns for NO_2-BK, NH_2-BK, I-BK, and ^{125}I-BK. An aliquot of ^{125}I-BK coeluted with nonradioactive I-BK in a TFA solvent gradient. Perfect coelution of I-BK and ^{125}I-BK is evidence of their chemical identity. *AcN*, acetonitrile.

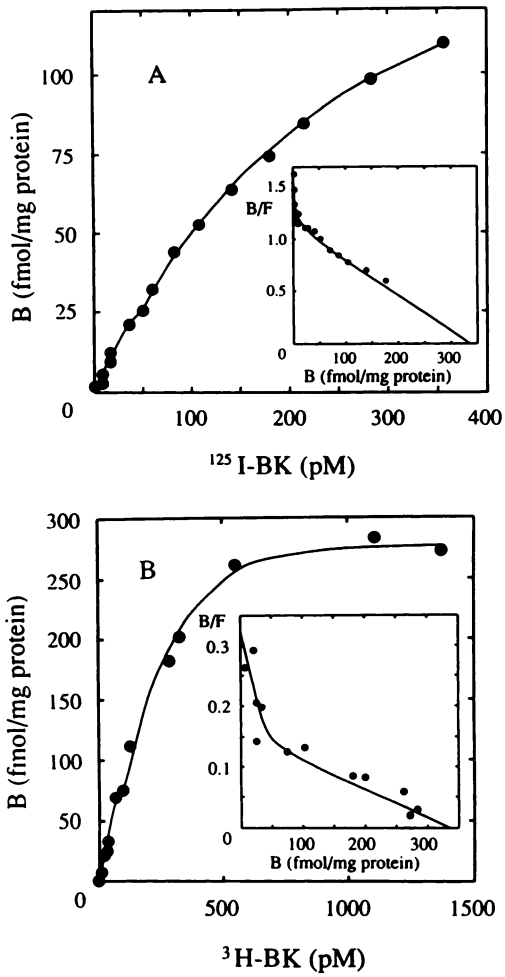

Fig. 2. Saturation binding of ^{125}I-BK (A) and [^{3}H]BK (B) to GPI membranes. Increasing concentrations of ^{125}I-BK (0.5 pM to 0.4 nM) or [^{3}H]BK (1 pM to 1.5 nM) were incubated with GPI membranes (0.2–0.3 mg of protein/assay), as described in Experimental Procedures. The saturation binding curves and the respective Scatchard plots (*insets*) are shown. The graphs are representations of single experiments assayed in duplicate, which were each repeated twice with comparable results. *B*, bound; *F*, free.

character, indicating heterogeneity in binding sites. The observed association rate constants (k_{obs}) were 1.25×10^{-3} sec^{-1} for the fast phase and 5.83×10^{-4} sec^{-1} for the slow phase. Thus, the k_{obs} values for ^{125}I-BK under our assay conditions (4°) were approximately 10 times lower than those for [^{3}H]BK (fast phase, 2.6×10^{-2} sec^{-1}; slow phase, 3×10^{-3} sec^{-1}) determined at 25° (9). The dissociation of specifically bound ^{125}I-BK was again biphasic, with rates evaluated as $k_{-1} = 1.04 \times 10^{-4}$ sec^{-1} and $k_{-1} = 4.66 \times 10^{-4}$ sec^{-1}. A similar biphasic dissociation pattern was observed earlier with [^{3}H]BK (9), but the difference between the dissociation rates was clearly larger (9×10^{-5} sec^{-1} and 1.4×10^{-3} sec^{-1}), compared with the dissociation of ^{125}I-BK at 4°. The calculated second-order rate constants of association (k_{+1}) were 5.05×10^7 M^{-1} sec^{-1} for the B_{HA} site and 5.10×10^5 M^{-1} sec^{-1} for the B_{LA} site. When the K_d values were calculated from the k_{-1}/k_{+1} ratio, we obtained values of 2 pM and 910 pM, which are in reasonable agreement with the parameters determined by saturation analysis.

The competition with [^{3}H]BK (102 Ci/mmol) by unlabeled BK revealed approximately 15% of total binding sites as B_{HA} sites and 85% as B_{LA} sites, with K_i values of about 11 pM and

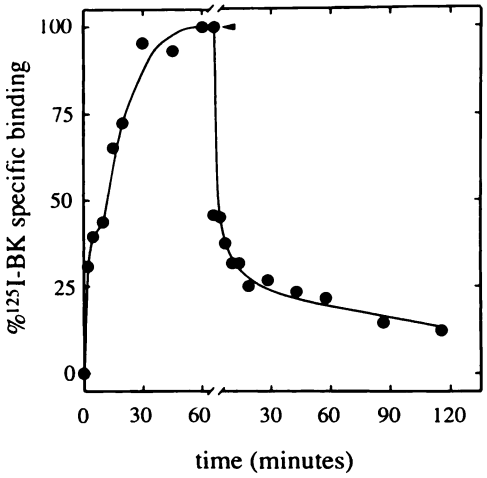

Fig. 3. Association and dissociation of ^{125}I-BK (0.3 nM) binding to GPI membranes (0.45 mg of protein/assay) as a function of time. Incubations were performed as described in Experimental Procedures. Dissociation was initiated by addition of 1 μM unlabeled BK (final concentration) (*arrowhead*). At the indicated times, the respective samples were filtered as described. Specific binding was defined as the difference between binding in the absence and in the presence of 1 μM unlabeled BK, after incubation to equilibrium (60 min). Nonspecific binding was always determined in parallel samples.

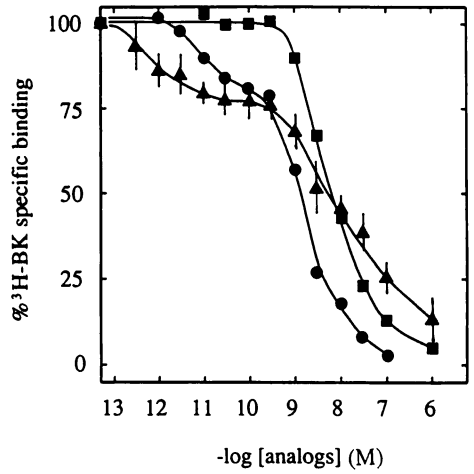

Fig. 4. Competition with specific [^{3}H]BK (30 pM) binding by BK (●), I-BK (▲), and NO_2-BK (■) in GPI membranes. Data points are the means of three independent experiments with duplicate determinations, with <10% interassay variability. Because of clustered data, error bars are shown only for the I-BK curve. The standard error was comparable in all cases.

1.2 nM, respectively (Fig. 4; Table 1). Comparable amounts of B_{HA} and B_{LA} sites were identified with I-BK, but with enhanced affinity for the B_{HA} site and lowered affinity for the B_{LA} site. In contrast, the nitro precursor NO_2-BK showed a monophasic displacement curve for all available binding sites and and bound with low (nanomolar) affinity to an apparent single site. The competition with ^{125}I-BK by increasing concentrations of unlabeled BK analogues (Fig. 5) yielded similar curve shapes but with more distant K_i values. As shown in Table 1 and Figs. 4 and 5, I-BK revealed a significantly increased affinity for the B_{HA} site and a decreased affinity for the B_{LA} site, compared with native BK, just as did ^{125}I-BK, compared with [^{3}H]BK. NO_2-BK demonstrated a lower affinity than BK with both tracers. The B_1 receptor agonist des-Arg^9-BK possessed a very weak affinity, with a K_i value higher than 10 μM. Vasopressin was clearly inactive at concentrations up to 1 μM, whereas we observed a slight cross-reactivity with angiotensin II.

In addition, inhibition studies were performed with ^{125}I-BK in physiological (high ionic strength) buffer, to provide an estimation of binding affinity under the conditions used for the determination of biological activity (17). In these experiments, I-BK was again found to bind to a heterogeneous population of binding sites (Fig. 6) but the specific binding was clearly reduced. Thus, the total number of binding sites was approximately 10% of that measured in TES buffer. In the high ionic strength buffer used, the affinity of the B_{HA} site for I-BK was lowered from approximately 0.4 pM to about 4 pM, whereas the B_{LA} site affinity remained almost unchanged. BK failed to reveal the B_{HA} site in physiological buffer.

For comparison, some inhibition experiments were performed using rat myometrial membranes labeled with ^{125}I-BK (Fig. 7). Under those conditions I-BK displayed binding to a single binding site, with a K_i value of 20 nM, compared with the higher affinities of BK (K_i = 0.4 nM) and NO_2-BK (K_i = 0.7 nM). A similar K_i value for I-BK (16 nM) was obtained when myometrial receptors were labeled with [^{3}H]BK, whereas BK itself recognized two binding sites, with K_i values of 13 pM and 0.7 nM (data not shown).

For further characterization of the ^{125}I-BK binding sites, we performed displacement experiments with the B_2 receptor antagonists Hoe 140 and NPC 349. Hoe 140 has been reported to be about 2 orders of magnitude more potent than NPC 349 in antagonizing BK-induced contraction in the isolated GPI (23). In GPI membranes labeled with ^{125}I-BK, both antagonists displayed clearly biphasic displacement curves (Fig. 8), indicating interaction with two binding sites. The respective binding parameters are summarized in Table 2. With [^{3}H]BK as radioligand, in contrast, only the B_{LA} site was recognized by both antagonists.

As shown in Fig. 9, under our assay conditions BK caused a concentration-dependent and biphasic variation of [^{35}S]GTP[S] binding to GPI membranes. For the first increase, half-maximal and maximal stimulations (up to 135% of control binding) were observed with 0.5–1.0 pM and 10–30 pM BK, respectively. At BK concentrations higher than 30 pM the binding of [^{35}S]GTP[S] was continously reduced, followed by a second increase at BK concentrations of approximately 0.3 nM for half-maximal [^{35}S]GTP[S] binding and 10–20 nM for maximal [^{35}S]GTP[S] binding (up to 140% of control binding). When I-BK was used to stimulate [^{35}S]GTP[S] binding to GPI membranes, we obtained a first increase at lower peptide concentrations (0.1 pM for half-maximal stimulation), followed by an extended range of I-BK concentrations where the peptide had no effect or a decreased stimulatory effect. To obtain the second stimulation, higher I-BK concentrations were needed, compared with BK, which is reflected by the half-maximal stimulation at 30 nM. Finally, NO_2-BK, which bound with a single affinity constant, also activated G proteins, as measured by [^{35}S]GTP[S] binding, but only at higher concentrations and in a monophasic manner, with about 1 nM required for half-maximal stimulation. The effect of I-BK on [^{35}S]GTP[S] binding was completely prevented by the BK B_2 receptor antagonist Hoe 140.

The biological potencies of BK, I-BK, and NO_2-BK to contract the isolated GPI were very similar (Fig. 10), and all peptides were full agonists. The order of potency was as follows:

TABLE 1

Potencies of BK, I-BK, and NO_2-BK for inhibition of [^{3}H]BK and ^{125}I-BK binding to GPI membranes

The affinity constants for the B_{HA} site (with the affinity constant K_H) and B_{LA} site (with the affinity constant K_L) were determined as described in Experimental Procedures and calculated with the LIGAND program (21). The K_i values presented are the means ± standard errors from three separate experiments, with duplicate determinations. Each set of three curves was sequentially evaluated by fitting models of increasing complexity. With the exception of NO_2-BK, the two-site fit was superior (F test) to the one-site model. The number of experimental data points wsa not sufficient for a higher resolution of the curves (three- or four-site models corresponding to the interconvertible affinity states of two independent G protein-coupled receptors). RA is the relative affinity of the analogues, compared with BK (1.0). The values in parentheses are the binding site concentrations as percentage of the total binding site number labeled by the respective radioligand. The selectivity is expressed as the logarithm of the quotient K_L/K_H for the amount of [^{3}H]BK displaced by BK versus the amount of ^{125}I-BK displaced by I-BK.

Compound	[^{3}H]BK				^{125}I-BK				Selectivity, log K_L/K_H
	B_{HA} site		B_{LA} site		B_{HA} site		B_{LA} site		
	K_H	RA	K_L	RA	K_H	RA	K_L	RA	
	pM		nM		pM		nM		
BK	11 ± 1.5 (15%)	1.0	1.2 ± 0.4 (85%)	1.0	5 ± 2 (20%)	1.0	0.5 ± 0.2 (80%)	1.0	2.04
I-BK	3 ± 2 (18%)	3.7	5.6 ± 0.4 (82%)	0.2	0.7 ± 0.1 (28%)	7.4	2.0 ± 0.9 (72%)	0.16	3.63
NO_2-BK			3.0 ± 0.2 (100%)	0.4			0.4 ± 0.1 (100%)	1.25	

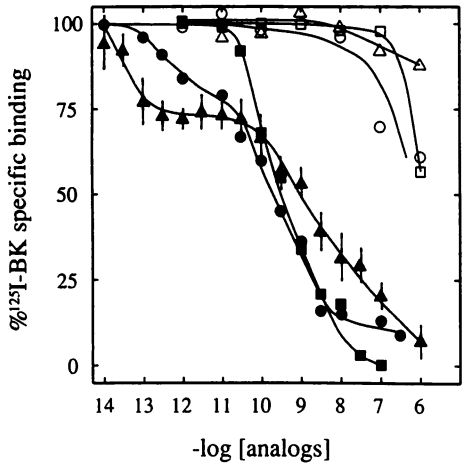

Fig. 5. Inhibition of specific ^{125}I-BK (20 pM) binding to GPI membranes by BK (●), I-BK (▲), NO_2-BK (■), des-Arg^9-BK (□), and, for comparison, angiotensin II (○) and vasopressin (△). Each value is the average of two (□, ○, △) or three (●, ▲, ■) experiments assayed in duplicate, which differed by <10%. Error bars are shown for I-BK and were comparable for the other curves.

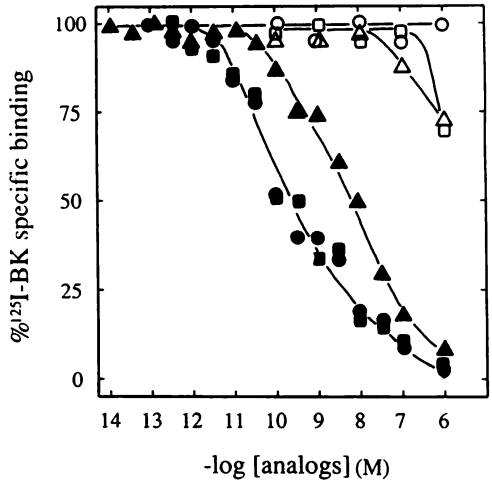

Fig. 7. Inhibition of specific ^{125}I-BK (30 pM) binding to rat myometrial membranes by BK (●), I-BK (▲), NO_2-BK (■), des-Arg^9-BK (□), angiotensin I (○), and substance P (△). Each data point is the mean of two experiments with duplicate determinations. Interassay determinations differed by <5%.

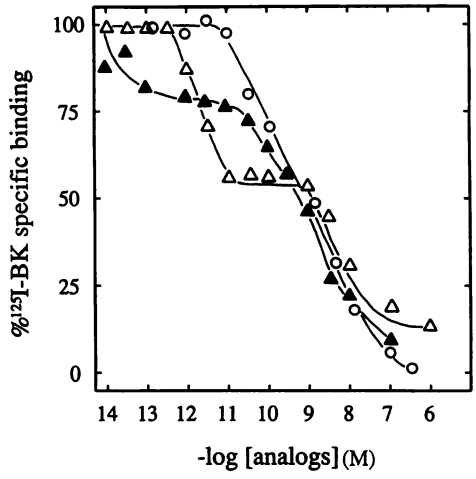

Fig. 6. Inhibition of specific ^{125}I-BK (20 pM) binding to GPI membranes by I-BK (▲, △) or BK (○). The assays were performed either in TES standard assay buffer (▲) or in physiological, high ionic strength buffer (△, ○). Each data point is the mean of duplicate determinations that varied by <10%. The experiment was repeated twice, giving similar results.

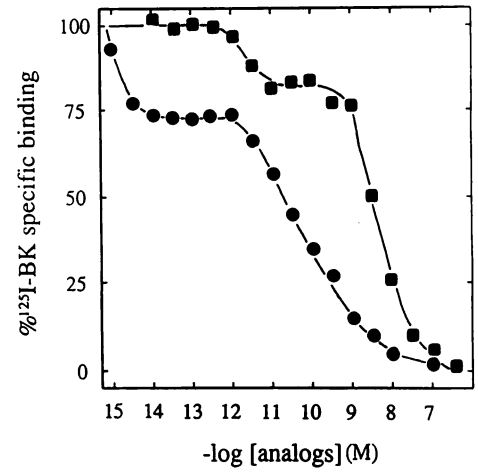

Fig. 8. Inhibition of ^{125}I-BK (20 pM) binding to GPI membranes by the B_2 receptor antagonists Hoe 140 (●) and NPC 349 (■). Shown are the means of two separate experiments assayed in duplicate. The interassay variations were <5%.

TABLE 2

Binding affinities and biological potencies of the B_2 receptor antagonists Hoe 140 and NPC 349 in the GPI

The inhibition constants (K_i values) were experimentally determined as described and calculated with the LIGAND program (21). A two-site model best described the data ($p < 0.001$). Points are the means from two separate experiments with duplicate determinations. The antagonistic potencies were taken from the report of Hock *et al.* (23) and are expressed in terms of IC_{50}, the antagonist concentration that inhibits by 50% the contraction induced by 40 nM BK (23).

Compound	Binding affinity		Antagonistic potency
	[^{3}H]BK	^{125}I-BK	
Hoe 140	180 pM	8 fM, 100 pM	11 nM
NPC 349	20 nM	8 pM, 26 nM	30 μM

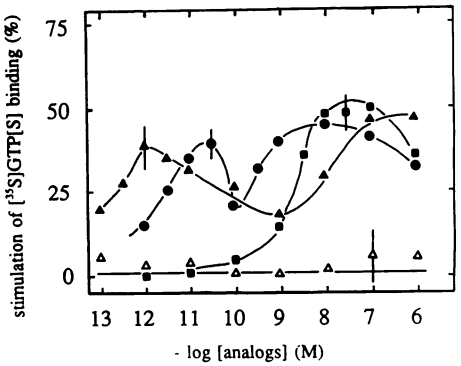

Fig. 9. Stimulation of [^{35}S]GTP[S] binding to GPI membranes by BK (●), I-BK (▲), NO_2-BK (■), and I-BK plus 1 nM Hoe 140 (△). *Ordinate*, stimulation of [^{35}S]GTP[S] binding over control [[^{35}S]GTP[S] binding without peptide additions (100% binding) was set at 0]. Values are the means of three separate experiments assayed in quadruplicate. For the sake of clarity, error bars are shown only for the maximal effects. The standard errors were comparable in all cases.

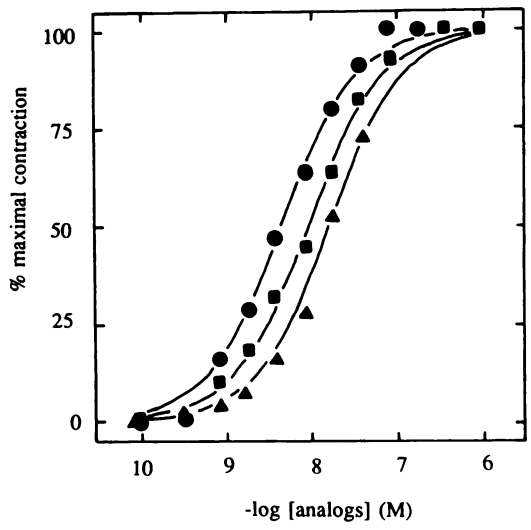

Fig. 10. Concentration-response curves for BK (●), I-BK (▲), and NO_2-BK (■) in the isolated GPI. *Abscissa*, concentration of the peptides; *ordinate*, contractile effects expressed as percentage of the maximal contraction. Data points are the means of four to six independent determinations.

BK ($pD_2 = 8.43$) > NO_2-BK ($pD_2 = 8.09$) > I-BK ($pD_2 = 7.80$). The apparent affinities (pD_2) were all within a range of 1 log unit, suggesting that the introduction of iodine into the 5-position did not dramatically change the contractile activity of BK, and correlated with low affinity binding only.

Discussion

To obtain a radiolabeled BK analogue with specific radioactivity higher than that of [^{3}H]BK, we have prepared an iodinated analogue by the direct introduction of ^{125}I into the *para*-position of Phe^5, without the replacement of Phe^5 or Phe^8 by tyrosine. The new radioligand, ^{125}I-BK, was prepared carrier-free in high yield and has a specific radioactivity varying between 1000 and 1500 Ci/mmol, depending on the specific radioactivity of the Na^{125}I utilized. Both the corresponding unlabeled compound I-BK and the nitro precursor NO_2-BK act as full agonists and contract the isolated GPI with potencies similar to, but slightly lower than (pD_2 values of 7.80 and 8.09, respectively), that of BK ($pD_2 = 8.43$).

Binding of [^{3}H]BK to GPI plasma membranes has been measured by several groups. Thus, Manning *et al.* (9) reported two binding sites, with K_d values of 13 pM and 910 pM and a density of the B_{LA} site almost twice that of the B_{HA} site. In contrast, Farmer *et al.* (16) and Ransom *et al.* (17) described a single class of binding sites, with K_d values of 65 pM and 16 pM, respectively. A completely contradictory result was reported by Tousignant *et al.* (14), who assayed BK binding with [^{125}I-Tyr^8]BK. They identified a single binding site, with a K_d value of 1600 pM. Our results with both [^{3}H]BK and ^{125}I-BK indicate the existence of two BK binding sites with well separated affinities in GPI membranes and, thus, confirm the conclusion of Manning *et al.* (9). Using [^{3}H]BK of high specific activity (102 Ci/mmol), we measured K_d values of 24 pM (K_H) for the B_{HA} site and 200 pM (K_L) for the B_{LA} site. In saturation experiments, the new label ^{125}I-BK yielded affinity constants of 3 pM (K_H) and 192 pM (K_L). It should be noted that the goodness of fit for a two-site model is further improved in the case of ^{125}I-BK, compared with [^{3}H]BK. A very good correlation was found between the K_H values obtained from saturation (3 pM) and kinetic (2 pM) studies, whereas the K_i value for the B_{HA} site estimated in competition experiments was clearly lower (0.7 pM). For the B_{LA} site, we obtained similar values from kinetic (0.91 nM) and competition (2.0 nM) studies but a lower K_d value from saturation experiments (0.19 nM).

The identification of two binding sites from the inhibition of [^{3}H]BK binding with unlabeled BK is very difficult (9) and quite speculative. A successful separation of B_{HA} and B_{LA} sites depends on good selectivity of the ligand (separation of the K_d values) and high specific radioactivity of the tracer. In our approach, we have compared the competition with [^{3}H]BK binding, as well as ^{125}I-BK binding, by the respective nonradioactive ligands. In both assay systems, BK showed flat displacement curves yielding two K_i values, which were comparable to the affinity constants for the B_{HA} and B_{LA} sites obtained from the respective saturation experiments. When unlabeled I-BK was assayed for competition with [^{3}H]BK binding the inhibition curve was clearly biphasic, showing higher affinity for the B_{HA} site, compared with BK. With ^{125}I-BK as label, the corresponding unlabeled compounds displayed the highest affinity and selectivity towards the B_{HA} site, allowing the best discrimination between the two BK binding sites in GPI membranes. Very surprisingly, the nitro precursor NO_2-BK failed to discriminate the B_{HA} site but bound with equal affinity to all available sites. It may be assumed, therefore, that the introduction of the relative large, hydrophobic, iodine atom into the *para*-position of phenylalanine in the 5-position of BK favors ligand-receptor interactions that produce higher affinity

for the B_{HA} site and lower affinity for the B_{LA} site, compared with native BK. In contrast, more hydrophilic substitutions in the *para*-position of Phe[5], such as -NO_2 or -OH (9), abolish this selectivity and even reduce overall affinity, compared with BK.

Interestingly, in rat myometrial membranes, a tissue reported to bear the B_2 receptor, ^{125}I-BK did not show any binding site selectivity, although a similar B_{HA}/B_{LA} distribution had been proposed (11, 24). The specific binding capacity of those membranes was approximately 180 fmol/mg of protein for [^{3}H]BK but only 20 fmol/mg of protein for ^{125}I-BK, i.e., >10-fold lower, compared with GPI membranes (270 fmol/mg of protein). Several BK derivatives were weak agonists or inactive in rat uterus but antagonized the BK-induced contraction of GPI, or vice versa (16, 25). The hypothesis that there are tissue differences between the B_2 receptors in GPI and in rat uterus (1, 2) is also supported by our findings.

Competition with ^{125}I-BK binding to GPI membranes by the B_2 receptor antagonists Hoe 140 (23) and NPC 349 (26) confirmed the presence of two binding sites (Fig. 8). Hoe 140 displayed affinities approximately 1000-fold higher than those of NPC 349 against ^{125}I-BK, which correlates with the 300-fold difference in antagonistic potency. A similar affinity difference (100-fold) was also apparent with [^{3}H]BK as tracer, although only a single site (K_i = 180 pM) could be clearly assessed (Table 2). Recently, a 4-[^{125}I]iodobenzoate conjugate of Hoe 140, referred to as *para*-[^{125}I]iodophenyl-Hoe 140, was presented as a new radiolabel that bound with high affinity (K_d = 15 pM) to a homogeneous receptor population in GPI membranes (27). In contrast, another analogue of Hoe 140, in which the thienylalanine in position 5 was replaced by *p*-iodophenylalanine (D-Arg[0]-[Hyp[3],*p*-I-Phe[5],D-Tic[7],Oic[8]]BK), also showed a clearly biphasic displacement curve against [^{3}H]BK in GPI membranes, displaying K_i values of approximately 30 pM and 20 nM, compared with Hoe 140 (0.4 nM).[1]

There remains a marked difference of about 2 orders of magnitude between binding parameters and biological values of pD_2 (or pA_2), but such discrepancies commonly exist (14, 17). The lower potencies observed in intact tissue may be attributed to the use of physiological (high ionic strength) buffers, higher incubation temperature, and/or diffusion barriers (14, 17). However, these factors cannot account for the large difference, of about 6 orders of magnitude, between the B_{HA} site affinities of the antagonists and their antagonistic potencies in the GPI. The same conclusion can be drawn when the B_{HA} and B_{LA} site binding parameters of the agonists BK, I-BK, and NO_2-BK are compared with their biological potencies, in terms of pD_2 in the GPI (8.43, 7.80, and 8.09, respectively). This leads to the assumption that the B_{HA} sites are not directly involved in the mediation of BK-induced contraction.

Ransom *et al.* (17) suggested that the B_{HA} site, with picomolar affinity for BK, might be an artifact due to the use of nonphysiological (low ionic strength/low pH) incubation buffers. With [^{3}H]BK as label, those authors found a single class of sites, with a K_d of 16 pM, in GPI membranes in TES buffer. Using a physiological buffer (Krebs' solution), they obtained a 20-fold reduction in the binding affintiy, yielding a better but still not good correlation between binding affinity (0.3 nM) and the EC_{50} for stimulation of phosphoinositide hydrolysis (13 nM). In our displacement experiments with the new tracer ^{125}I-BK and the more selective unlabeled compound I-BK as competitor, we showed that both the B_{HA} site and the B_{LA} site also occur under physiological conditions. However, both the measurable binding site concentration and the binding affinity for B_{HA} are clearly reduced. In contrast, native BK, with lower affinity and selectivity for the B_{HA} site, failed to recognize the B_{HA} site and bound only to an apparent single class of sites in physiological buffer. This shows again that discrimination between the two BK binding sites depends on the assay conditions.

The question of whether one or the other of the two sites has a biological importance or represents an artifact remains. Because B_2 receptors belong to the G protein-coupled receptor family (4, 5), we studied the degree of G protein activation by BK and its derivatives in GPI membranes using [^{35}S]GTP[S] binding, a biochemical measure of ligand-induced stimuli before conversion into intracellular signals. We show here that BK and I-BK induce in GPI membranes a biphasic stimulation of [^{35}S]GTP[S] binding, with maximal binding at BK concentrations corresponding to the respective B_{HA} and B_{LA} site affinities. In addition, the nonselective NO_2-BK causes monophasic G protein activation that corresponds to its binding site affinity. These results indicate that both binding sites are able to transduce BK-induced G protein activation and represent, therefore, separate G protein-coupled BK receptors.

Because both sites seem to be real receptors, we have to ask whether both, or which, of them may be linked to the BK-induced contraction of GPI. All hitherto known pD_2 and pA_2 values for BK agonists and antagonists, respectively, suggest a correlation with the receptor with nanomolar affinity (B_{LA}), rather than with the site with picomolar affinity (B_{HA}) (1, 2). Furthermore, BK has been reported to stimulate phosphoinositide metabolism at nanomolar concentrations (EC_{50} = 13 nM) (17). It should be assumed, therefore, that the BK-induced contraction of the GPI is mediated via the receptor with nanomolar affinity (B_{LA}) and that the B_{HA} receptor is not directly involved in this process. This B_{HA} receptor certainly does not reflect an interaction with the BK-degrading angiotensin I-converting enzyme, because, firstly, angiotensin I-converting enzyme has an affinity for BK in the nanomolar concentration range (28), secondly, in our binding assay system angiotensin I-converting enzyme was blocked by an excess of captopril, and, thirdly, the binding of the B_2 receptor antagonist Hoe 140 indicates its receptor nature.

Now we have the first evidence that BK, at picomolar concentrations exactly corresponding to the B_{HA} site affinity, inhibits GPI adenylate cyclase activity.[2] In several studies inhibitory actions of cAMP-elevating agents on phosphoinositide hydrolysis have been described (29, 30). It might be speculated, therefore, that low picomolar concentrations of BK act via the B_{HA} receptor to inhibit adenylate cyclase activity, which may be stimulated by other simultaneously arriving signals and thus sensitisizes the smooth muscle cell to nanomolar concentrations of BK that induce contraction via the B_{LA} receptor after stimulation of phosphoinositide hydrolysis.

In summary, the use of ^{125}I-BK as a probe for BK receptors has enabled us to confirm the existence of two separate BK binding sites in GPI membranes. The new tracer and the corresponding unlabeled BK analogue displayed enhanced affinity and selectivity for the low picomolar B_{HA} site, which

[1] Liebmann *et al.* Manuscript in preparation.

[2] Liebmann *et al.* Submitted for publication.

exists in addition to the low nanomolar B_{LA} site. The B_{LA} site presumably represents the classical B_2 receptor that stimulates phosphoinositide metabolism (17) and mediates BK-induced smooth muscle contraction. The B_{HA} site seems to be a B_2 receptor subtype, because it is recognized by classical B_2 receptor antagonists. We further suggest that this is a physiological receptor, based on its ability to activate G proteins as the first step of a signal transduction pathway. Additional studies are needed to elucidate the second messenger systems involved in this signal transduction pathway and the functional role of this B_{HA} receptor in the GPI.

References

1. Farmer, S. G., and R. M. Burch. Biochemical and molecular pharmacology of kinin receptors. *Annu. Rev. Pharmacol. Toxicol.* **32**:511–536 (1992).
2. Hall, J. M. Bradykinin receptors: pharmacological properties and biologcal roles. *Pharmacol. Ther.* **56**:131–190 (1992).
3. Regoli, D., D. Jukic, C. Tousignant, and N.-E. Rhaleb. Kinin receptor classification. *Agents Actions Suppl.* **38**:475–486 (1992).
4. McEachern, A. E., E. R. Shelton, S. Bhakta, R. Obernolte, C. Bach, P. Zuppan, J. Fujisaki, R. W. Aldrich, and K. Jarnagin. Expression cloning of a rat B_2 bradykinin receptor. *Proc. Natl. Acad. Sci. USA* **88**:7724–7728 (1991).
5. Hess, J. F., J. A. Borkowski, G. S. Young, C. D. Strader, and R. W. Ransom. Cloning and pharmacological characterization of a human bradykinin (BK-2) receptor. *Biochem. Biophys. Res. Commun.* **184**:260–268 (1992).
6. Eggerickx, D., E. Raspe, D. Bertrand, G. Vassart, and M. Parmentier. Molecular cloning, functional expression and pharmacological characterization of the human bradykinin B_2 receptor gene. *Biochem. Biophys. Res. Commun.* **187**:1306–1313 (1992).
7. McIntyre, P., E. Phillips, E. Skidmore, M. Brown, and M. Webb. Cloned murine bradykinin receptor exhibits a mixed B_1 and B_2 pharmacological selectivity. *Mol. Pharmacol.* **44**:346–355 (1993).
8. Hess, J. F., J. A. Borkowski, T. Macneil, G. Y. Stonesifer, J. Fraher, C. D. Strader, and R. W. Ransom. Differential pharmacology of cloned human and mouse B_2 bradykinin receptors. *Mol. Pharmacol.* **45**:1–8 (1994).
9. Manning, D. C., R. Vavrek, J. M. Stewart, and S. H. Snyder. Two bradykinin binding sites with picomolar affinities. *J. Pharmacol. Exp. Ther.* **237**:504–512 (1986).
10. Leeb-Lundberg, L. M., and S. A. Mathis. Guanine nucleotide regulation of B_2 kinin receptors: time-dependent formation of a guanine nucleotide-sensitive receptor state from which [^{3}H]bradykinin dissociates slowly. *J. Biol. Chem.* **265**:9621–9627 (1990).
11. Liebmann, C., S. Offermanns, K. Spicher, K.-D. Hinsch, M. Schnittler, J. L. Morgat, S. Reissmann, G. Schultz, and W. Rosenthal. A high-affinity bradykinin receptor from rat myometrium is coupled to pertussis toxin-sensitive G proteins of the G_i family. *Biochem. Biophys. Res. Commun.* **167**:910–917 (1990).
12. Trifilieff, A., E.-B. Haddad, Y. Landry, and J.-P. Gies. Evidence for two high-affinity bradykinin binding sites in guinea-pig lung. *Eur. J. Pharmacol.* **207**:129–134 (1991).
13. Odya, C. E., T. L. Goodfriend, and C. Pena. Bradykinin receptor-like binding studied with iodinated analogues. *Biochem. Pharmacol.* **29**:175–185 (1980).
14. Tousignant, C., G. Guilemette, J. Barabé, N.-E. Rhaleb, and D. Regoli. Characterization of kinin binding sites: identity of B_2 receptors in the epithelium and the smooth muscle of the guinea pig ileum. *Can. J. Physiol. Pharmacol.* **69**:818–825 (1991).
15. Keravis, T. M., H. Nehlig, M.-F. Delacroix, D. Regoli, C. R. Hiley, and J.-C. Stoclet. High-affinity bradykinin B_2 binding sites sensitive to guanine nucleotides in bovine aortic endothelial cells. *Eur. J. Pharmacol.* **207**:149–155 (1991).
16. Farmer, S. G., R. M. Burch, C. J. Dehaas, J. Togo, and L. Steranka. [Arg^1-D-Phe^7]-substituted bradykinin analogs inhibit bradykinin- and vasopressin-induced contractions of uterine smooth muscle. *J. Pharmacol. Exp. Ther.* **248**:677–681 (1989).
17. Ransom, R. W., C. B. Goodman, and G. S. Young. Bradykinin stimulation of phosphoinonositide hydrolysis in guinea-pig ileum longitudinal muscle. *Br. J. Pharmacol.* **105**:919–924 (1992).
18. Bossé, R., and E. Escher. Synthesis of radio-iodinated ligands with high affinity and selectivity for μ- and δ-opioid receptors, in *Peptides 1990* (E. Giralt and D. Andren, eds.). ESCOM Science Publishers, Leiden, The Netherlands, 632–634 (1990).
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
20. Liebmann, C., M. Schnittler, S. Nawrath, and K. H. Jakobs. High-affinity bradykinin receptor-catalyzed G protein activation in rat myometrium. *Eur. J. Pharmacol.* **207**:67–71 (1991).
21. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107**:220–239 (1980).
22. Tobler, H. J., and G. Engel. Affinity spectra: a novel way for the evaluation of equilibrium binding experiments. *Naunyn-Schmiedebergs Arch. Pharmacol.* **322**:183–192 (1983).
23. Hock, F. J., K. Wirth, U. Albus, W. Linz, H. J. Gerhards, G. Wiemer, S. Henke, G. Breipohl, W. König, J. Knolle, and B. A. Schölkens. Hoe 140, a new long acting bradykinin antagonist: *in vitro* studies. *Br. J. Pharmacol.* **102**:769–773 (1991).
24. Liebmann, C., S. Nawrath, B. Ludwig, and I. Paegelow. Pharmacological and molecular actions of the bradykinin B_2 antagonist, Hoe 140, in the rat uterus. *Eur. J. Pharmacol.* **235**:183–188 (1993).
25. Bathon, J. M., and D. Proud. Bradykinin antagonists. *Annu. Rev. Pharmacol. Toxicol.* **31**:129–162 (1991).
26. Stewart, J. M., and R. J. Vavrek. Bradykinin competitive antagonists: design and applications. *Protides Biol. Fluids* **34**:473–476 (1986).
27. Brenner, N. J., G. Y. Stonesifer, K. A. Schneck, H. D. Burns, and R. W. Ransom. [^{125}I]PIP-Hoe 140, a high affinity radioligand for bradykinin B_2 receptors. *Life Sci.* **53**:1879–1886 (1993).
28. Odya, C. E., F. P. Wilgis, R. J. Vavrek, and J. M. Stewart. Interactions of kinins with angiotensin I converting enzyme (kininase II). *Biochem. Pharmacol.* **32**:3839–3847 (1983).
29. Gardner, S. D., G. Milligan, J. E. Rice, and M. J. O. Wakelam. The effect of cholera toxin on the inhibition of vasopressin-stimulated inositol phospholipid hydrolysis is a cyclic AMP-mediated event at the level of receptor binding. *Biochem. J.* **259**:679–684 (1989).
30. Guillon, G., M.-N. Balestre, C. Lombard, F. Rassendren, and C. J. Kirk. Influence of bacterial toxins and forskolin upon vasopressin-induced inositol phosphate accumulation in WRK 1 cells. *Biochem. J.* **260**:665–672 (1989).

Send reprint requests to: Emanuel Escher, Department of Pharmacology, Faculty of Medicine, University of Sherbrooke, Sherbrooke, Quebec, J1H 5N4, Canada.